Biochemical Activities of Propolis-Extracts
III. Inhibition of Dihydrofolate Reductase

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Ethanolic and aqueous extracts of the natural compound PROPOLIS indicate substantial antiinflammatory functions as well as antibiotic activities *in vitro* and *in vivo*. The exact mode of physiological or biochemical mechanisms responsible for the medical effects, however, is all but clear. The standardization on the basis of quantitative determination of prominent components of these extracts have been substituted recently by simple biochemical model reactions including photodynamic properties. In this communication we report on the inhibitory activity of an aqueous extract of propolis on the enzyme dihydrofolate reductase. This activity may at least partially be due to the content of caffeic acid, as revealed by HPLC chromatography and comparative activity tests of representative ingredients of the propolis extract. This result may explain some of the protective functions of propolis, similar to those shown for several "non-steroidal antiinflammatory drugs", NSAIDs.

Introduction

Honey bees collect a resinous substance from the buds of different trees. They convert it to the bee glue, called "Propolis", and use it to seal their hive. Propolis extracts have widely been used in folk medicine for the treatment of infections of the skin, mucosa and mouth. Propolis contains numerous aliphatic and aromatic compounds such as terpenes, phenylpropane derivatives, flavonoids, amino acids and different aldehydes and ketones, altogether more than 150 substances [1–6].

The antibacterial and antifungal activities and thus antiseptic properties [7–13] especially warranted the use of these extracts in medicine [14, 15]. Immune modulatory activities of aqueous extracts from propolis seem to be responsible for the increased protection against gram negative infections *via* macrophage activation [16, 17]. These properties as well as antiinflammatory and antiphlogistic effects may be due to several components of these extracts of propolis which may mediate their beneficial medical effects through the antioxidant properties reported in the preceding communications [18, 19].

Dihydrofolate reductase (DHFR) plays an important role in intermediary metabolism especially in rapidly dividing cells such as bacterial cultures or uncontrolled growing tissues like tumors. This enzyme transfers H^+ to N-5 of dihydrofolate (DHF). As a consequence, the more electrophilic DHF gets a hydrid ion from the ternary DHFR-NADPH-DHF complex and regenerates tetrahydrofolate (THF). This molecule, in turn, transfers activated C-1 units necessary for the biosynthesis of purines, pyrimidines and certain essential amino acids such as methionine. It is conceivable that inhibition of this enzyme provokes cytostatic and antibacterial effects [20, 21].

Methotrexate (4-amino-10-methylfolic acid) is a chemical analogon of DHF and a well known inhibitor of DHFR. Thereby, the pteridine ringsystem of methotrexate binds to the active centre of the enzyme, which is surrounded by a hydrophobic protein moiety. The N-1 of the coenzyme forms a hydrogen bridge with the protonated Glu-30 in the active site of the enzyme [22].

Methylbenzoprim is another strong inhibitor of this enzyme. This substance also forms hydrogen bonds between its diamino pyrimidine ringsystem and the amino acids Glu-30, Thr-136, Ile-7 and Val-112 in the active centre of the enzyme. Both aromatic rings of methylbenzoprim fit into the hydrophobic pocket of DHFR [23].

Baggott *et al.* [21] showed that several non-steroidal antiinflammatory drugs (NSAIDs) inhibited

DHFR. This fact may thus explain part of their anti-inflammatory effect. In this communication we report on significant inhibition of DHFR activity by an aqueous extract of propolis. HPLC-chromatographic separation of this extract and subsequent activity tests showed that caffeic acid was the most active component with an I_{50}-value of approximately 80 μM. Cinnamic acid, vanillin and ferulic acid showed only negligible activities. The aromatic amino acid dihydroxyphenylalanine (DOPA) is a structurally related compound to caffeic acid. DOPA exhibits approximately 40% activity as compared to caffeic acid. This effect and the lack of activity of cinnamic acid allows the conclusion that an *o*-dihydroxy configuration in addition to the double bond in the C-3 side chain may represent the important structural features for the inhibitory effect of these phenylpropane (C_6-C_3) derivatives.

Materials and Methods

Materials

An aqueous extract (WSD) of propolis was obtained from Fa. Medice (Iserlohn).

DHFR (E.C. 1.5.1.3), DHF and NADPH were obtained from Sigma (Deisenhofen). All other chemicals were purchased from Merck (Darmstadt), Sigma and Roth chemicals (Karlsruhe).

a) Lipophilic extraction

The WSD was treated with petrol ether (1:1, vol/vol) for 30 min at room temperature in order to extract lipophilic components. After centrifugation for 20 min at $3000 \times g$, the lipophilic phase was evaporated to dryness and redissolved in a corresponding equivalent of water. The two resulting aqueous solutions were tested for their inhibitory activity in the DHFR reaction.

b) Charcoal treatment

10 ml of the aqueous WSD extract were mixed with 400 mg charcoal (Merck, Art. No. 2186). After shaking for 30 min at room temperature, the suspension was filtered thus obtaining an aqueous effluent (charcoal filtrate). The filtrated charcoal was dried and washed with ethanolic ammonia (95% EtOH and 5% NH_4OH) in order to get rid off the bounded aromatic derivatives. This ethanolic solution was evaporated to dryness and redissolved with an equivalent amount of water (charcoal wash). This new aqueous extract and the filtrate of the charcoal treatment were comparatively tested in the DHFR system.

Methods

a) HPLC separation

5 μl of the different preparations (Materials, a, b) were injected into a Hypersil-ODS column (octadecyl-5 μm, reversed phase, 12.5 cm length, 4.6 mm diameter) and separated with a flow rate of 1 ml/min by the following solvents: A: 50 mM phosphate buffer pH 2.0; B: 100% methanol (MeOH).

Program: 1 min equilibration at 10% MeOH; increase of MeOH from 10% to 17% within 15 min; increase of MeOH from 17% to 50% within 10 min; 50% MeOH constant for another 9 min.

The elution profile of untreated WSD shows a wealth of compounds. Altogether 16 distinct and sharp peaks can be detected at 280 nm. The following compounds with known retention times have been identified: caffeic acid: 12.01 min (Roth chemicals); vanillin: 13.14 min (Sigma); ferulic acid: 22.01 min (Sigma); cinnamic acid: 27.81 min (Sigma).

b) Dihydrofolate reductase activity was determined as follows

The test solution contained in 1 ml: 500 μl 15 mM phosphate buffer pH 7.4; 100 μl 1 mM NADPH; 100 μl DHFR (0.1 U/ml); different concentrations of the propolis preparations or phenolic derivatives.

This reaction mixture was preincubated for 5 min at 37 °C and started with 100 μl DHF (600 μM). Incubation time was 4 h at 37 °C in the dark. One unit will convert 1.0 μmole of 7.8-dihydrofolate and NADPH to 5.6.7.8-tetrahydrofolate and $NADP^+$ per min at pH 6.5 at 25 °C.

c) Enzyme activity was determined according to Bratton-Marshall [24]

After incubation time, the reaction was stopped by the addition of 0.6 ml sulfuric acid (5 M) and 1.1 ml NaCl (0.3 M).

Then, the test solutions were transfered to an ice bath and the following reagents were added: 0.2 ml sodium nitrite (0.1%), 0.2 ml ammonium sulfamate (0.5%), 0.2 ml α-naphthylethylene diamine (0.1%).

The reaction mixture was incubated for 75 min at room temperature in the dark in order to develop the red azo-dye with an absorption maximum at 540 nm. The extinctions in the absence of an inhibitor were set as 100% activity. All reactions were carried out with five parallels and mean values with standard deviations were calculated for the tables and figures.

Results and Discussion

Inhibition of dihydrofolate reductase by untreated aqueous extract of propolis (WSD)

The DHFR reaction is inhibited by increasing amounts of added WSD (Fig. 1). A 50% reduction of activity is obtained by *ca.* 1.2 vol.% of the extract.
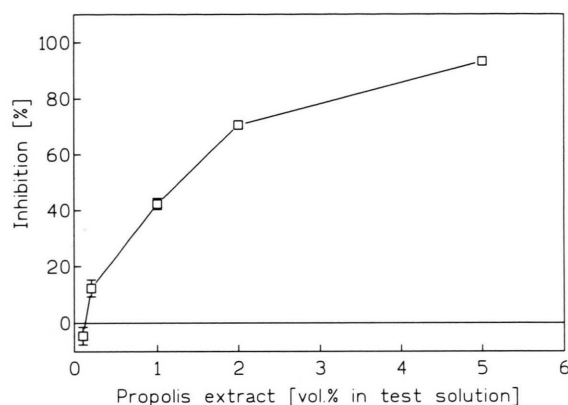

Fig. 1. Inhibition of dihydrofolate reductase activity by the aqueous extract of propolis (100% activity correspond to about 0.40 ± 0.01 E).

Extraction of the untreated aqueous extract of propolis with petrol ether

Extraction of the WSD with petrol ether yields two new aqueous preparations, namely the aqueous phase derivative and the lipophilic phase derivative (see Materials, a). The absorption spectrum between 200 nm and 800 nm of the aqueous petrol ether supernatant shows no change as compared to untreated WSD (Fig. 2a). Both extracts exhibit high absorption in the UV with a small peak at 295 nm and a strong decrease between 300 and 350 nm. Nearly no absorption was measured at higher wavelengths. 100% of the activity is left in this new extracted aqueous phase, whereas the redissolved, lipophilic petrol ether phase has no effect on DHFR activity (data not shown).

Charcoal treatment of the aqueous extract of propolis

The filtrate after charcoal treatment exhibits an absorption spectrum strongly different from the untreated WSD. The spectrum shows a considerable loss of absorption between 210 nm and 290 nm and a distinct and broad absorption maximum around 295 nm. The loss of absorption at 250 nm is approximately 70%. Despite this altered spectrum, the charcoal filtrate contains the same inhibitory activity compared to the untreated WSD. In contrast, the EtOH-ammonia-treated aqueous charcoal wash is inactive (Fig. 2b).

Separation of different preparations of propolis by HPLC

The elution profile of untreated WSD was not significantly changed in the filtrate after charcoal treatment. The molarities of caffeic acid, vanillin, ferulic acid and cinnamic acid in the extracts (see Materials, b) before and after charcoal treatment are compared in Table I.

Table I. Concentrations of aromatic compounds in aqueous extracts of propolis before and after charcoal treatment.

[mM]	Untreated aqueous extract	Charcoal filtrate	Charcoal wash
Caffeic acid	0.055 ± 0.008	0.043 ± 0.002	0
Vanillin	0.110 ± 0.005	0.036 ± 0.004	0.012 ± 0.001
Ferulic acid	0.282 ± 0.029	0.121 ± 0.016	0.004 ± 0.001
Cinnamic acid	0.727 ± 0.053	0.263 ± 0.003	0.004 ± 0.002

The comparison of the concentrations of different identified compounds with the corresponding activities before and after charcoal treatment clearly indicates that caffeic acid only suffers from an approximately 20% loss by the treatment, while vanillin is reduced by *ca.* 70%, ferulic acid by roughly 60% and cinnamic acid by 65%. In order to test whether these compounds contribute to the inhibition of DHFR we tested the authentic chemicals in the DHFR assay.

As shown in Fig. 3 only caffeic acid is significantly inhibiting DHFR activity. An I_{50}-value of 80 μM can be calculated.
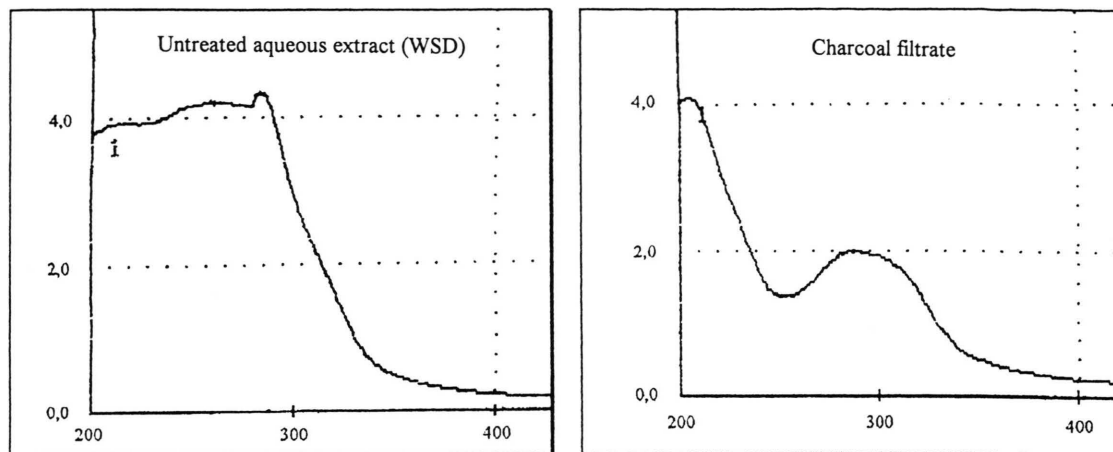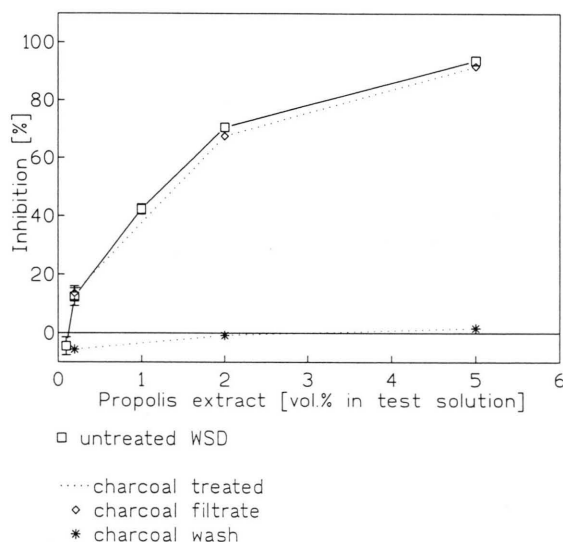
Fig. 2. Comparison of spectral properties and dihydrofolate reductase inhibition by aqueous extracts before and after charcoal treatment. a) Absorption spectra of charcoal filtrate and untreated aqueous extract.

b) Inhibition of DHFR activity by charcoal filtrate and untreated aqueous extract (the 100% values range between 0.39 and 0.41 E).
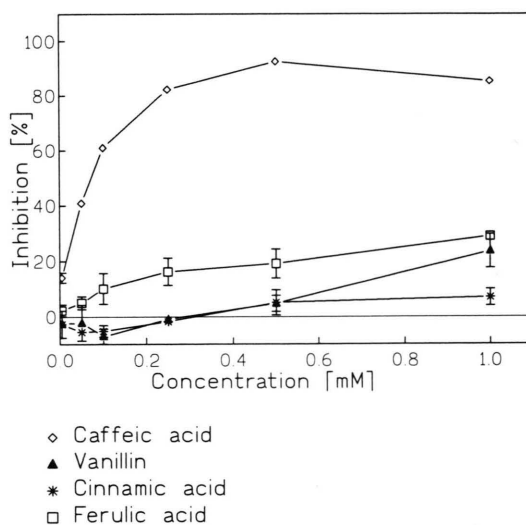
Fig. 3. Comparison of the inhibition of dihydrofolate reductase activity by caffeic acid, vanillin, ferulic acid and cinnamic acid (the 100% values correspond to about 0.40 ± 0.01 E).

Since none of the above compounds besides caffeic acid has an unsubstituted *o*-3.4-dihydroxy aromatic moiety we compared DOPA (*o*-3.4-dihydroxy phenylalanin) on its influence on DHFR activity. In comparison to caffeic acid, DOPA shows reduced inhibitory activity exhibiting an I_{50}-value at approximately 200 μM (Fig. 4). Thus, the unsaturated side chain itself as the substituent of an aromatic body (ferulic acid, cinnamic acid) is not essential for DHFR inhibition. The *o*-dihydroxy configuration in the absence of the unsaturated C-3 side chain (DOPA) shows reduced activity as compared to caffeic acid configuration. This fact renders the *o*-3.4-dihydroxy-1-propene carboxylate moiety as the important structural feature for maximal inhibitory activity.
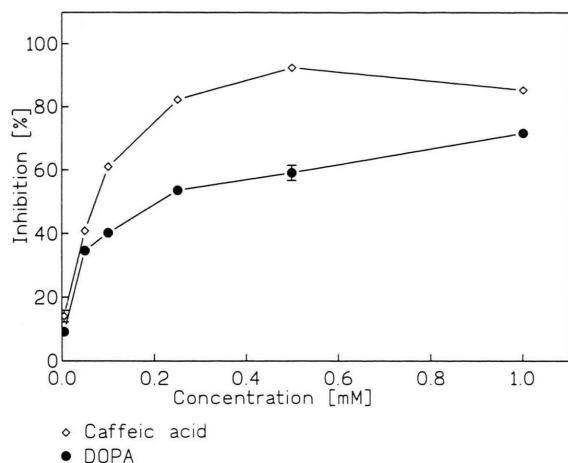
Fig. 4. Comparison of the inhibitory activity of DOPA and caffeic acid on dihydrofolate reductase (an extinction of about 0.41 E corresponds to 100%).

Conclusion

From the presented results we conclude that WSD of propolis may exert some of its antiinflammatory and antiinfectuous properties *via* the inhibition of DHFR activity similar to the report on well known NSAIDs [21]. This enzyme plays an important role in intermediary C-1 metabolism and is one basic enzymic requirement in fast growing and/or dividing cells (bacteria) and tissues (tumours). This property is in part due to caffeic acid as one of at least 16 major (aromatic or heterocyclic) components of WSD of propolis, detectable after HPLC separation at 280 nm.

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