Biochemical Activities of Propolis Extracts
II. Photodynamic Activities

Regina Volpert and Erich F. Elstner
Institut für Botanik und Mikrobiologie, Biochemisches Labor, Technische Universität München, Arcisstraße 21, D-80290 München, Bundesrepublik Deutschland

Z. Naturforsch. **48 c**, 858–862 (1993); received July 7/August 16, 1993

Propolis, Photodynamic Reactions, Riboflavin, Rose Bengal, Hematoporphyrin

Ethanolic and aqueous extracts of the "bee glue" Propolis exhibit antioxidative properties and are used as antiinflammatory drugs in folk medicine. In order to standardize the principle activities of prominent components of these extracts, simple biochemical tests have been introduced in the preceding paper. These activity tests prove the high antioxidative and inhibitory capacities of aqueous and ethanolic extracts of propolis *in vitro*. In the present communication we report on experiments documenting photodynamic quenching properties of these extracts. Using riboflavin, rose bengal or hematoporphyrin as photoactivators and ketomethylthiobutyric acid or crocin as indicators, the protective functions of propolis preparations can be demonstrated. The results indicate that the aqueous extracts are more active than the corresponding ethanolic preparation.

Introduction

Ethanolic and aqueous extracts of the "bee glue", called "Propolis" have been shown to inhibit several oxidative reactions, thus representing significant antioxidative capacities [1]. The treatment of infections of the skin, mucosa and other tissues may be explained by these properties intrinsic to the numerous compounds identified. Propolis in general contains many different bioflavonoids, aromatic acids, phenols, terpenes, amino acids, aldehydes and ketones as well as metal ions [2–7].

The antibacterial, antifungal and thus antiseptic properties may represent the basis for the historical and present use of these extracts in dermatology, against inflammatory processes and common colds [11]. A recent report on immune modulatory activities of aqueous extracts of propolis documented that protection from gram-negative infections may probably operate *via* macrophage activation [8]. Juan *et al.* [9] used rat stomach and rabbit ear tissues for demonstrating the inhibition of the release of proinflammatory prostaglandins as well as the inhibition of arachidonic acid-induced platelet aggregation and thromboxane formation. The mentioned publications clearly indicate the immune modulatory properties of ethanolic and aqueous extracts of propolis.

In this communication we report on biochemical model reactions documenting the inhibition of light-driven oxidations by different propolis extracts. These activities suggest that preparations of propolis may also function in UV protection by quenching excited states in addition to their radical scavenging capacities for superoxide and OH· type oxidants [1].

Materials and Methods

Materials

The different preparations of propolis were obtained as described in the preceding paper [1]. The following three different extracts were tested: WSDs: a) untreated, particle-containing, aqueous PWE-13 derivative, b) lyophilized and particle-free, aqueous PWE-13 derivative; ESD: lyophilized, aqueous PEE-40, but primary ethanolic derivative. All chemicals used were of the highest grade of purity available (Merck, Serva, Sigma).

Methods

a) Propolis-catalyzed KMB fragmentation in the light

The test solution contained in 2 ml: 1 ml 100 mM phosphate buffer, pH 7.4; 100 µl extract of propolis (0.5 vol.%, 2.0 vol.% and 5.0 vol.% of ESD or

Abbreviations: WSDs, water-soluble derivatives; ESD, ethanol-soluble derivative; RB, rose bengal; RF, riboflavin; HP, hematoporphyrin; KMB, 2-ketomethylthiobutyric acid.

Reprint requests to E. F. Elstner.

Verlag der Zeitschrift für Naturforschung, D-72072 Tübingen
0939–5075/93/1100–0858 $ 01.30/0

WSDs); 100 µl 25 mM KMB. The reaction mixture was incubated for 30, 60 or 90 min at 37 °C in a water bath under illumination (30 klux, white light). After incubation time, 1 ml gas from the headspace of the reaction vessels was withdrawn and analyzed in the gas chromatograph as described previously [1, 10].

b) Riboflavin-, rose bengal- or hematoporphyrin-catalyzed photodynamic fragmentation of KMB

The test solution contained in 2 ml: 1ml 100 mM phosphate buffer, pH 7.4; 100 µl 20 µM RF or RB or 100 µM HP; 100 µl 25 mM KMB; 100 µl of different concentrations of ESD or WSDs. After illumination time (30 klux, white light) for 5 min (RF), 30 min (RB) or 45 min (HP) at 37 °C, ethylene release from KMB was quantified as described above (Methods, a).

c) Crocin-bleaching by riboflavin, rose bengal or hematoporphyrin

The test solution contained in 2 ml: 1ml 100 mM phosphate buffer, pH 7.4; 100 µl crocin solution (E_{440} = 1.0); 100 µl 100 µM RF, RB or HP; 100 µl of different concentrations of ESD or WSDs. The reductive bleaching of the carotenoid derivative crocin (maximal absorption at 440 nm) was determined photometrically after illumination in a water bath for 10 min (RB) or 45 min (RF and HP) at 37 °C.

Results and Discussion

Propolis-catalyzed ethylene release from ketomethylthiobutyric acid

The three different propolis derivatives exhibit very weak photodynamic properties, visible as ethylene formation from KMB in the light (data not shown). This photodynamic activity is extremely low with a maximal ethylene production of about 900 pmol by the WSDs and about 280 pmol by the ESD after 90 min in the light. This represents less than 1% of the activities of riboflavin, rose bengal or hematoporphyrin.

Effects of different propolis preparations on riboflavin-driven oxidations

The photoactivator riboflavin (RF) generates superoxide *via* charge separation in the excited state (photodynamic reaction type I) as well as singlet oxygen (photodynamic reaction type II) [10]. Fragmentation of KMB forming ethylene is a sensitive indicator for photodynamic reactions [13].

Illumination of a test solution with 1 µM RF and 1.25 mM KMB produces approximately 22.5 nmol ethylene after 5 min at 30 klux. Derivatives of propolis inhibit this photodynamic fragmentation of KMB to different extents (Fig. 1).

The following I_{50} values were calculated: ESD: 1.2 vol.%; particle-free WSD: 0.4 vol.%; particle-containing WSD: 0.3 vol.%.

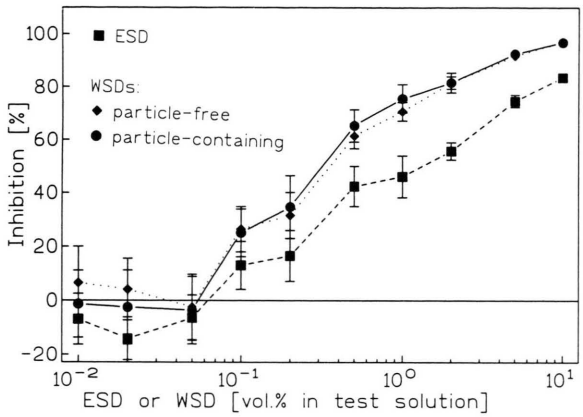

Fig. 1. Inhibition of riboflavin-induced ethylene formation from ketomethylthiobutyric acid by derivatives of propolis.

Crocin is a water-soluble digentiobiose diester of the carotenoid acid crocetin. It represents a model substance for polyunsaturated compounds such as fatty acids [10].

None of the tested concentrations of the three extracts of propolis affected the light-absorbing properties of crocin solutions in the dark or after incubation in the light for 45 min (data not shown). After this incubation time crocin is bleached about 0.40 E in the presence of 5 µM riboflavin.

Below 1 vol.% none of the three extracts of propolis significantly affected crocin bleaching by RF within 45 min. In contrast, higher concentrations of WSDs inhibited the reaction, whereas higher concentrations of ESD slightly stimulated the bleaching reaction (Fig. 2). Because of this difference between the ESD and the both WSDs, we varied incubation time from 5 min to 45 min. Since in the RF-catalyzed fragmentation of KMB the aqueous extracts are about twice as effective as the primary

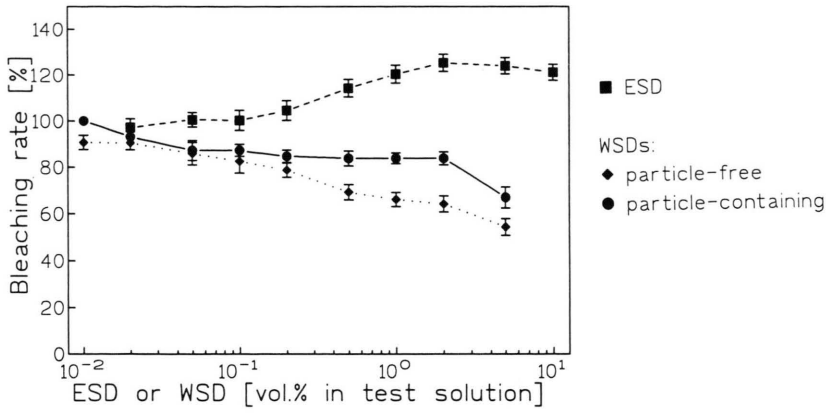

Fig. 2. Effects of propolis derivatives on riboflavin-dependent bleaching of crocin.

ethanolic extract, we compared the effects of 5 vol.% particle-free and particle-containing WSD with 10 vol.% ESD. As shown in Fig. 3, all preparations stimulated crocin bleaching after short incubation times (up to 10 min). After longer incubation times this stimulation was converted into inhibition, where only the aqueous derivatives yielded really net inhibition of *ca.* 40% as compared to the riboflavin controls.

This result indicates that compounds in the propolis preparations interact with the photoactivator RF and polyunsaturated compounds such as crocin. After the time-dependent consumption or destruction of stimulating compounds the aqueous derivatives seem to function as "protectors". For these extracts the equilibration between stimulating and inhibitory properties seems to be terminated after illumination for 30 min with RF. In contrast, the ethanolic extract is less effective in scavenging the reactive species of oxygen and RF even at longer incubation times.

Effects of different propolis extracts on rose bengal-dependent reactions

In contrast to riboflavin, the photoactivator rose bengal (RB) is a relatively specific singlet oxygen ($^1O_2$) generator [12]. The RB-catalyzed fragmentation of KMB is significantly stimulated by the ethanolic extract in the concentration range between 0.01 and 1.0 vol.%, whereas the riboflavin-driven ethylene release is not affected. At concentrations higher than 1 vol.% the stimulation is converted into inhibition reaching 60% at 5.0 vol.%. None of the WSDs stimulated the RB-driven ethylene release. The particle-free WSD is inhibitory from *ca.* 0.2 vol.% upwards and the particle-containing WSD at slightly lower concentrations (Fig. 4).

The results shown in Fig. 4 indicate that compounds present in the ethanolic extract are activated by $^1O_2$ yielding derivatives which support fragmentation of KMB. At higher concentrations of ESD, the inhibitors present either quench excited

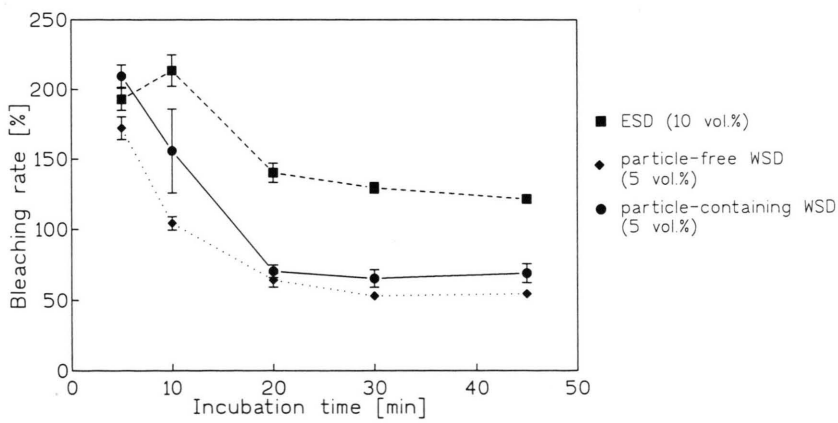

Fig. 3. Time-dependent stimulation and inhibition of riboflavin-driven crocin bleaching by the different preparations of propolis.

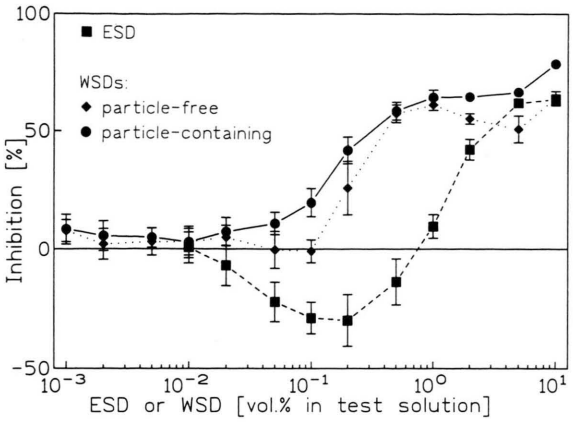

Fig. 4. Effects of propolis derivatives on rose bengal-catalyzed ethylene formation from ketomethylthiobutyric acid (about 5300 pmol ethylene correspond to 100%).

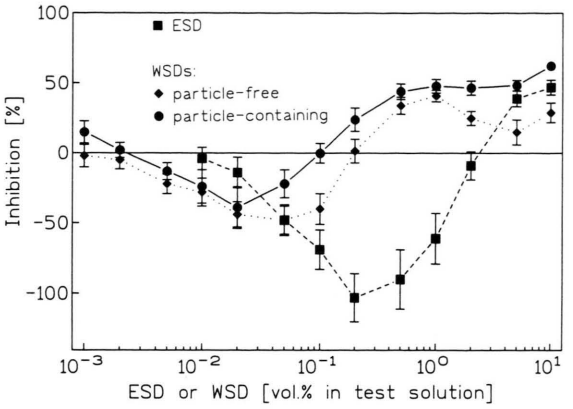

Fig. 5. Effects of propolis derivatives on hematoporphyrin-catalyzed ethylene formation from ketomethylthiobutyric acid.

states of $^1O_2$ or react with the activating derivatives of converted ESD. In contrast, both aqueous extracts lack components which can be activated by $^1O_2$. Thus only the quenching or radical scavenging properties are expressed.

After 10 min crocin is bleached about 0.31 E by RB. This reaction is not significantly influenced by the three derivatives of propolis indicating that the interaction between crocin and RB is faster than the reactions between these derivatives and the active intermediates responsible for crocin bleaching (data not shown).

Effects of different preparations of propolis on hematoporphyrin-catalyzed photoreactions

Hematoporphyrin (HP) derivatives are frequently used as activators in photodynamic therapy [12]. Analogous to the action of riboflavin, the products of light activation of HP are mixed type including both photodynamic reactions of type I and II. Fragmentation of KMB by HP yields about 2600 pmol ethylene after 45 min of illumination. Similar to the observation with rose bengal, this reaction is strongly stimulated by low concentrations of the ethanolic extract (0.02–1 vol.%). An inhibitory activity is observed only at the highest concentrations of 5 and 10 vol.%. In contrast, the WSDs show stimulatory effects at lower concentrations (between 0.002 and 0.1 vol.%) and better inhibitory effects above 0.2 vol.% (Fig. 5).

Crocin bleaching is only slightly inhibited by high concentrations of the WSDs, whereas the ESD has no effect (data not shown).

Conclusions

The differential behaviour of the ethanolic extract in the rose bengal and hematoporphyrin systems as compared to the aqueous extracts clearly indicates the presence of photoactivatable compounds in the ESD. These substances interact with the corresponding photoactivators, RB and HP, respectively. The reduced activity of ESD in comparison to the particle-free and particle-containing WSDs in all systems indicates the lack of radical scavengers. This was already demonstrated in diaphorase- or xanthine oxidase-catalyzed model reactions [1].

The results reported in this communication support the view that the "bee glue" contains a mixture of phenolic compounds exhibiting pronounced antioxidative properties including excited state quenchers.

[1] R. Volpert, E. F. Elstner, Z. Naturforsch. **48 c,** 851–857 (1993).
[2] V. S. Bankova, S. S. Popov, N. L. Marekov, Phytochemistry **28,** 871–873 (1989).
[3] V. S. Bankova, Al. Dyulgerov, S. S. Popov, N. L. Marekov, Z. Naturforsch. **42 c,** 147–151 (1987).
[4] B. König, J. H. Dustmann, Naturwissenschaften **72,** 659–661 (1985).
[5] J. Gabrys, J. Konecki, W. Krol, S. Scheller, J. Shani, Pharmacol. Res. Commun. **18,** 513–518 (1986).
[6] P. Walker, E. Crane, Apidologie **18,** 327–334 (1987).
[7] J. Verge, Apiculteur **95,** 13 (1951).
[8] V. Dimov, N. Ivanovska, N. Manolova, V. Bankova, N. Nikolov, S. Popov, Apidologie **22,** 155–162 (1991).
[9] H. Juan, W. Sametz, S. Diethart, personal communication (1993).
[10] W. Bors, M. Saran, E. F. Elstner, in: Modern Methods of Plant Analysis (H. F. Linskens, J. F. Jackson, eds.), New Series, **Vol. 13,** 277–295, Springer Verlag, Berlin 1992.
[11] S. Scheller, F. Pawlak, Heilkunst **94,** 222–233 (1981).
[12] E. F. Elstner, in: Der Sauerstoff: Biochemie, Biologie, Medizin (E. F. Elstner, ed.), B. I. Wissenschaftsverlag, Mannheim, Wien, Zürich 1990.
[13] R. J. Youngman, P. Schieberle, H. Schnabl, W. Grosch, E. F. Elstner, Photobiochem. Photobiophys. **6,** 109–119 (1983).