Biochemical Activities of Propolis Extracts
I. Standardization and Antioxidative Properties of Ethanolic and Aqueous Derivatives

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Z. Naturforsch. **48 c**, 851–857 (1993); received June 21/August 16, 1993

Propolis, Peroxidase, Xanthine Oxidase, Diaphorase, Indole Acetic Acid Oxidation

Ethanolic extracts of Propolis are used as antiinflammatory and wound healing drugs since ancient times. In order to facilitate a comparison of different extracts, the standardization on the basis of quantitative determination of prominent components of these extracts has been substituted for simple biochemical "activity" tests. One of these activity tests bases on the inhibition of peroxidase-catalyzed oxidation of indole acetic acid indicating the presence of a defined mixture of monophenolic and diphenolic compounds. Other tests (diaphorase-catalyzed reductions and xanthine oxidase-catalyzed oxidations) demonstrate significant radical scavenging properties. Water-soluble extracts of propolis exhibit higher antioxidative and inhibitory activities as compared to the ethanolic extract.

Introduction

Ethanolic extracts (ESDs) of the "bee glue", a resinous substance collected by honey bees called "Propolis", have widely been used in folk medicine. The successful treatment of certain infections of the skin, mucosa and mouth is due to one or more of the numerous compounds identified in these extracts. All together, at least 150 different structures are known chemically belonging to terpenes, various phenylpropane derivatives such as caffeic acid esters, flavonoids, amino acids and a vast amount of aldehydes and ketones [1–6].

The antibacterial and antifungal activities [7–10] and thus antiseptic properties [11–13] especially warranted the use of these extracts in dermatology [14] and against dental complications [15]. Very recently, several striking reports on immune modulatory activities of aqueous extracts of propolis have been published [16, 17]. Dimov *et al.* [16] showed that a water-soluble extract of propolis (WSD) increases the protection from gram-negative infections probably *via* macrophage activation. Juan *et al.* [17] used pieces of the rat stomach and perfused rabbit ears for their experimental approach. They documented the inhibition of the release of proinflammatory prostaglandins as well as the inhibition of arachidonic acid-induced platelet aggregation and thromboxane formation. From the mentioned reports it seems clear that antibiotic activities, immune modulatory properties as well as antiinflammatory, wound healing and antiphlogistic effects may be due to different components of the individual ethanolic or aqueous extracts. However, since the beginning of the research on propolis ingredients [18] a wealth of data on possible functions and health effects of protective compounds typical for propolis has been published [19–23]. Both in plant and medical sciences these compounds have been shown to mainly act as antioxidants and/or as regulators of specific cellular functions. In this communication we wish to report on very sensitive and specific reactions including: a) diaphorase in the presence of NADH as electron donor and several electron acceptors; b) xanthine oxidase with xanthine as electron donor and oxygen as electron acceptor producing $O_2^{\cdot -}$, H_2O_2 and $OH^{\cdot}$ as strong oxidants; c) peroxidase in the presence of indole acetic acid but in the absence of peroxide; under these conditions peroxidase acts like an indole acetic acid "oxidase" [24].

Several of the mentioned reactions allow the standardization and differentiation of ethanolic and aqueous extracts of propolis. Especially the indole acetic acid oxidation is very sensitive to different concentrations of propolis. Taken together, enzy-

Abbreviations: IAA, indole acetic acid; BPDS, bathophenanthroline-disulfonic acid; DCPIP, 2,6-dichlorophenolindophenol; KMB, 2-ketomethylthiobutyric acid; WSD, water-soluble derivatives; ESD, ethanol-soluble derivative.

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Verlag der Zeitschrift für Naturforschung,
D-72072 Tübingen
0939–5075/93/1100–0851 $ 01.30/0

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matic activity tests are more suitable for standardization than the quantitative determination of a few prominent components of these extracts. Furthermore these reactions clearly indicate the antioxidative properties of the different propolis extracts potentially responsible for their beneficial medical effects.

In two following papers, inhibitory effects of propolis preparations on photodynamic reactions and dihydrofolate reductase are reported. These reactions show the broad spectrum of antioxidative and potentially also antiinflammatory activities of propolis.

Materials and Methods

Materials

Two different extracts of propolis have been obtained from Fa. Medice (Iserlohn): an ethanolic extract (PEE-40) and an aqueous extract (PWE-13). Both extracts were lyophilized and resolved in water in order to get comparable solutions in aqueous media. After freeze drying the following weight of the powders were obtained per ml original extracts: PEE-40: 0.29 ± 0.01 mg; PWE-13: 0.0775 ± 0.0012 mg.

The freeze-dried powders were reextracted for 30 min at 60 °C with 13.0 ml of distilled water. After centrifugation for 30 min at 27,000 × *g* and filtration through a glass filter, two new particle-free and aqueous preparations were obtained. Taken together the following aqueous solutions 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 extract.

Xanthine oxidase and peroxidase (horse radish) were purchased from Boehringer (Mannheim), and diaphorase *(Clostridium kluyveri)* from Serva (Deisenhofen); all other chemicals were of the highest grade of purity available (Merck, Serva, Sigma).

Methods

a) Diaphorase-catalyzed reactions

The test solution contained in 2 ml: 1 ml 100 mM phosphate buffer, pH 7.4; 100 µl 2 mM NADH; 100 µl 1 mM juglone or 100 µl 1 mM dichlorophenolindophenol (DCPIP); 100 µl diaphorase (3 U/ml); different concentrations of ESD and WSDs. If 200 µl $FeCl_3$–EDTA (4 mM $FeCl_3$; 6 mM EDTA) are used as electron acceptor, another buffer is necessary (1 ml 200 mM phosphate buffer, pH 6.0).

The reductive bleaching of the blue dye DCPIP (maximal absorption at 600 nm) to its colourless leuco form was determined photometrically. $FeCl_3$–EDTA is reduced to Fe^{2+} by diaphorase and this free ion yields an inactive red complex with BPDS (100 µl 4 mM). The red dye can be quantified photometrically at 530 nm [27].

Juglone-dependent oxygen reduction was either followed potentiometrically with an oxygen electrode (Rank-brothers, England), or gas chromatographically by determining ethylene release from 2-ketomethylthiobutyric acid (KMB, 100 µl 25 mM), withdrawn from the headspace of the reaction vessels with 1 ml gas-tight plastic syringes [22].

b) Xanthine oxidase reactions

The test solution contained in 2 ml: 1 ml 200 mM phosphate buffer, pH 7.8; 100 µl 10 mM xanthine (dissolved in 10 mM NaOH); 100 µl xanthine oxidase (0.8 U/ml); different concentrations of ESD and WSDs.

The reactions were followed: 1) potentiometrically with an oxygen electrode by observing oxygen uptake; 2) by determining nitrite formation from hydroxylamine (100 µl 10 mM NH_2OH) [25]; 3) by following ethylene release from KMB as described above (Methods, a).

c) Peroxidase-catalyzed IAA oxidation

The test solution contained in 1 ml: 500 µl 150 mM citrate-phosphate buffer, pH 5.6; 100 µl 3 mM IAA (dissolved in 10 mM NaOH); 100 µl peroxidase (3 U/ml); different concentrations of ESD and WSDs. The reaction mixture was incubated for 30 min at 37 °C in a water bath. After incubation time, the non-oxidized IAA was photometrically (520 nm) quantified by forming a coloured complex with $FeCl_3$ [24].

Results and Discussion

Effects of different propolis derivatives on diaphorase-catalyzed reactions

Oxygen uptake by the NADH-diaphorase-juglone system is rather differently influenced by the three types of extracts. A 50% inhibition of the

corresponding basal rates is obtained with about 14.3 vol.% of the ESD, 1.4 vol.% of the particle-free WSD or 0.6 vol.% of the particle-containing WSD (Fig. 1).

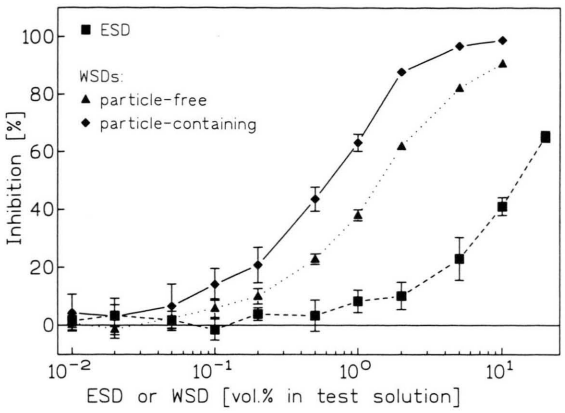

Fig. 1. Inhibition of oxygen consumption of the NADH-diaphorase-juglone system (about 75 nmol O_2 uptake/min corresponds to 100%).

If the NADH-diaphorase-juglone system is supplemented with KMB, ethylene release is inhibited in a concentration-dependent manner with increasing amounts of extracts. In this case, however, the striking difference between the three different preparations of propolis is almost completely lost (Fig. 2). The I_{50} values are 0.66 vol.% for the ESD, 0.47 vol.% for the particle-free WSD and 0.45 vol.% for the particle-containing WSD.

Substitution of juglone for DCPIP as electron acceptor yields an oxygen-independent redox process where the blue colour of the dye is bleached. This reaction can also be suppressed by the three extracts indicating a direct inhibition of the enzyme itself. In contrast to the fragmentation of KMB (Fig. 2) the individual differences between ESD and WSDs also observed during oxygen reduction (*cf.* Fig. 1) are retained (Fig. 3). The individual I_{50} values are calculated as follows: ESD: 8.0 vol.%; particle-free WSD: 1.4 vol.%; particle-containing WSD: 0.9 vol.%.

In a similar manner as oxygen and DCPIP reduction, Fe^{3+} reduction is also inhibited by the different extracts. In this reaction, the WSDs are almost equally active with I_{50} values of 0.25 vol.% (particle-containing WSD) and 0.26 vol.% (particle-free WSD) while the ESD is one order of magnitude less active exhibiting an I_{50} of 2.5 vol.% (Fig. 4).

The presented results indicate that the diaphorase-catalyzed interaction between NADH and different electron acceptors such as juglone, DCPIP or Fe^{3+} is more influenced by the WSDs as compared to the ESD. Thereby the most active preparation is the non-centrifuged, unfiltered and thus particle-containing WSD. The diaphorase-catalyzed ethylene release from KMB is inhibited by all three extracts to nearly the same extent. This result suggests that the rate-limiting step is the formation of the $OH^{\cdot}$ type oxidant and not the interaction between NADH, diaphorase and acceptor. Certain

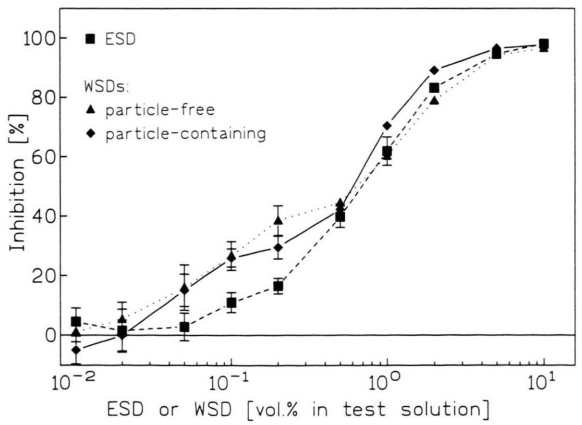

Fig. 2. Inhibition of ethylene release from ketomethylthiobutyric acid of the NADH-diaphorase-juglone system (the 100% values range between 5600–6300 pmol ethylene formation per assay).

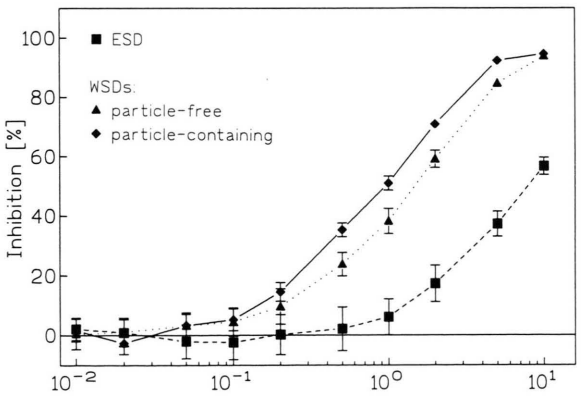

Fig. 3. Inhibition of dichlorophenolindophenol reduction of the NADH-diaphorase system (the 100% value for the initial bleaching ranges between 0.65 and 0.48 E/min).

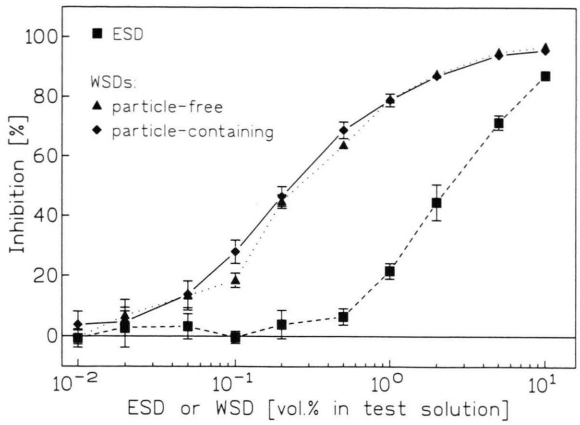

Fig. 4. Inhibition of Fe^{3+} reduction of the NADH-diaphorase system (the 100% value of the initial reaction rate ranges between 0.13 and 0.10 E/min).

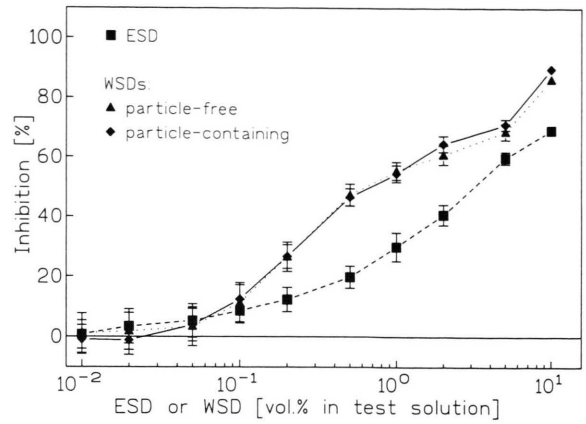

Fig. 5. Inhibition of superoxide-dependent hydroxylamine oxidation by xanthine oxidase (an extinction of about 0.88 E corresponds to 100%).

compounds present in ESD seem to compensate for the lack of enzyme-inhibitory components of the primary electron transfer. Probably the ESD exhibits a higher affinity for OH· than the WSDs thus acting as a potent free radical scavenger.

Effects of different prpolis preparations on xanthine oxidase-catalyzed reactions

Similarly to the diaphorase-catalyzed reaction, the xanthine oxidase-catalyzed reaction can be followed by measuring oxygen reduction, forming superoxide, H_2O_2 and OH·. The direct enzyme inhibition is measured potentiometrically, whereas about 65 nmol O_2 uptake corresponds to 100%. The I_{50} values are about 68 vol.% for the ESD, 11.5 vol.% for the particle-free WSD and 4.0 vol.% for the particle-containing WSD (data not shown). Thus xanthine oxidase itself is apparently not very sensitive towards the extracts as compared to diaphorase or peroxidase.

The superoxide generation by xanthine oxidase can be detected as nitrite formation from hydroxylamine. The inhibition of this reaction by superoxide dismutase proves $O_2^{\cdot -}$ production. Therefore the inhibition of nitrite formation by propolis extracts indicates their $O_2^{\cdot -}$-scavenging capacities.

Following I_{50} values were calculated as: ESD: 3.2 vol.%; particle-free WSD: 0.8 vol.%; particle-containing WSD: 0.6 vol.%. Again the water-soluble extracts exhibit some stronger inhibitory activities as compared to the ESD (Fig. 5).

Like the diaphorase-catalyzed fragmentation of KMB, the xanthine oxidase-driven reaction is inhibited by increasing amounts of added extracts. In contrast to the diaphorase reaction, however, low concentrations of both particle-free and particle-containing WSD stimulate ethylene production. Concentrations above *ca.* 0.15 vol.% show fast decrease of KMB fragmentation. The ESD causes a slow increase of inhibition above 1.0 vol.% (Fig. 6) and no stimulation at lower concentrations. The calculated I_{50} values are about 0.5 vol.% for the two WSDs and 1.3 vol.% for the ESD.

From the xanthine oxidase data we conclude, that in addition to a low inhibition of enzyme activity

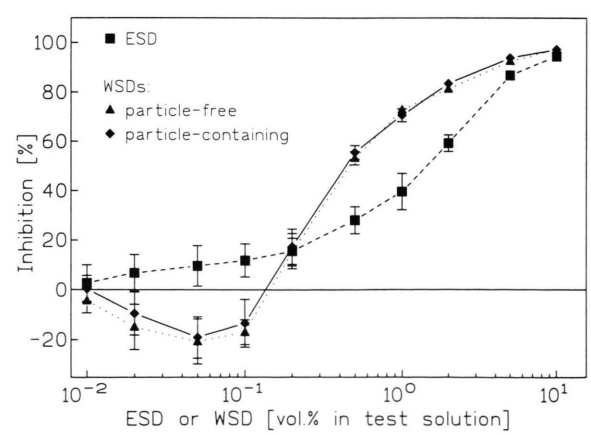

Fig. 6. Influence of the different preparations of propolis on xanthine oxidase-driven fragmentation of ketomethylthiobutyric acid (the ethylene release of the 100% reaction ranges between 5000 and 5500 pmol per assay).

(oxygen uptake) especially the WSDs contain more effective superoxide scavengers, inhibiting hydroxylamine oxidation, than the ESD. The stimulation of xanthine oxidase-dependent fragmentation of KMB by low concentrations of the water-soluble extracts may be due to their content of Fe ions and corresponding "activating" chelators. This hypothesis is supported by the fact that the xanthine oxidase-catalyzed reaction is strongly stimulated by the addition of EDTA and Fe ions catalyzing a "Haber-Weiss" type OH· radical formation [20–23]. At higher concentrations of the water-soluble derivatives, the scavenging of $O_2^{\cdot -}$ strongly inhibits OH· radical formation.

Effects of different propolis preparations on peroxidase-catalyzed indole acetic acid oxidation

In the absence of H_2O_2 peroxidase functions like an "oxidase" with indole acetic acid (IAA) as electron donor. This reaction is stimulated by certain monophenols and inhibited by diphenols [24]. The three extracts of propolis interact with IAA oxidation in a characteristic manner: in low concentrations a slight stimulation of IAA oxidation is observed, but at a certain concentration – very spe-

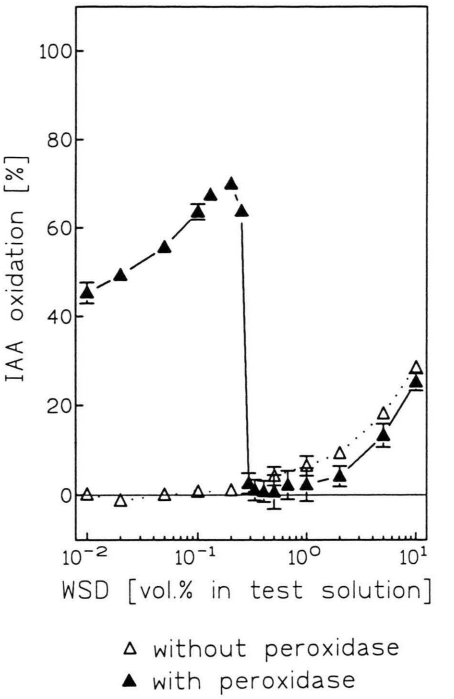

Fig. 7b. Effect of the particle-free WSD on IAA oxidation with and without peroxidase.

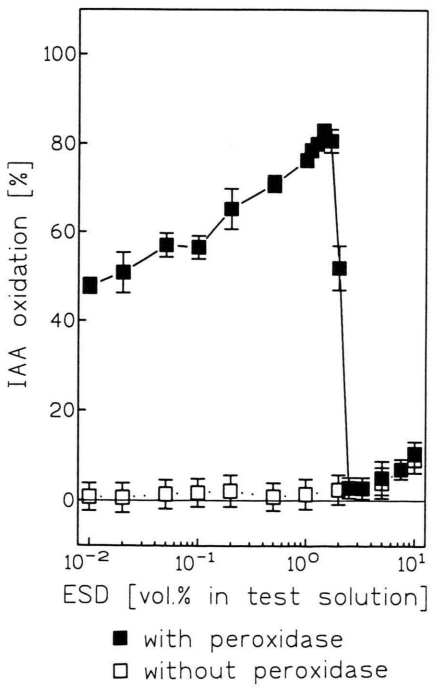

Fig. 7a. Effect of the ESD on IAA oxidation with and without peroxidase.

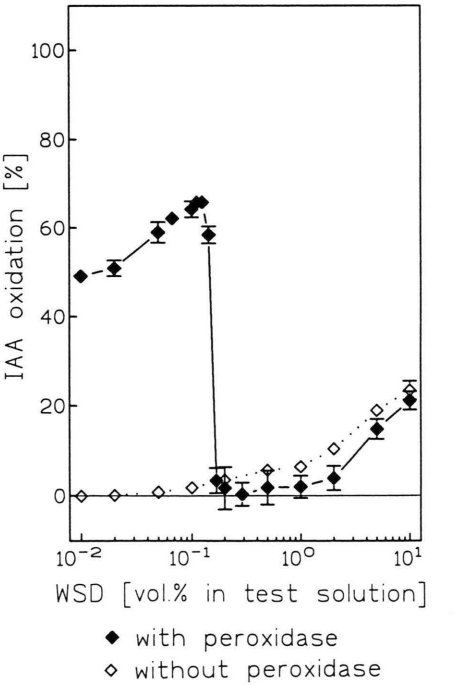

Fig. 7c. Effect of the particle-containing WSD on IAA oxidation with and without peroxidase.

cific for the individual preparation – the stimulation is abruptly converted into almost 100% inhibition of IAA oxidation (Fig. 7 a – c). The 100% value of IAA oxidation corresponds to *ca.* 0.3 E.

As shown in Fig. 7 a, the rapid onset of inhibition by the ESD occurs between 1.9 and 2.1 vol.% in the reaction mixture. In the case of the particle-free WSD, the decline of oxidation occurs between 0.25 and 0.29 vol.% (Fig. 7 b) and in the case of the particle-containing WSD already between 0.14 and 0.16 vol.%. Thus, this sensitive reaction allows a simple and reliable standardization and differentiation of different propolis extracts.

Conclusions

Relevance of the presented test systems and their modification by extracts of propolis for biological activities and potential drug functions

a) Diaphorases are ubiquitous in all living cells and function as redox catalysts between NAD(P)H and different acceptors. Naturally they reduce iron chelates and are therefore responsible for the vital iron sequestration by microorganisms. The inhibition of Fe^{3+} reduction may thus reflect one possible mode of function of propolis derivatives, especially the water-soluble extracts. Furthermore, the inhibition of the KMB-coupled NADH-diaphorase-juglone reaction clearly reflects the free radical scavenging activities of components [22] present in both ESD and WSDs.

b) Xanthine oxidase is derived from xanthine dehydrogenase under ischemic or inflammatory conditions [20, 21, 23] in different cell types such as endothelial cells. The products of its oxygen-reducing capacity are similar to the ones produced by activated leukocytes and thus responsible for oxidative damage in the corresponding tissues [19 – 21, 23]. Inhibition of hydroxylamine oxidation, a reaction indicative for superoxide-scavenging activities, can be shown especially for the water-soluble derivatives of propolis (Fig. 5). This scavenger function may also reflect a possible mode of action *in vivo i.e.* antiinflammatory activities.

The utility of the presented model reactions for standardization of propolis derivatives

Clear differences are obtained between the activities of the different preparations of propolis (for an exception see Fig. 2).

The best and simplest way to standardize propolis may be presented by the peroxidase-dependent IAA oxidation (Fig. 7) and the NADH-diaphorase-Fe^{3+} reaction (Fig. 4), where at least a difference of one order of magnitude of the concentrations necessary for 50% inhibition of the corresponding reaction exists between ESD and WSDs. Especially the sensible peroxidase reaction, reflecting a sophisticated balance between the presence of monophenolic and diphenolic compounds (especially representing $C_6 - C_3$ cinnamic acid derivatives) and thus different types of antioxidants, may be valuable for this standardizing purpose. This may represent an advantage to just determining one or two major "leading" ingredients, irrespective of their biological activities or functions.

Comparison of the aqueous extracts with the ethanolic extract of propolis

The basis of the presented data indicates the medical superiority of the WSDs as compared to ESD, due to a) their multifunctional properties as both enzyme inhibitors and radical scavengers; b) the lack of ethanol in the drugs and thus the possibility of an oral administration especially in pediatrics or in ethanol-sensitive patients and c) the lack of producing allergic reactions indicated in some cases for the ESDs [26].

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