Assessment by Survival Analysis of the Radioprotective Properties of Propolis and Its Polyphenolic Compounds

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The radioprotective effects of propolis and polyphenolic compounds from propolis on the radiation-induced mortality of mice exposed to 9 Gy of γ-irradiation were studied. Intraperitoneal (i.p.) treatment of mice at doses of 100 mg kg⁻¹ body weight of propolis (water or ethanolic extract; WSDP or EEP) or its polyphenolic compounds (quercetin, naringin caffeic acid, chrysin) consecutively for 3 d before irradiation, delayed the onset of mortality and reduced the symptoms of radiation sickness. All test compounds provided protection against hematopoietic death (death within 30 d after irradiation). The greatest protection was achieved with quercetin; the number of survivors at the termination of the experiment was 63%. According to statistical analyses by the Kaplan–Meier method and the log-rank test, a significant difference between test components and control was found (p<0.001). Treatment with test components after lethal irradiation was ineffective. These results suggest that propolis and its polyphenolic compounds given to mice before irradiation protect mice from the lethal effects of whole-body irradiation.

Key words radioprotection; propolis; polyphenolic compound; mice

Ionizing radiation is any electromagnetic wave or particle capable of producing ions in its passage through the matter, causing immediate chemical alterations in biological tissues. These alterations produce a metabolic disarrangement which after days or weeks can lead either to cell damage or ultimately to cell or organism death. Ionizing radiation damage is caused by either a direct interaction with target molecules or indirect action by formation of chemically and pharmacologically active elements produced mainly by water molecules. Water radiolysis generates molecules of hydrogen peroxide (H₂O₂), molecular hydrogen (H₂), and a number of highly active radicals such as hydrogen radical (H·), hydroxyl radical (OH·), hydroperoxyl radical (HO₂·), and superoxide anion radical(O₂⁻); all together free radicals and reactive oxygen species (ROS). These molecules cause radiation injury to living cells, to a large extent, due to oxidative stress.1,2 Chemical alterations of nucleic acids such as breaks in the hydrogen bonds, breaks in base-sugar bonding, sugar oxidation, breaks in nucleotide strands, and release of terminal phosphates are caused by reaction of free radicals; all of these are followed by single-strand breaks of DNA that undergo repair processes relatively easily3 and double-strand breaks that cause more serious consequences. Double-strand breaks are closely correlated with the cytotoxic effects of ionizing radiation and are considered to be the primary lesion involved in cellular death.4 If DNA repair mechanisms, which are induced after exposure to ionizing radiation, are inefficient, the damaged DNA strands that are copied during replication lead to mutagenesis and/or carcinogenesis.5 The damaging effects of ionizing radiation lead to the cell death and are associated with an increased risk for numerous genetically determined diseases.6,7

Exposure of mammals to ionizing radiation causes the development of a complex, dose-dependent series of potentially fatal physiologic and morphologic changes, known as hematopoietic syndrome.8 Radiation induced destruction of lymphoid and hematopoietic systems is the primary cause of septicemia and death. Enhanced susceptibility to infections with opportunistic microbes occurs in parallel with progressive radiation induced atrophy of lymph nodes, spleen, and bone marrow.9 It has been suggested that a great deal of the biological activities of propolis are mainly mediated by the presence of flavonoids in it.10—13 Flavonoids are reported to induce activities of the immune system.11,13—16 One important effect of flavonoids that has been reported is oxygen radical scavenging.17,18 Several studies have confirmed the role of flavonoids in the deactivation of free radicals,19—21 however, there is a paucity of data on the effects of propolis and its polyphenolic compounds in protection from whole-body irradiation.5 It was also demonstrated that propolis and some of its active substances have pronounced antimicrobial, cytostatic, anticarcinogenic, and antitumor effects both in "in vitro" and "in vivo".22—29 Since the increased hematopoietic activity could account for the improved hematopoietic tolerance to radiotherapy,30,31 data on the influence of propolis and polyphenolic components from propolis on radioprotection could shed more light on this problem.

Here, we report the radioprotective ability of two preparations of propolis and several of its polyphenolic components in mice exposed to an acute whole-body gamma radiation dose of 9 Gy using survival studies for the observation of total body injury at the organism level.

MATERIALS AND METHODS

Animal Studies Animal studies were carried out according to the guidelines in force in the Republic of Croatia (Law...
on the Welfare of Animals, N. N. #19, 1999) and in compliance with the Guide for the Care and Use of Laboratory Animals, DHHS (NIH) Publ. # 86-23 (1986). Male and female CBA inbred mice from our conventional animal facility were used. In all experiments, the mice were three months old, approximately 20 g body weight at initiation of the experiment, and were housed at 22 ± 1°C and 50—70% humidity with a 12/12 h light/dark cycle photoperiod. All animals were maintained on a standard diet (4 RF 21, Mucedola s.r.l., Italy and water ad libitum.

Irradiation Whole-body irradiation (WBI) was performed with a cobalt-60 γ-radiation source (Teratron 780, Canada). Five to six mice were placed in Plexiglass cages and irradiated simultaneously. The source-to-skin distance was 291 cm with a dose rate of 0.0233 Gy/s at room temperature (23 ± 2°C). Mice were irradiated with a total dose of 9 Gy; the duration of irradiation was 390 s.

Water-Soluble Derivative of Propolis (WSDP) Preparation and Treatment A WSDP was prepared by a method described elsewhere.

Briefly, Croatian propolis from bees was kept in the outskirts of Zagreb, Croatia was extracted with 96% ethanol and the extract was filtered and evaporated to dryness in a vacuum evaporator. The resultant resinous product was added to a stirred solution of 8% L-lysine (Sigma Chemie, Deisenhofen, Germany) and freeze-dried to yield the WSDP, a yellow-brown powder. The WSDP was stored under sterile conditions at −20°C to minimize bacterial contamination. Before use the WSDP was dissolved in distilled water. The solutions were filtered through Whatman paper No. 1. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. The coefficient of determination was r² = 0.9984. The flavones and flavonols were calculated as a percentage of crude, lyophilized propolis extracts.

Reagents and Solvents Ethanol, methanol, potassium acetate, potassium hydroxide and sodium carbonate used in the studies were of analytical grade and were purchased from Kemika (Croatia). AlCl₃ and 2,4-dinitrophenyldiazine purchased from Merck (Germany) were also of analytical grade.

Instruments The measurements were carried out using a UV 160 UV–Visible spectrophotometer diode-array (Shimadzu, Japan).

Determination of Flavones and Flavonols in Propolis Samples Flavones and flavonols were determined by an aluminium chloride method described elsewhere. Flavones and flavonols in propolis were expressed as quercetin (3,3′,4′,5,7-pentahydroxyflavone-dehydrate) equivalent. Quercetin-dihydrate (Sigma, Germany) was used to make the calibration curve with five standard solutions ranging from 12.5 to 100 μg ml⁻¹ in 80% ethanol (v/v). Propolis extracts (0.5 ml) were mixed with 1.5 ml of 95% ethanol (v/v), 0.1 ml of 10% aluminum chloride (m/v), 0.1 ml of 1 mol l⁻¹ potassium acetate, and 2.8 ml of water. The volume of 10% (m/v) aluminum chloride was substituted by the same volume of distilled water in blanks. The solutions were filtered through Whatman paper No. 1. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. The coefficient of determination was r² = 0.9984. The flavones and flavonols were calculated as a percentage of crude, lyophilized propolis extracts.

Determination of Flavanones and Dihydroflavonol in Propolis Sample Flavanones were analyzed using the 2,4-dinitrophenylhydrazine method described elsewhere. Flavanones in propolis were expressed as the naringenin (3,3′,4′,5,7-pentahydroxyflavone-dehydrate) equivalent. Naringenin (Sigma, Germany) was used to make the calibration curve with five standard solutions ranging from 0.25 to 2.00 mg ml⁻¹ in methanol. One milliliter of each propolis solution was separately mixed with 2 ml of 1% 2,4-dinitrophenyldiazine (m/v) and 2 ml of methanol at 50°C in a water-bath for 50 min. After cooling at room temperature, the solution was mixed with 5 ml of 1% potassium hydroxide (m/v) in 70% ethanol (v/v). The volume of 1% (m/v) potassium hydroxide (m/v) in 70% ethanol (v/v) was substituted by the same volume of distilled water in blanks. Then, 1 ml of the mixture was filtered twice through Whatman No. 4 and 1 filter papers. The filtrate was adjusted to 25 ml. The absorbance of
the filtrate was measured at 495 nm. The coefficient of determination was \( r^2 = 0.9989 \). The flavanones and dihydroflavonols were calculated as a percentage in crude, lyophilized propolis.

**Total Flavonoid Content in Propolis Samples**

Total flavonoid content was calculated as the sum of the two complementary spectrophotometric methods for determination of flavones/flavonols and flavanones/dihydroflavonols according to previous recommendations. \(^3^4,^3^5\) The sum of the results represents the real content of total flavonoids in propolis samples, and was expressed as a percentage in crude, lyophilized propolis.

**Determination of Total Polyphenols in Propolis Sample**

Total polyphenol contents in propolis extracts were determined by the Folin–Ciocalteau colorimetric method. \(^1^8\) Total polyphenol contents expressed as the tannic acid (galloptannin) equivalent. Propolis extracts (0.5 ml) were mixed with 0.5 ml of the freshly diluted Folin–Ciocalteau reagent (Kemika, Croatia) with distilled water (50:50, v/v), and 0.5 ml of 10% (m/v) sodium carbonate was added. The absorbance was measured at 760 nm after 1 h incubation at room temperature. Tannic acid (Merek, Germany) was used for building calibration curves with five different concentrations of tannic acid ranging from 1.70 to 6.81 mg/g. Total polyphenol content is expressed as a % (m/m) of the tannic acid equivalent.

**Experimental Procedure**

In two experiments performed in the study groups consisting of 11 mice each were treated intraperitoneally (i.p.) with test components and thereafter irradiated to the whole-body with gamma source (WBI). One hour after treatment, all mice were returned to the animal facility; they were inspected twice daily, at least 6 h apart for morbidity and mortality. Experiments were terminated on day 60th after treatment.

**Survival Analysis**

For survival analysis, the mice were treated with test components (100 mg kg\(^{-1}\) body weight) for 3 consecutive days, before or after lethal WBI. The endpoint of an experiment defined either by spontaneous death of an animal or by elective killing of an animal showing signs of pain or suffering according to established criteria. Results are expressed as a percent of mean survival time of the treated animals over mean survival time of the control group (treated vs. control, \( T/C \)). The percentage of increased lifespan (ILS\%) was calculated according to the formula: ILS\% = \((T−C)/C\times100\) where \( T \) represents mean survival time of the treated animals and \( C \) is the mean survival time of the control group.

**Statistical Analysis**

Treatment-dose specific survival curves were calculated according to the Kaplan–Meier method and log rank test; the survival curves were compared using STATA 7.0 statistical software (Stata Press, College station, Texas, U.S.A.).

**RESULTS**

**Radioprotective Ability of Two Preparations of Propolis and Its Polyphenolic Compounds**

The radioprotective effects of two preparations of propolis (WSDP or EEP) and their polyphenolic compounds (caffeic acid, naringin, chrysin, quercetin) were studied in the lethally whole-body irradiated mice; test substance was administered to mice i.p. at a dose of 100 mg kg\(^{-1}\) body weight, for 3 consecutive days, before or after WBI. Administration before WBI significantly increased the survival time of mice (Table 2, Fig. 1). Table 2 shows the number of long-term surviving mice and increased life span (ILS\%) in all groups treated with a test substance before or after lethal WBI. Mean survival time of the irradiated control groups ranged from 5—14 d. In the groups treated with WSDP, EEP or chrysin, which received WBI, 13.63, 18.18 and 22.72% of the mice survived throughout the observation period; in these groups ILS\% was increased (94.82, 58.37 or 89.98). Long-term survivor rates in the groups treated with caffeic acid, naringin or QU were 27.27%, 40.90%, or 63.60%. Statistical analyses revealed a significant difference between the treated and control groups (\( p < 0.001 \)) (Fig. 1). The effect on long-term survival of mice achieved with WSDP treatment was similar to that achieved by EEP and could be explained by the almost equal contents of polyphenolic compounds (Table 1) in both preparations.

Treatment with test compounds after irradiation was ineffective considering survival time (data not shown), as expected.

**DISCUSSION**

These studies showed statistically significant differences in the survival times of WBI mice pretreated with test compounds as compared with control (solvent: H\(_2\)O or ethanol). The most effective compound regarding survival of mice was QU, showing protection similar to that achieved by the AET; such a huge protective effect of QU could result from its chemical structure, which consist at the most suitable form for scavenging free radicals. \(^3^6,^3^7\) All other polyphenolic compounds were also effective in protection against radiation induced damage as well as propolis preparations (EEP and WSDP); the similarity in radiation protection between WSDP and EEP could be explained by higher contents of polyphenols present in both WSDP and EEP preparations, as shown in Table 1.

A single whole-body exposure to ionizing radiation results in a complex set of symptoms whose onset, nature, and severity are a function of both total radiation dose and radiation quality. \(^3^8\) The hematopoietic syndrome occurs at a dose 2.5—8 Gy; it is manifested by hematopoietic stem cell depletion, and ultimately by depletion of mature hematopoietic and immune cells. \(^3^9\) In this study, the pathological cellular emptiness of bone marrow and spleen in mice which died on day 5 after lethal WBI indicated that their deaths could have been prescribed to the hematopoietic syndrome. Administration of several immunomodulators including propolis and propolis derived compounds has been shown to stimulate hematopoietic recovery and enhance the survival of irradiated animals. \(^8^,^1^5,^1^6,^2^5—^2^7,^2^9,^3^1\) Dimov et al. \(^2^5,^2^6\) observed that oral and parenteral administration of WSDP enhanced the survival rate and the mean survival time in experimental bacterial and fungal infections in normal and immunodepressed mice. The authors suggested that the broad therapeutic spectrum of propolis includes a pronounced immunomodulatory activity directed mainly toward augmenting of non-specific anti-infectious resistance via macrophage activation. Our recent findings imply that the antitumor activity of WSDP and polyphenolic compounds of propolis enhanced host resist-
Fig. 1. The Kaplan–Meier Survival Curves for Mice (n=22) Treated with WSDP (A), EEP (B), Quercetin (C), Caffeic Acid (D), Chrysin (E), Naringin (F) and AET (G) before Irradiation

The test compounds were given to mice i.p. daily for 3 consecutive days, and the daily dose contained 100 mg/kg body weight. 1 h after the last treatment, mice were exposed to an acute whole-body gamma radiation dose of 9 Gy. The results of log rank test show that test compounds significantly reduced the total body injury at the organism level (p<0.001) and increased the life span of the mice after irradiation at a dose of 9 Gy.
ance in the Ehrlich ascites tumor model, increasing the activities of macrophages, cytotoxic T cells, B cells and NK cells as well. Moreover, WSDP stimulated peritoneal macrophages to produce IL-1, serving as a differentiation-and maturation-inducing agent for a variety of cells. There are also indications that IL-1 could serve as a signal that initiates radioprotective events in vivo. Our recent observations and those by others proved the protective effect of propolis on bone marrow and lymphoid tissues of mice to cytotoxic drugs and radiation. Augmented immunological activity as seen in increased activity of macrophages, cytotoxic T cells, B cells and NK cells by propolis and related compounds seems to play a central role in preventing secondary infections associated with irradiation, contributing to further acceleration of hemopoietic regeneration and increasing survival following radiation-induced lympho- and myelo-suppression.

Our unpublished results have shown that pretreatment with flavonoid compounds, both in vitro and in vivo, could protect human and mouse blood lymphocytes from gamma radiation-induced genetic damage (manuscript in preparation). The exact mechanism of action in protecting mice from the lethal effects of acute whole-body irradiation by propolis and related flavonoids is not known. In addition to modulation of immunohematopoiesis, scavenging of radiation-induced free radicals could also be one of the important mechanisms of radiation protection by test components, which was confirmed by Chen et al. who showed that propolis and related flavonoids exercise their activity through the scavenging of hydroxyl, superoxide free radicals, and lipid peroxides. The antioxidant activities of propolis and its polyphenolic/flavonoid components are related to their ability to chelate metal ions and to scavenge singlet oxygen, superoxide anions, peroxyl radicals, hydroxyl radicals and peroxynitrite. Jeon et al. showed that flavonoids from propolis elevate catalase, superoxide dismutase and glutathione peroxidase mRNA synthesis; the elevation of these enzymes by flavonoids was considered to be responsible for the observed protection against radiation-induced damage. By increasing the activities of antioxidant enzymes, flavonoids from propolis reduce the number of free radicals and ROS and increase the production of molecules capable of protecting against oxidative stress. It is possible that propolis and its polyphenolic/flavonoid components may influence the survival of the WBI mice via increased activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione (GSH) through the free radical scavenging ability, tissue regeneration properties, and immunostimulatory effects. It is likely that more different co-operative and synergistic mechanisms of propolis and its polyphenolic compounds are included in the protection of the whole organism against radiation.

Since the test components showed effectiveness in radioprotective studies, it appears that propolis and its related compounds should be considered for protection against radiation in humans. These results suggest that propolis and its polyphenolic compounds may be a promising adjunct treatment for patients exposed to radiation as well as to a hazardous radiation environment.

### Table 1. Contents of Flavonoids and Polyphenols in Propolis Extracts

<table>
<thead>
<tr>
<th>Extract of propolis</th>
<th>Flavone and flavanol content (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Flavanone and dihydroflavonol content (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Total flavonoids (%)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Total polyphenols (%)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanolic extract (EEP)</td>
<td>1.60</td>
<td>38.60</td>
<td>40.20</td>
<td>84.40</td>
</tr>
<tr>
<td>Water extract (WSDP)</td>
<td>2.13</td>
<td>9.06</td>
<td>11.19</td>
<td>70.48</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed as quercetin equivalent in lyophilized crude extract.  
<sup>b</sup> Expressed as naringenin equivalent in lyophilized crude extract.  
<sup>c</sup> Sum of the flavone/flavanol and flavanone/dihydroflavonol content in lyophilized crude extract.  
<sup>d</sup> Expressed as tannic acid equivalent in lyophilized crude extract.

### Table 2. Radioprotective Ability of Propolis and Its Polyphenolic Compounds

<table>
<thead>
<tr>
<th>Group&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Mean survival time in days (Range)</th>
<th>ILS%</th>
<th>T/C%</th>
<th>Long-term survivors (LTS %)</th>
<th>Mean survival time in days (Range)</th>
<th>ILS%</th>
<th>T/C%</th>
<th>Long-term survivors (LTS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.27 (5—13)</td>
<td></td>
<td></td>
<td></td>
<td>9.63 (7—13)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent (H2O)</td>
<td>9.09 (5—12)</td>
<td></td>
<td></td>
<td></td>
<td>9.40 (9—12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent (ethanol)</td>
<td>10.09 (6—14)</td>
<td></td>
<td></td>
<td></td>
<td>10.04 (6—12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AET</td>
<td>16.94 (13—27)</td>
<td>94.82</td>
<td>194.82</td>
<td>3 (13.63)</td>
<td>16.94 (13—27)</td>
<td>94.82</td>
<td>194.82</td>
<td>3 (13.63)</td>
</tr>
<tr>
<td>EEP</td>
<td>13.00 (7—21)</td>
<td>44.60</td>
<td>140.60</td>
<td>6 (27.27)</td>
<td>13.00 (7—21)</td>
<td>44.60</td>
<td>140.60</td>
<td>6 (27.27)</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>17.08 (10—27)</td>
<td>89.98</td>
<td>189.98</td>
<td>5 (22.72)</td>
<td>17.08 (10—27)</td>
<td>89.98</td>
<td>189.98</td>
<td>5 (22.72)</td>
</tr>
<tr>
<td>Chrysin</td>
<td>13.0 (11—17)</td>
<td>44.60</td>
<td>144.60</td>
<td>9 (40.90)</td>
<td>13.0 (11—17)</td>
<td>44.60</td>
<td>144.60</td>
<td>9 (40.90)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>15 (12—19)</td>
<td>48.66</td>
<td>148.66</td>
<td>14 (63.60)</td>
<td>15 (12—19)</td>
<td>48.66</td>
<td>148.66</td>
<td>14 (63.60)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mice (n=22) were exposed to an acute whole-body gamma radiation dose of 9 Gy; test compounds were given to mice i.p. before or after irradiation, daily for 3 consecutive days, and the daily dose contained 100 mg kg<sup>-1</sup> body weight. T/C, treated vs. control. ILS% (increased life span %)=(T—C)/C×100. T, mean survival days of treated group; C, mean survival days of control group. LTS, long-term survivors; mice surviving more than 60 d after treatment.

### REFERENCES