# STIMULATION OF HEMATOPOIESIS BY A WATER - SOLUBLE DERIVATIVE OF PROPOLIS IN MICE

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**Summary**

We have investigated the effect of water - soluble derivative of propolis (WSDP) on hematopoiesis of CBA mice using exogenous spleen colony assay (CFUs assay). Given perorally (po) 50 mg / kg to mice for 20 consecutive days WSDP increased the number of exogenous CFUs as compared to control. WSDP given either for 20 or 40 days elevated the number of cells in hematopoietic tissue and increased the number of leucocytes in peripheral blood; prolonged treatment with WSDP also elevated histological appearance of myeloid and megakaryocytic types of CFUs.

Key words: Colony forming units (CFUs), Hematopoiesis, Mice, Propolis,

Propolis (bee glue) is the generic name for the resinous substance collected by honey bees from various plant sources (1). It is rich in biochemical constituents, including mostly a mixture of polyphenols, flavonoid aglycones, phenolic acid and their esters, and phenolic aldehydes and ketones, terpenes, sterols, vitamins, amino acids, etc (2). There have been many attempts to validate biological effects of propolis and elucidate its composition (3-8). It was shown that propolis and its constituents have strong antimicrobial effect, acting on viruses, bacteria and fungi (3). It was also demonstrated that propolis and some of its active substances have a pronounced cytostatic, anticarcinogenic and antitumor effect both, in “in vitro” and “in vivo” tumor models (4-7). Immunomodulatory effects of propolis have also been recorded (4-8). Since the increased hematopoietic activity could account for the improved hematopoietic tolerance to chemotherapy, at least to the cell - cycle nonspecific chemotherapeutic agents (9), data on the influence of propolis on hematopoiesis could shed more light on this problem. Here, we report the ability of water - soluble derivative of propolis (WSDP) to influence hematopoietic activity in mice.

## Material and methods

***Mice:*** We used CBA mice bred at our conventional animal facility. They were three months old, approximately 20 g body weight at the initiation of the experiment, and were maintained at $20^0$C and at 12L : 12 D photoperiod with a free access to food and water. All studies were carried out according to the guidelines in force in the Republic of Croatia (Law on the Welfare of the Animals; Narodne Novine No. 19, 1999) and in compliance to the Guide for the Care and Use of Laboratory Animals DHHS Publ. No. (NIH) 86 - 23.

***Propolis:*** A water - soluble derivative of propolis (WSDP) was prepared from the crude resin. The method of preparation was described previously (7). Briefly, crude propolis was dissolved in 96% ethanol with constant stirring overnight at room temperature. Ethanol was evaporated in vacuum and semisolid ethanol extract was slowly poured into the stirring solution of 5% solution of L - lysine at room temperature. After two hours, the solution was filtered and vacuum - evaporated to dryness. The resulting WSDP was bright yellow, crystalline powder. The WSDP was stored at room temperature until use. Mice were given WSDP *per os* (*po)* via gastric tube. The WSDP was given daily for 20 and 40 days respectively, and the daily dose contained 50 mg/kg body weight.

***Spleen hematopoietic colonies:*** The formation of exogenous hematopoietic colonies in the spleen (CFUs) was induced by method described elsewhere (10, 11). Briefly, 21 or 41 days after the initiation of the experiment, mice were intravenously (*iv)* injected with 50 IU of heparin (Sigma Chemical Company, Deisenhofen). Within 10 - 15 minutes, groups of mice were anesthetized, exsanguinated from axillary blood vessels and the number of white blood cells (WBC) was determined using a hemacytometer. Spleens were removed, passed through nylon mesh and syringed in and out through #20 gauge needles. The resultant cells suspension was washed twice by centrifugation in Hanks balanced salt solution. Bone marrow from each mouse was washed out from the shaft of femur. The cell viability of both bone marrow and spleen cell suspensions were determined by Trypan Blue exclusion assay and were found to be above 90%. The recipient mice were whole - body irradiated (WBI) with 9 Gy using a $^{60}$Co Gy ray source. After irradiation each recipient group was *iv* injected with either bone marrow, or spleen or whole blood from either normal or WSDP treated mice at concentrations 5 x $10^4$, 5 x $10^5$, and 5 x $10^6$ cells, respectively. Exogenous CFUs was determined 9 days following the irradiation; mice were killed their spleens removed and placed in Bouin solution for 24 hours. The hematopoietic colonies on the surface of the spleen were counted under a dissecting microscope. For histological examinations, spleens were fixed as described above, mounted using standard histological procedures and stained with hematoxylin - eosin.

The 5 µm slides were examined under the light microscope, and the presence of different types of hematopoietic colonies was determined using criteria described elsewhere (10).

***Blood cell counts:*** From the initiation of the experiment every seven days apart, WSDP - treated mice were bled from a tail vein and the number of erythrocytes, leukocytes, and thrombocytes was determined. Blood smears were prepared and stained by May Grünwald - Giemsa method.

## Results

To test the effect of WSDP on hematopoiesis, mice were given WSDP peroraly (*po*) at dose of 50 mg / kg for 40 consecutive days. WSDP greatly increased the spleen weight and cellularity of the spleen and bone marrow (Table 1). The increase occurred 3 days after treatment and was evident throughout the observation period of 40 days.

**Table 1.** Effect of the WSDP on the spleen weight and cellularity of the spleen and bone marrow in CBA mice

| Days after treatment with the WSDP [b] | Spleen weight (mg) [a] | Spleen cellularity ($\times 10^6$) [a] | Bone marrow cellularity ($\times 10^6$)[a] |
|---|---|---|---|
| No treatment | 58.3 ± 6.1 | 104.8 ± 9.1 | 9.5 ± 1.8 |
| 3 | 99.1 ± 7.2 | 191.4 ± 14.4 | 11.1 ± 2.2 |
| 7 | 122.5 ± 12.1 | 235.9 ± 12.1 | 12.2 ± 1.9 |
| 14 | 171.4 ± 13.7 | 281.2 ± 15.7 | 14.7 ± 1.8 |
| 21 | 272.2 ± 12.8 | 252.1 ± 9.2 | 13.1 ± 2.3 |
| 28 | 268.8 ± 14.7 | 237.9 ± 13.1 | 13.9 ± 1.6 |
| 34 | 241.2 ± 9.9 | 241.6 ± 11.7 | 15.2 ± 1.4 |
| 40 | 211.1 ± 11.7 | 211.7 ± 9.2 | 11.9 ± 1.7 |

[a] Mean ± SD. [b] 50 mg / kg WSDP was given po for 40 consecutive days. Groups comprised 7 - 10 mice each.

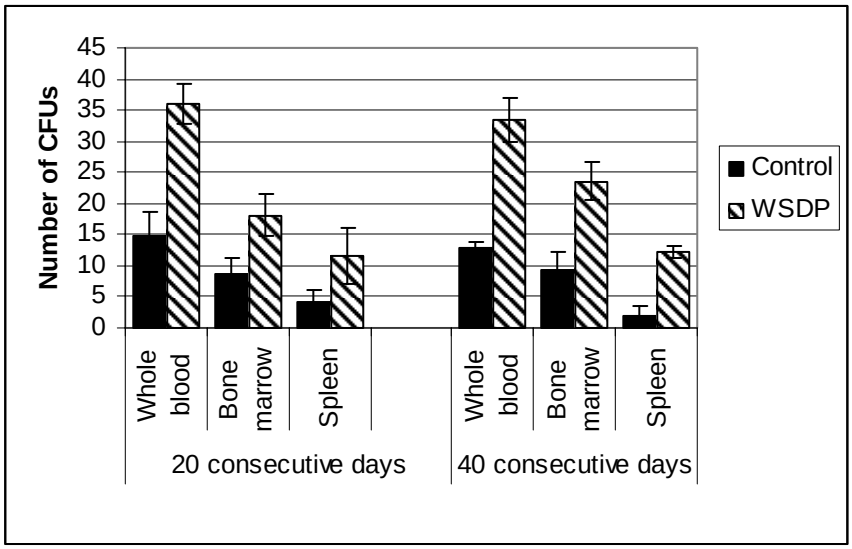


**Figure 1.** Number of CFUs (mean ± SD) in the whole blood cells ($5 \times 10^6$) bone marrow cells ($5 \times 10^4$) or spleen cells ($5 \times 10^5$) of normal or mice treated with WSDP. Mice were given WSDP throughout either 20 or 40 consecutive days. Groups comprised 7 - 8 mice each.

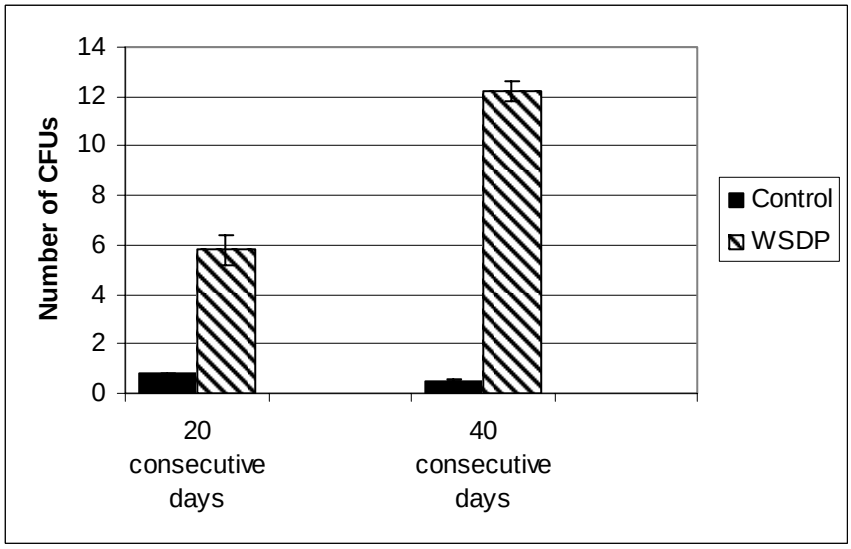


**Figure 2**.Calculated total number of CFUs (mean ± SD) in the spleen on the basis of cellularity and CFUs setting efficiency in the spleen of whole - body irradiated mice (f value) of 17 % (12). Mice were given WSDP throughout either 20 or 40 consecutive days. Groups comprised 7 - 8 mice each.

To test whether WSDP causes an increase in the number of CFUs in hematopoietic tissue of treated animals, mice receiving WSDP throughout either 20 or 40 consecutive days were killed and their whole blood, spleen, or bone marrow cell suspensions injected iv into 9 Gy WBI syngeneic recipients. Figure 1 shows that the number of CFUs originating from different sources of hematopoietic tissue of WSDP- treated mice was increased more than twofold as compared to control. However when the amount CFUs of the spleen was calculated on the basis of cellularity and CFUs setting efficiency (*f* value) the number of CFUs in the WSDP was increased by 7 and 10 folds, respectively (Figure 2).

To determine whether WSDP affects the differentiation of CFUs, spleen of 9 Gy WBI mice receiving either whole blood, bone marrow or spleen cells from normal or WSDP-treated mice were histologically inspected for the presence of different types of exogenous spleen colonies. Histology of colonies revealed that WASP treatment, besides elevation of the number of CFUs (Figures 1 and 2) induced a pronounced increase of myeloid colony type in both 20 and 40 days treated mice; the only exception being spleen of mice treated with WSDP for 20 days (Table 2).The megakaryocytic type of colonies increased in all tissue from mice receiving WSDP for 40 consecutive days (Table 2).

## Discussion

The WSDP, given orally throughout period of 20 consecutive days induced the extensive proliferation of nucleated cells in the spleen and bone marrow (Table 1) which are mainly macrophages (6, 7) and hematopoietic cells (Figure 1, Table 2). Stimulated hematopoietic activity in WSDP-treated mice, as evidenced by the increased number of cells capable of producing hematopoietic colonies in the spleen of lethally irradiated recipients (Figure 1, Table 2), has also been evidenced in animals treated with other biological response modifiers such as *C. parvum* (13, 14). WSDP given to mice caused a significant elevation of the number of leucocytes in peripheral blood of treated animals (data not shown). WBI of animals with either 5 Gy or 7 Gy decreased the number of leucocytes in same proportion in normal and WSDP - treated mice respectively. However, the recovery of leucopenia was three times faster in treated mice, as compared to control (not shown), suggesting thus that proliferation of leukocyte precursors from pluripotent stem cells is increased in mice after treatment with WSDP. Concerning the specific type of CFUs (Table 2), the only difference between untreated and WSDP - treated was noticed in mice receiving treatment for 40 consecutive days; their spleen cells injected to WBI recipients gave rise to more myeloid and megakaryocytic colonies.

**Table 2.** The effect of WSDP on the formation of types of spleen colonies in lethally irradiated mice

| Days | 20 | | | | | 40 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Colony type | Erythroid | Myeloid | Mega karyocytic | Undifferentiated | Mixed | Erythroid | Myeloid | Mega karyocytic | Undifferentiated | Mixed |
| Control | 3.8 ± 1.9 [a] (44.2%) | 3.4 ± 0.5 (39.5%) | 0.9 ± 0.3 (10.5%) | 0.1 ± 0.08 (1.2%) | 0.4 ± 0.02 (4.6%) | 5.7 ± 1.8 (60.6%) | 2.6 ± 0.4 (27.6%) | 0.3 ± 0.18 (3.2%) | 0.6 ± 0.2 (6.4%) | 0.2 ± 0.1 (2.1%) |
| Bone marrow | 5.8 ± 0.6 (32%) | 9.3 ± 1.3 (51.4%) | 0.4 ± 0.02 (2.2%) | 2.0 ± 1.1 (11%) | 0.6 ± 0.38 (3.3%) | 6.2 ± 0.9 (26.3%) | 10.2 ± 1.1 [b] (43.2%) | 3.4 ± 0.6 [b] (14.6%) | 1.2 ± 0.3 (5%) | 2.6 ± 0.1 (11%) |
| Control | 1.8 ± 1.2 (43.9%) | 1.4 ± 0.3 (34.1%) | 0.3 ± 0.19 (7.3%) | 0.2 ± 0.04 (4.9%) | 0.4 ± 0.2 (9.7%) | 1.9 ± 1.0 (73%) | 0.3 ± 0.19 (11.5%) | 0.25 ± 0.18 (9.6%) | 0.08 ± 0.07 (3%) | 0.07 ± 0.06 (2.7%) |
| Spleen | 5.7 ± 2.3 (49.1%) | 3.8 ± 1.2 (32.7%) | 0.8 ± 0.2 (6.9%) | 1.0 ± 0.4 (8.6%) | 0.3 ± 0.2 (2.6%) | 6.0 ± 0.6 (49.2%) | 3.5 ± 0.12 [b] (28.6%) | 2.1 ± 0.24 [b] (17.2%) | 0.26 ± 0.04 (2.1%) | 0.34 ± 0.1 (2.8%) |
| Control | 10.4 ± 2.9 (70.7%) | 3.2 ± 0.2 (21.8%) | 0.5 ± 0.42 (3.4%) | 0.2 ± 0.18 (1.4%) | 0.4 ± 0.2 (2.7%) | 10.3 ± 0.4 (81.1%) | 2.1 ± 0.6 (16.5%) | 0.21 ± 0.14 (1.6%) | 0.06 ± 0.04 (0.5%) | 0.03 ± 0.02 (0.2%) |
| Whole blood | 16.0 ± 1.4 (44.4%) | 14.3 ± 1.1 (39.7%) | 0.8 ± 0.11 (2.2%) | 2.7 ± 0.29 (7.5%) | 2.2 ± 0.3 (6.1%) | 12.3 ± 2.8 (36.7%) | 14.4 ± 1.2 [b] (42.9%) | 4.3 ± 0.2 [b] (12.8%) | 0.7 ± 0.24 (2%) | 1.8 ± 0.06 (5.4%) |

[a] Mean ± SD
[b] $P < 0.05$ (Mann - Whitney U test)

Moreover, the increase in glutathione after the treatment with WSDP (15, 16) suggests that the elevation of glutathione level in "normal" cell line may also spare normal hematopoietic cells from the effect of irradiation. These data are in agreement with other (17, 18), suggesting that intracellular glutathione level may be implicated in the control of cell proliferation. More CFUs in hematopoietic tissues of mice as shown by exogenous spleen colony assay indicate that WSDP given continuously to mice *po* has stimulative effect on hematopoiesis.

Our results demonstrate that WSDP has stimulative and regenerative properties on hematopoiesis and suggest a clinically potential use of WSDP in treatment of various cytopenias induced by radiation and/or chemotherapy. In addition, these studies provided the first evidence that WSDP acts directly on hematopoietic bone marrow and spleen cells and enhances their growth and differentiation into colony forming cells.

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