Beneficial Effect of Pollen and/or Propolis on the Metabolism of Iron, Calcium, Phosphorus, and Magnesium in Rats with Nutritional Ferropenic Anemia

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There has been considerable debate regarding the nutritional benefits of pollen and the propolis produced by bees, although most contributions have lacked scientific soundness. This paper describes the possible beneficial effect of their use in pharmacological products in cases of anemic syndrome. We studied the effect of these two natural products on the digestive utilization of iron, calcium, phosphorus, and magnesium, using control rats and rats with nutritional ferropenic anemia. The addition of these products to the diet produced a positive effect on weight gain; this fact could constitute a scientific basis for the application of pollen and propolis as fortifiers. They improve the digestive utilization of iron and the regeneration efficiency of hemoglobin, especially during recovery from an anemic syndrome. They also have a positive effect on phosphocalcic metabolism and maintain an appropiate level of magnesium metabolism. Furthermore, in iron-deficient rats, these natural products palliate, to a large extent, the adverse effects of iron deficiency on calcium and magnesium metabolism as a result of the improvement in the digestive utilization of these minerals.

Keywords: *Pollen; propolis; iron; calcium; phosphorus; magnesium; rat; iron deficiency*

INTRODUCTION

All cells contain iron, which is of fundamental biological importance, being a micronutrient that is active in oxidative metabolism, cell growth, and reproduction, as well as in oxygen transportation (hemoglobin) and storage (myoglobin). Nevertheless, levels of iron are not always sufficient, because they are influenced by physiological and dietary factors (Beutler, 1988).

According to the WHO, iron deficiency is the second most serious problem in public health, only surpassed by protein-caloric deficiency (malnutrition), and is the main cause of nutritional anemia. Between 15 and 20% of the world's inhabitants are affected by this syndrome, with a greater incidence being observed in poor countries than in rich ones. Irrespective of the level of development, population groups at risk of nutritional anemia are women of fertile age, especially those pregnant or lactating, and young children (Campos Guerra, 1992).

The use of natural products, such as multiflora pollen and propolis, as coadjuvants and supplements to therapies based on pharmacological products could speed up recovery from ferropenic anemia; indeed, it is hoped such products might even prevent the appearance of the syndrome. Moreover, as anemia affects the immune system, leading to an increased risk of infectious disease, the treatment of this pathology with propolis, which is known to have antibacterial, antiviral, and fungicidal properties (Murray et al., 1997; Park et al., 1998; Eley, 1999), is worth investigating.

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Apian pollen, obtained from the hive, is highly nutritious and is made by bees from flower pollen. It is, in fact, a complex mixture of flower pollen and substances secreted by the bees. The main features of this pollen are, both its high protein and amino acid content and its richness in B-group vitamins (Chauvin, 1959). Moreover, it is a good source of dietary fiber and minerals (mainly potassium, calcium, and magnesium, with significant quantities of phosphorus, iron, sodium, and silica) and is particularly rich in linoleic and linolenic acid, which are essential fatty acids. It also contains enzymes, antibiotics, and bioflavonoids.

Propolis in its raw form is a heterogeneous substance with a large number of constituents, such as resins and balsams, waxes, ethereal oils, flavonoids, aromatic and phenyl acids, etc. The active agents within propolis seem to be the flavonoids, which present antibacterial, fungicidal, and local anesthetic effects. Some of them also possess spasmolytic, antiinflammatory, antioxidant, anti-ulcerous, and cytostatic properties (Kepcija et al., 1981; González and Orzáez, 1997).

The aim of this paper is to study the effects of these natural products on the metabolism of minerals (iron, calcium, phosphorus, and magnesium). Moreover, on the basis of the mineral interactions described by O'Dell (1997), it is logical to seek to determine how the metabolism of these minerals is affected because multiflora pollen and propolis are known to interact with iron, the deficiency of which is the cause of ferropenic anemia.

MATERIALS AND METHODS

Experimental Design. We studied the influence of two natural products, multiflora pollen and propolis, when these were included in the diet on the bioavailability and concentra-

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Table 1. Composition of the Normal Diet

component	g/kg of dry wt
protein (casein)	200
DL-methionine	3
fiber (micronized cellulose)	50
fat (olive oil)	50
mineral supplement[a]	35
vitamin supplement[b]	10
choline chloride	2
sucrose	500
wheatstarch	150

[a] The mineral supplement contained the following (g/kg): calcium phosphate dibasic ($CaHPO_4$), 500.0; sodium chloride (NaCl), 74.0; potassium citrate, monohydrate ($K_3C_6H_5O_7 \cdot H_2O$), 220.0; potassium sulfate (K_2SO_4), 52.0; magnesium oxide (MgO), 24.0; manganous carbonate (43−48% Mn), 3.5; ferric citrate (16−17% Fe), 6.0; zinc carbonate (70% ZnO), 1.6; cupric carbonate (53−55% Cu), 0.3; potassium iodate (KIO_4), 0.01; sodium selenite ($Na_2SeO_3 \cdot 5H_2O$), 0.01; chromium potassium sulfate [$CrK(SO_4)_2 \cdot 12H_2O$], 0.55; and sucrose, finely powdered, to make up to 1000 g (American Institute of Nutrition, 1977). [b] The vitamin supplement contained the following (g/kg): nicotinic acid, 3.0; calcium panthothenate, 1.6; pyridoxine hydrochloride (vitamin B_6), 0.7; thiamin hydrochloride, 0.6; folic acid, 0.2; D-biotin, 0.02; vitamin K (phylloquinone), 0.005 g/kg; vitamin B_{12} (cyanocobalamin) 0.001; and vitamin E, 50 UI; vitamin A, 4000 UI; vitamin D_3 (cholecalciferol) 1000 UI; and sucrose, finely powdered, to make up to 1000 g (American Institute of Nutrition, 1977).

tion of iron in the body (liver, femur, and sternum), together with the possible interaction with the metabolism of other minerals (calcium, phosphorus, and magnesium). An effective methodology was sought, and thus eight experiments were performed; four with iron-deficient rats and four with control rats. In each of the experiments, we determined the food intake, body weight, dietary concentration and fecal concentration of Ca, P, Mg, and Fe, hematologic parameters (hemoglobin regeneration efficiency, coefficient of apparent digestibility, etc.), biochemical parameters (iron, calcium, and phosphorus in serum, total bilirrubin), and the concentration of iron, calcium, phosphorus, and magnesium in the liver, femur, and sternum.

Diets. Four types of diet were used: standard diet, following the guidelines of the American Institute of Nutrition (AIN, 1977) (Table 1); standard diet plus 10 g of multiflora pollen/kg of diet; standard diet plus 1 g of propolis/kg of diet; and standard diet plus 10 g of multiflora pollen and 1 g of propolis/kg of diet. The quantities of multiflora pollen and propolis were calculated according to the weight of the animals, using the doses employed for humans as a reference.

Animals. The complete study consisted of eight experiments, carried out on 64 animals (white male rats; *Ratus norvergicus*, Wistar albino breed), with an initial weight of ∼45−60 g. The rats had been recently weaned and were obtained from the Laboratory Animal Service of the University of Granada. The animals were kept in individual metabolism cages, and fecal and urine samples were collected separately. The cages were located in a well-ventilated and temperature-controlled room (21 ± 2 °C), with 12-h periods of light and dark.

The rats of the control group were fed a semisynthetic "standard diet" prepared according to the recommendations of the American Institute of Nutrition (AIN, 1977). To obtain anemic rats, we used the technique of totally eliminating iron from the diet for a period of 40 days (Campos et al., 1998), which is the most suitable method to produce the syndrome experimentally; this diet, termed "Diet 0", comprised the same semisynthetic diet as the standard diet except for the absence of iron, and was administered for the same period. After 40 days, the Thomas-Mitchell biological technique (1923) was applied.

The Thomas-Mitchell biological technique was used between days 40 and 50. The first 3 days were for adaptation to the diet, and the remaining 7 days constituted the experiment itself. During this 10-days period, the corresponding diet was given to each animal. From day 43, we measured food intake and body weight and obtained samples of feces for subsequent analysis of the digestive utilization of the different minerals being studied. The quantity of food eaten by each rat was calculated from the amount provided less what was refused or spilled.

At the beginning and end of each experiment (days 40 and 50, respectively) and after a 12-h fast, an aliquot of venous blood was extracted into a tube with EDTA to determine the hematologic parameters. Before the animals were killed, they were anesthetized with sodium pentobarbital (5 mg/100 g bodyweight, administered intraperitoneally), and the abdominal aorta was cannulated. The total volume of blood was centrifuged to extract the serum, which was frozen at −30 °C until it was used to determine the various biochemical parameters. Subsequently, the liver, femur, and sternum were extracted and frozen for later determination of the mineral content, after sample preparation.

Experiment C_1: Standard Diet. The standard diet of ferric citrate was supplied both for the first 40 days and for the following 10 days.

Experiment D_1: Standard Diet. Diet 0 was supplied for 40 days, following which the animals received the standard diet for 10 days.

Experiment C_2: Standard Diet + Multiflora Pollen. The standard diet was supplied for 40 days, after which 10 g of multiflora pollen/kg of diet was added to the standard diet for an additional 10 days.

Experiment D_2: Standard Diet + Multiflora Pollen. Diet 0 was supplied for 40 days, after which the standard diet plus 10 g of multiflora pollen/kg of diet was supplied for an additional 10 days.

Experiment C_3: Standard Diet + Propolis. The standard diet was supplied for 40 days, after which 1 g of propolis/kg of diet was added to the standard diet for an additional 10 days.

Experiment D_3: Standard Diet + Propolis. Diet 0 was supplied for 40 days, after which 1 g of propolis/kg of diet was added to the standard diet for an additional 10 days.

Experiment C_4: Standard Diet + Multiflora Pollen + Propolis. The standard diet was supplied for 40 days, after which 10 g of multiflora pollen plus 1 g of propolis/kg of diet were added to the standard diet for an additional 10 days.

Experiment D_4: Standard Diet + Multiflora Pollen + Propolis. Diet 0 was supplied for 40 days, after which the standard diet plus 10 g of multiflora pollen and 1 g of propolis/kg of diet was supplied for an additional 10 days.

Biological Indices. The apparent digestibility coefficient (ADC) was calculated according to the following formula:

$$\text{percentage ADC} = \frac{\text{absorbed}}{\text{intake}} \times 100 \quad (1)$$

where nutrient absorption = intake−fecal excretion.

The hemoglobin regeneration efficiency (HRE) was calculated according to the following formula (Mahoney and Hendricks, 1982):

$$\text{HRE} = \frac{\text{mg hemoglobin Fe final} - \text{mg hemoglobin Fe initial}}{\text{mg Fe consumed}} \times 100 \quad (2)$$

$$\text{hemoglobin Fe (mg)} = \text{body wt (g)} \times \frac{\text{mL blood}}{\text{g body wt}} \times \frac{\text{g hemoglobin}}{\text{mL blood}} \times \frac{\text{mg Fe}}{\text{g hemoglobin}} \quad (3)$$

where mL blood/g body wt → (assumed 0.067 mL), and mg Fe/g hemoglobin → (assumed 3.35 mg).

Analytical Methods. The moisture content of the diet, feces, liver, femur, and sternum was determined by drying the samples to a constant weight at a temperature of 105 ± 2 °C. One or two grams of the resulting sample (in the case of diet, feces and liver) or the entire sample (femur and sternum) was

Table 2. Body Weight and Food Intake in Control and Iron-Deficient Rats Fed Different Diets (Mean Values with Standard Errors)

group	*n*	Body wt (g) At day 43 (initial wt)	Body wt (g) At day 50 (final wt)	wt change (g/rat/day)	food intake (g/rat/day)
C-standard	8	276 ± 6	307 ± 5	4.3 ± 0.2[a,b,c]	19.0 ± 0.5
D-standard	8	276 ± 8	294 ± 8	2.6 ± 0.2[d,e,f]	20.8 ± 0.6
C-standard + pollen	8	269 ± 7	313 ± 9	6.3 ± 0.5	20.0 ± 0.8
D-standard + pollen	8	257 ± 7	287 ± 10	4.3 ± 0.5	19.0 ± 0.9
C-standard + propolis	8	258 ± 5	296 ± 6	5.4 ± 0.2	17.9 ± 0.6
D-standard + propolis	8	247 ± 8	276 ± 9	4.1 ± 0.3	18.6 ± 0.4
C-standard+ pollen + propolis	8	259 ± 9	301 ± 10	6.3 ± 0.3	19.5 ± 0.6
D-standard + pollen + propolis	8	248 ± 7	287 ± 7	5.6 ± 0.3	21.0 ± 0.6

[a] Significant difference between C-standard and D-standard. [b] Significant difference between C-standard and C-standard + pollen. [c] Significant difference between C-standard and C-standard + pollen + propolis. [d] Significant difference between D-standard and D-standard + pollen. [e] Significant difference between D-standard and D-standard + propolis. [f] Significant difference between D-standard and D-standard + pollen + propolis.

oven-baked at 450 °C. The residue obtained was weighed and then diluted in a 5 N solution of hydrochloric acid to which double-distilled water was added to a predetermined volume for subsequent analysis.

The concentrations of iron, calcium, and magnesium in the diet, feces, liver, femur, and sternum were determined by atomic absorption spectrophotometry (Perkin-Elmer 1100B) and compared to a series of standard values. The concentration of phosphorus in the diet, feces, liver, femur, and sternum was analyzed by visible spectrophotometry (Perkin-Elmer UV/vis spectrometer lambda 16) using the Fiske-Subbarow technique (1925). Hemoglobin concentration was determined with a Symex CC-130 automatic cell counter. The hemogram and platelet counts were obtained using peripheral blood extracted from the caudal vein and channelled through a tube with EDTA. The analyses were carried out using an H-1 autoanalyzer supplied by Technicon.

The biochemical parameters were analyzed on the basis of the blood obtained by cannulation of the abdominal aorta at the end of each experiment. The serum levels of iron, calcium, and phosphorus were determined by colorimetry: iron, by the Trinder method (1956), calcium by the method of Sarkar and Chauvan (1967), and phosphorus by the method of Drewes (1972). Total bilirrubin was determined by a color test, the DPD method (Boehringer Mannheim GmbH Diagnostica).

Quality Control. Bearing in mind the importance of accurately determining the different parameters studied, a quality control assessment was made of the processes involved. This control included an analysis of a set of primary standards and problematic samples. There were two types of primary standards: those particular to each measurement and control samples of liophylized serum. In the present case, neither the standard deviation between the mean values of the primary standards nor those of the problematic samples were significant at any moment during the course of the experiments.

Statistical Analysis. We calculated the mean and the standard error of the mean (SEM) for each parameter studied. Variance analysis (the ONEWAY method of the SPSSPC) and the Bonferroni *post hoc* test were used to compare the different diets supplied to the two groups of animals (control and iron-deficient rats). To compare the two groups given the same diet, we used a Student *t* test for independent samples (the SPSSPC TTEST procedure). Values of $p < 0.05$ were considered significant.

RESULTS

Chemical Analysis. The content of the iron-deficient diet was as follows (mg/kg of diet): Fe, 4.43; Ca, 4769; P, 5139; and Mg, 514. The mineral content of the standard diet was as follows: Fe, 41.76 (as ferric citrate), Ca, 5166; P, 5535; and Mg, 554. The mineral content of the standard diet plus multiflora pollen was as follows: Fe, 39.31; Ca, 4955; P, 4500; and Mg, 540. The mineral content of the standard diet plus propolis was as follows: Fe, 42.25; Ca, 4876; P, 4356; and Mg, 540. The mineral content of the standard diet plus multiflora pollen and propolis was: Fe, 43.22; Ca, 5102; P, 4721; and Mg, 564.

Biological Analysis. Among the control rats, the addition of multiflora pollen to the diet produced a 43% greater weight gain than with the standard diet alone. When the diet contained propolis, there was a 23% greater weight gain than with the standard diet alone. When both natural products were added to the standard diet, the weight gain was similar to that when only pollen was added (Table 2).

In the rats with nutritional ferropenic anemia, both with the addition of multiflora pollen and that of propolis, the weight gain was greater than with the standard diet alone, despite a lower intake of nutrients. When both natural products were added and the food intake was similar to that of the rats given a standard diet, the weight gain was almost 50% greater. It is noteworthy, too, that the presence of these two products together in the diet has an additive effect on weight gain, as the presence of just one produces a weight gain of only 35% (Table 2).

In the control rats and, particularly, in the iron-deficient ones, an increase in the apparent digestibility coefficient of the iron was observed when the natural products were added; this increase was greater in the case of propolis (Table 3). This improvement in iron absorption produced by multiflora pollen and propolis, either alone or in combination, is evidenced as a higher level of hemoglobin regeneration efficiency together with an increase in the number of erythrocytes and in the levels of serum iron in comparison with the rats receiving just the standard diet (Table 4).

In the iron-deficient rats, the concentration of platelets increased as a consequence of the deficiency; however, the addition of pollen and/or propolis to the standard diet led to a marked decrease in the blood platelet count (~19% in the case of the standard diet plus multiflora pollen, 29% when propolis was added; and 32% when both products were added, in every case with respect to the iron-deficient rats fed with the standard diet; Table 4).

The levels of iron in serum also reflect the improvement in iron absorption produced by the addition of multiflora pollen and/or propolis in control and iron deficient rats (Table 4). The concentration of iron in the liver, femur, and sternum is not significantly different from that of the animals given the standard diet (Table

Table 3. Digestive Utilization of Fe, Ca, P, and Mg in Control and Iron-Deficient Rats Fed Different Diets (Mean Values with Standard Errors)

group	*n*	absorbed Fe (μg/rat/day)	ADC Fe (%)	absorbed Ca (mg/rat/day)	ADC Ca (%)	absorbed P (mg/rat/day)	ADC P (%)	absorbed Mg (mg/rat/day)	ADC Mg (%)
C-standard	8	95 ± 5	12 ± 1[a,c,d]	44 ± 3	44 ± 2[b,c,d]	66 ± 3	62 ± 2[b,c,d]	5.9 ± 0.4	56 ± 2[a]
D-standard	8	145 ± 6	17 ± 1[e,f,g]	48 ± 3	44 ± 2[e,f,g]	75 ± 3	64 ± 2	4.4 ± 0.3	38 ± 2[e,f,g]
C-standard + pollen	8	116 ± 11	14 ± 1	54 ± 3	51 ± 1	65 ± 3	72 ± 1	5.9 ± 0.3	54 ± 1
D-standard + pollen	8	373 ± 18	50 ± 1	47 ± 3	50 ± 1	51 ± 2	60 ± 1	4.9 ± 0.3	48 ± 1
C-standard + propolis	8	164 ± 12	21 ± 1	53 ± 2	60 ± 2	58 ± 2	74 ± 1	5.2 ± 0.2	53 ± 1
D-standard + propolis	8	419 ± 13	53 ± 1	47 ± 1	52 ± 1	49 ± 2	60 ± 2	4.5 ± 0.3	45 ± 2
C-standard+ pollen + propolis	8	171 ± 8	20 ± 1	56 ± 2	56 ± 1	72 ± 3	78 ± 1	5.9 ± 0.3	54 ± 1
D-standard + pollen + propolis	8	475 ± 22	50 ± 1	56 ± 3	52 ± 1	60 ± 2	61 ± 1	5.6 ± 0.3	47 ± 1

[a] Significant difference between C-standard and D-standard. [b] Significant difference between C-standard and C-standard + pollen. [c] Significant difference between C-standard and C-standard + propolis. [d] Significant difference between C-standard and C-standard + pollen + propolis. [e] Significant difference between D-standard and D-standard + pollen. [f] Significant difference between D-standard and D-standard + propolis. [g] Significant difference between D-standard and D-standard + pollen + propolis.

Table 4. Hemoglobin (Hb) Values, Erythrocytes (RBC), Hemoglobin Regeneration Efficiency (HRE), Serum Values of Iron and Platelets in Control and Iron-Deficient Rats Fed Different Diets (Mean Values with Standard Errors)

group	*n*	Hb (g/L) initial	Hb (g/L) final	HRE (%)	RBC (10^6/μL)	serum Fe (μg/L)	platelets (1000/μL)
C-standard	8	147 ± 2	155 ± 3[a,b,c]	43 ± 1[a,b,c]	7.9 ± 0.1	1200 ± 80	854 ± 27
D-standard	8	72 ± 3	106 ± 2[d]	38 ± 1[d,e,f]	7.3 ± 0.2	280 ± 40[d,e,f]	2196 ± 129[d,e,f]
C-standard + pollen	8	137 ± 2	141 ± 4	49 ± 2	7.6 ± 0.2	1390 ± 170	776 ± 43
D-standard + pollen	8	86 ± 3	133 ± 3	46 ± 1	7.8 ± 0.2	710 ± 90	1776 ± 103
C-standard + propolis	8	132 ± 4	141 ± 4	49 ± 2	7.6 ± 0.2	1210 ± 120	775 ± 43
D-standard + propolis	8	87 ± 4	127 ± 4	45 ± 2	7.8 ± 0.3	910 ± 70	1568 ± 89
C-standard + pollen + propolis	8	143 ± 2	146 ± 2	50 ± 1	7.9 ± 0.1	1310 ± 180	819 ± 33
D-standard + pollen + propolis	8	84 ± 5	135 ± 4	47 ± 1	8.1 ± 0.3	810 ± 140	1498 ± 149

[a] Significant difference between C-standard and C-standard + pollen. [b] Significant difference between C-standard and C-standard + propolis. [c] Significant difference between C-standard and C-standard + pollen + propolis. [d] Significant difference between D-standard and D-standard + pollen. [e] Significant difference between D-standard and D-standard + propolis. [f] Significant difference between D-standard and D-standard + pollen + propolis.

Table 5. Iron Concentration in Several Organs in Control and Iron-Deficient Rats Fed Different Diets (Mean Values with Standard Errors)

group	*n*	liver (μg/g of dry wt)	femur (μg/g of dry wt)	sternum (μg/g of dry wt)
C-standard	8	320 ± 22[a]	64 ± 2[a]	96 ± 2[a]
D-standard	8	130 ± 4[b]	39 ± 1[b,c,d]	116 ± 3[b,c]
C-standard + pollen	8	303 ± 10	70 ± 2	90 ± 5
D-standard + pollen	8	169 ± 4	56 ± 2	98 ± 4
C-standard + propolis	8	309 ± 11	63 ± 2	93 ± 5
D-standard + propolis	8	140 ± 4	54 ± 2	101 ± 4
C-standard+ pollen + propolis	8	300 ± 8	57 ± 2	100 ± 4
D-standard + pollen + propolis	8	134 ± 4	60 ± 2	117 ± 3

[a] Significant difference between C-standard and D-standard. [b] Significant difference between D-standard and D-standard + pollen. [c] Significant difference between D-standard and D-standard + propolis. [d] Significant difference between D-standard and D-standard + pollen + propolis.

5) and is within the normal range described in the bibliography for this species.

In the control rats, the joint addition of pollen and propolis to the standard diet had a beneficial effect on the absorption of calcium and phosphorus, whereas that of magnesium remained unchanged and within the normal limits for this species (Table 3).

In the control rats, the mineral content in the liver, femur, and sternum increased when pollen and/or propolis was added to each of the three diets tested (Table 6). The concentrations of calcium and phosphorus in the liver, femur, and sternum reflect the digestibility of each.

In the iron-deficient rats given the multiflora pollen and/or propolis-supplemented diet, the ADC increased for calcium and magnesium, but the ADC for phosphorus remained unchanged (Table 3).

The greater digestibility of calcium is reflected in the concentration of calcium in the liver and femur, compared with that in the iron-deficient rats given just the standard diet. On comparing these results with those obtained for the respective control animals, we find a lower concentration of calcium in the femur and a higher concentration in the sternum, with only minor differences in the liver (Table 6).

The concentration of phosphorus in the femur and liver in the iron-deficient rats was not affected by the addition of multiflora pollen or propolis, whereas the concentration in the sternum increased slightly (Table 6). Compared with the control rats, the concentrations of phosphorus in the femur and liver fell and those in the sternum remained unchanged (Table 6).

The content of magnesium in the liver, femur, and sternum was not affected by the addition of multiflora pollen or propolis, remaining within the normal range for this species (Table 6).

The serum levels of calcium and phosphorus in the control rats and in the iron-deficient ones were unaffected by the addition of multiflora pollen or propolis to the standard diet (Table 7).

Table 6. Ca, P and Mg Concentrations in Several Organs in Control and Iron-Deficient Rats Fed Different Diets (Mean Values with Standard Errors)

		liver (per g of dry wt)			femur (per g of dry wt)			sternum (per g of dry wt)		
group	*n*	Ca (μg)	P (mg)	Mg (mg)	Ca (mg)	P (mg)	Mg (mg)	Ca (mg)	P (mg)	Mg (mg)
C-standard	8	$82 \pm 3^{a,c}$	$8.0 \pm 0.3^{a,b,c}$	0.66 ± 0.03	228 ± 4^{b}	108 ± 2	3.7 ± 0.08	107 ± 3	$46 \pm 1^{a,b,c}$	2.3 ± 0.1
D-standard	8	80 ± 4	8.4 ± 0.1	0.62 ± 0.02	$195 \pm 3^{d,e}$	95 ± 2	3.9 ± 0.17	$158 \pm 5^{d,e,f}$	$54 \pm 3^{e,f}$	2.2 ± 0.2
C-standard + pollen	8	102 ± 2	10.2 ± 0.1	0.71 ± 0.01	231 ± 2	115 ± 4	3.9 ± 0.07	115 ± 3	60 ± 1	2.4 ± 0.1
D-standard + pollen	8	90 ± 2	8.5 ± 0.1	0.60 ± 0.01	210 ± 2	97 ± 3	3.9 ± 0.06	140 ± 3	60 ± 1	2.7 ± 0.2
C-standard + propolis	8	85 ± 2	9.8 ± 0.2	0.72 ± 0.01	240 ± 3	116 ± 2	3.7 ± 0.07	112 ± 4	60 ± 2	2.4 ± 0.2
D-standard + propolis	8	89 ± 2	8.7 ± 0.04	0.67 ± 0.01	208 ± 2	91 ± 4	3.5 ± 0.04	139 ± 3	63 ± 1	2.9 ± 0.2
C-standard+ pollen + propolis	8	104 ± 3	9.7 ± 0.1	0.67 ± 0.01	235 ± 3	119 ± 4	3.8 ± 0.11	115 ± 4	64 ± 2	2.6 ± 0.2
D-standard + pollen + propolis	8	106 ± 2	8.7 ± 0.1	0.65 ± 0.01	200 ± 3	93 ± 3	3.8 ± 0.06	136 ± 3	61 ± 1	2.7 ± 0.2

[a] Significant difference between C-standard and C-standard + pollen. [b] Significant difference between C-standard and C-standard + propolis. [c] Significant difference between C-standard and C-standard + pollen + propolis. [d] Significant difference between D-standard and D-standard + pollen. [e] Significant difference between D-standard and D-standard + propolis. [f] Significant difference between D-standard and D-standard + pollen + propolis.

Table 7. Serum Values of Calcium and Phosphorus in Control and Iron-Deficient Rats Fed Different Diets (Mean Values with Standard Errors)

group	*n*	serum calcium (mg/dL)	serum phosphorus (mg/dL)
C-standard	8	10.1 ± 0.1	8.2 ± 0.1
D-standard	8	10.3 ± 0.2	8.6 ± 0.2
C-standard + pollen	8	9.8 ± 0.1	7.8 ± 0.2
D-standard + pollen	8	10.0 ± 0.1	8.5 ± 0.3
C-standard + propolis	8	9.7 ± 0.3	7.6 ± 0.4
D-standard + propolis	8	10.0 ± 0.1	8.2 ± 0.4
C-standard+ pollen + propolis	8	10.0 ± 0.1	7.9 ± 0.2
D-standard + pollen + propolis	8	10.1 ± 0.1	7.9 ± 0.5

DISCUSSION

In the control rats, the addition of multiflora pollen, propolis, or a mixture of the two to the diet had a positive effect on weight gain. This effect was more evident when just multiflora pollen was added, the effect not being reinforced by the addition of both. In the case of multiflora pollen, the weight gain could be due to the beneficial effects of its macro- and micronutrient content (Metcalf and Schmitz, 1996; Horwits, 1975; Pellet and Young, 1980; Navarro et al., 1988) and would also depend on the proportion in which it was added to the diet (10 g/kg of diet, which is equivalent to the recommended intake for humans). With this proportion, a positive effect on weight gain was observed and no sign of any negative effects by antinutritional substances content was noted (Aykroyd and Doughty, 1982; Abreu, 1987).

The addition of propolis (1 g/kg of diet) was positive in terms of its weight-gain effects. In part, this result could be attributed to the presence of micronutrients, such as certain vitamins and oligoelements (Greceanu, 1975; Foucher, 1982; Gardelle, 1983; González and Orzáez, 1997).

In rats with nutritional ferropenic anemia, both the multiflora pollen and the propolis increased the weight gain, despite the lower level of food intake.

In control and, above all, ferropenic rats, the addition of these natural products to the diet has a positive effect on weight gain. This benefit could be attributed to the fact that digestive functions are favored by this dietary supplement. These results could constitute the scientific basis for the application of multiflora pollen and propolis as fortifiers in the treatment of nutritional ferropenic anemia, which is accompanied by a weight loss originating from a diminution in food intake.

In the control rats given a standard diet plus multiflora pollen, the coefficient of apparent digestibility of iron is about the same as in the control rats fed with the standard diet, although a slight improvement in iron absorption was observed, which led to a higher hemoglobin regeneration efficiency. However, no differences in the concentration of iron in the liver, femur, or sternum were observed. The presence in multiflora pollen of iron-absorption inhibitory factors such as polyphenols (Dev Choudhury and Goswami, 1983), phytic acid (Oberleas, 1971), and fiber (Cummings, 1980), in the dose applied, had no negative effect on the metabolism of iron in the control rats.

If propolis is added to the standard diet, the digestive utilization of iron is 43% greater than with the standard diet alone, which thus produces a higher level of hemoglobin regeneration efficiency. This positive effect could be due to the potent antioxidant action of the flavonoids found in propolis (Kepcija et al., 1981; Jaiswal et al., 1997). This action favors the presence of ferrous iron, which is the optimum form for absorption (Beutler, 1988), whereas the inhibitory effects described by Cook and Samman (1996) and Brune et al. (1989) for phenolic compounds were not observed.

The addition of multiflora pollen and propolis to the standard diet improved the digestive utilization of iron by 40% and hemoglobin regeneration efficiency, these improvements were similar to these derived by adding just propolis to the standard diet. This positive effect can be considered a consequence of the presence of propolis in the diet and due to the antioxidant effect of the flavonoids.

In the iron-deficient rats, the addition of multiflora pollen, propolis, or the two together to the standard diet increased the digestive utilization of iron by ~66% in each case, in comparison to the iron-deficient rats given the standard diet. It is apparent that when there exists a state of iron deficiency, iron absorption is increased. This phenomenon has been described by numerous authors (Hallberg, 1981; Beutler, 1988; Bezkorovainy, 1989; Schümann et al., 1990; Scrimshaw, 1991), irrespective of the source of iron (Pallares et al., 1993; Campos et al., 1996). This effect was reinforced by the addition of the two natural products, multiflora pollen and propolis, either alone or jointly.

We believe the positive effect of multiflora pollen can be attributed to its content of substances that favor iron absorption, such as free amino acids (histidine), fructose, vitamin C, and bioflavonoids; in the case of propolis, the benefit is derived from its content of bioflavonoids and vitamin C (Chauvin, 1959; Stanley and Linskens, 1974; Foucher, 1982; Gozálbez, 1984; Serra et al., 1985). The explanation for this result might lie in the fact that

these constituents favor the union of iron within the mucins, according to the hypothesis of Conrad and Umbreit (1993). The mucins make the iron soluble and available for absorption, carrying it toward the integrins of the surfaces of the absorptive cells and facilitating the transfer of the iron through the cell membrane to join the mobilferrin that transports it to the capillaries.

In the iron-deficient rats, the concentration of platelets increased as a consequence of the deficiency; however, the addition of pollen and/or propolis to the standard diet led to a marked decrease in the blood platelet count. These results demonstrate the improved recovery of iron metabolism when the diet contains either or both of the products and confirm the theory proposed by Rodríguez-Matas et al. (1998) that platelet concentration constitutes a hematic parameter that reflects the state of the iron within an organism. This effect, with regard to the hematic parameters, is reflected in the utilization of iron in the hematopoietic organs, because the iron content in the sternum is higher in the iron-deficient rats than in the respective control animals for each of the three diets tested. Thus, the iron is preferentially allocated to the formation of hemoglobin (Milne et al., 1990), while the levels of iron in organs such as the liver remain low. This result could be because the repletion period was just 10 days, as in previous studies (Campos et al., 1998; Lisbona et al., 1999).

The greater digestibility of calcium and phosphorus in the control rats fed with the standard diet plus multiflora pollen could be due to the free amino acid content (Chauvin, 1959) and protein content (Gozálbez, 1984), because amino acids, such as lysine (Murillo et al., 1972), aspartate, glutamate, and ornithine (Gozálbez, 1984) are known to favor the absorption of calcium. Furthermore, taking into account the studies carried out by Abreu (1992), the pollen protein is of high biological value, approaching that of casein. According to Pointillart and Guenguen (1989), the quality of the protein is one of the factors that favors the absorption of phosphorus. The increase in the digestibility of calcium and phosphorus when propolis is added to the standard diet could be due to the acid derivates, such as benzoic, 4-hydroxy-benzoic, etc., which are found in propolis (Foucher, 1982) and that favor the solubility of calcium and phosphorus salts in the diet, thus increasing the absorption of the latter minerals.

The fact that no additive effect was found when the standard diet was supplemented with both multiflora pollen and propolis could be because, having attained high levels of calcium and phosphorus digestibility in the rat (Greger, 1989; Pallarés et al., 1996; Campos et al., 1998), it would be difficult to surpass these levels by the fine adjustment of the mineral metabolism.

In rats with nutritional ferropenic anemia, the addition of multiflora pollen, propolis, or a mixture of the two improves the digestive utilization of calcium. This improvement could be because these natural products contain substances that favor the absorption of calcium (Foucher, 1982) and also because the greater absorption of iron leads to improved hemoglobin recovery (Hill and Matrone, 1970). Thus, the quantity of available oxygen increases and the active iron-absorption mechanisms could be recuperated.

In the iron-deficient rats given the standard diet plus multiflora pollen and/or propolis, the levels of calcium in the different organs tested are consequence of its greater digestibility. The lower concentration of calcium in the femur and a higher concentration in the sternum, in iron-deficient rats in comparison with respective control rats, coincide with the results obtained by Pallarés et al. (1993) and Campos et al. (1996) for the iron-deficient rats provided with different types of diet.

The fine regulation of calcium homeostasis means that the concentration of serum calcium remains constant, which is also observed at the hepatic level, because the calcium content is very low in the liver, which is not a reserve organ for this cation. In the femur, however, where calcium is usually stored, there is a slight transfer of the cation toward the sternum where it is needed because hematopoiesis increases in rats with ferropenic anemia and a greater supply of calcium is needed for this purpose. These results lead us to believe that for anemic rats, the addition of multiflora pollen and/or propolis to the standard diet constitutes a factor that would palliate the interference between iron and calcium (Mahoney et al., 1985; Dallman, 1986; Hallberg et al., 1991; Cook et al., 1991) by increasing the absorption and utilization of these two cations.

In relation to phosphorus, the absence of a positive effect by multiflora pollen and/or propolis on the absorption of this anion in iron-deficient rats with respect to the control rats coincides with the findings of various authors who have shown that the digestive efficiency of phosphorus is not affected by the protein level (Pallarés et al., 1993), by the source or levels of iron (Pallarés et al., 1993; Campos et al., 1996), or by the type of diet (Gordon and Godber, 1989). The hepatic concentration of phosphorus in iron-deficient rats when either or both products are added to the standard diet is within the normal range described for this species (Barrionuevo et al., 1989; Pallarés et al., 1996) although these values are lower than those found in the control rats given one or both products. This result is due to the greater utilization of phosphorus by the latter rats, which is reflected in a higher hepatic phosphorus content. The levels of phosphorus in serum coincide with those given in the bibliography (Gordon and Godber, 1989; Barrionuevo et al., 1989; Campos et al., 1996).

In the control rats, the addition of either or both products to the standard diet did not affect the absorption of magnesium; however, in the iron-deficient rats, the absorption of this mineral was increased. This improvement could be because adding multiflora pollen and propolis produces a better recovery of iron metabolism, as indicated by the coefficient of apparent digestibility and the efficiency of haemoglobin regeneration. Thus, the amount of available oxygen and ATP increases, and the magnesium receptors recover more efficiently, which favors their absorption by active transport, as has been described by other authors (Zhang et al., 1989; Conrad and Umbreit, 1993). Another consequence is that the passive absorption component of magnesium is less influenced by the presence of free citrate in the lumen, which produces magnesium citrate, a low-solubility complex (López de Novales, 1974). This result is because the fact that part of the citrate is absorbed by active transport as an iron complex. Moreover, this explanation is supported by the results obtained by Campos et al. (1996) in which a ferric protein diet resulted in a greater recovery of iron absorption accompanied by a greater absorption of magnesium. The concentrations of magnesium in the

liver, femur, and sternum for each of the three diets tested were about the same as those found for the respective control animals, which confirms that the addition of multiflora pollen and/or propolis to the standard diet produces a correct metabolism of magnesium.

In conclusion, this study shows the beneficial effects of multiflora pollen and/or propolis as coadjutants in the treatment of nutritional ferropenic anemia. Addition of these products to the diets of rats led to a substantial improvement in the digestive utilization of iron, calcium, phosphorus, and magnesium.

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