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Study report

STUDY ON THE HEMATOLOGIC EFFECTS OF FLAVIN7, FRUIT EXTRACT WITH HIGH POLYPHENOL CONTENT IN MICE WITH DAMAGED BONE MARROW *IN VIVO*

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TITLE: **STUDY ON THE HEMATOLOGIC EFFECTS OF FLAVIN7®, FRUIT EXTRACT WITH HIGH POLYPHENOL CONTENT IN MICE WITH DAMAGED BONE MARROW *IN VIVO***

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CONTENTS

1. INTRODUCTION

Cancer is the second leading cause of death behind heart diseases.

Cancer incidence shows that a new cancer disease appears in about 10 % of the population each year in USA. Cancer rates could further increase by 50 % until 2020 all over the world according to the WHO. About half of the cases need systemic treatment by chemotherapy alone or together with irradiation. The most common dose-limiting side effect of cytostatic drugs is bone marrow suppression. The major mechanism of bone marrow suppression is inhibition of the proliferation of stem and progenitor cells which have a function in the continuous compensation of lost mature blood cells possessing a short life-span (Bond et al. 1965). Among these progenitors granulocyte-macrophage colony forming units (CFU-GM) are capable of forming colonies of granulocytes and/or macrophages in cell cultures. CFU-GM are supposed to be the major target of agents damaging bone marrow (Lohrmann and Schreml 1982). Damage of the CFU-GM pool has a great importance, as resulting neutropenia and severe infections with high mortality may develop in patients. Leading causes of mortality of patients with malignant tumours are infections associated with neutropenia (Harrison 2001).

Numerous plant-derived polyphenolic compounds with antioxidant and free radical scavenging properties can prevent tumour formation (Surh 1999). Cacao liquid polyphenols inhibited DNA strand cleavage induced by mitomycin C, an antitumour antibiotic *in vitro* (Yamagishi et al. 2001). Doxorubicin, an another antitumour antibiotic, is used much more frequently in many different types of malignant diseases. If the polyphenolic fruit extract we investigated could also decrease DNA damage, it might protect bone marrow from damage caused by doxorubicin. The experimental doxorubicin-induced damage of bone marrow has been a widely accepted

pharmacological tool for studying the preventive action of various, potentially useful chemoprotectant agents.

2. AIM OF THE STUDY

The aim of this study is to investigate whether

the fruit extract with high polyphenol (FLAVIN7®), can protect bone marrow from damage caused by doxorubicin *in vivo* in mice.

3. SUMMARY

Effects of a special fruit extract FLAVIN7® were analysed on hemopoiesis of mice with damaged bone marrow *in vivo*. Bone marrow damage was induced by doxorubicin, a widely used cytostatic drug. During anticancer chemotherapy myelotoxicity is the dose-limiting side effect of cytostatic agents, which hinders the successful treatment of malignant diseases.

Bone marrow damage was characterized by its cellularity and the features of granulopoiesis. Granulocyte-macrophage progenitor cells (CFU-GM) are supposed to be the major target, thus we measured directly their damage. To determine frequency of CFU-GM special soft-gel bone marrow cell cultures were prepared under steril conditions, in which only the CFU-GM cells could proliferate and formed colonies, because their descendants remained together in the soft gel. Number of colonies grown from 10^5 bone marrow cells shows intensity of granulopoiesis, which maintains the CFU-GM pool to supply the body with macrophages and monocytes. Whole CFU-GM content of femoral bone marrow was also studied. Results of the myelo- and granulopoiesis, the mature blood cells were also measured from the peripheral blood of the mice.

A single doxorubicin dose a caused dose-dependent decrease in the characteristics of myelo- and granulopoiesis described previously. After the choose of the 50 mg/kgbw doxorubicin dose we investigated whether pretreatment with the fruit extract FLAVIN7® could influence bone marrow damage caused by doxorubicin. The fruit extract was administered per orally for 7 consecutive days before the single doxorubicin i.p. dose.

Neither cellularity of bone marrow nor white blood cell counts were influenced by the fruit extract. This special fruit extract FLAVIN7®, however, specifically protected the granulocyte-macrophage progenitor cells. In the group pretreated with 4 ml/kgbw of the fruit extract before doxorubicin CFU-GM colony numbers were significantly higher than in the group treated with doxorubicin alone ($P < 0.01$). Due to the more intensive proliferation replacing the damaged CFU-GM pool was not only faster but CFU-GM pool expanded. It was greater than even the values of the control untreated group ($P < 0.01$). This could decrease the extent and duration of granulocytopenia namely neutropenia. Even this early time the absolute neutrophil counts were a little higher but not significantly in the group pretreated with 4 ml/kgbw of fruit extract. Effects on the mature blood cells appear after a latent period when the differentiation of the progenitor cells is completed.

From these results we can conclude that FLAVIN7® could protect granulocyte-macrophage progenitor cells of bone marrow against damage caused by doxorubicin. It is of great importance, as this could prevent developing of serious infections associated to neutropenia. It can be concluded that FLAVIN7® can be a promising product in the complex therapy of malignant diseases or immunological disorders, but a number of *in vitro and in vivo* experiments are needed to test its effectiveness. By supplying more progenitors for the more intensive granulopoiesis this fruit extract could decrease the immuno- and myelosuppressive effects of cytostatic drugs or other toxic molecules, which can decrease mortality.

4. METHODS

4.1. Materials

adriamycin (Pallagicin BIOGAL-TEVA, 10 mg of lyophilised doxorubicin hydrochloride/amp.) = DOXO

FLAVIN7® fruit extract with high polyphenol content (Crystal Institute Ltd)

Stock solution was prepared by acidic Aqua distillate in the following concentration: 40 ml extract/100 ml vehicle

pH of the vehicle was about 2-3

Stock solutions were prepared freshly before each experiment.

4.2. Mice

C57black x DBA hibrid BDF_1 male mice – weight: 20-35 g - obtained from the National Institute of Oncology, Budapest, Hungary. Mice were given standard laboratory food and water *ad libitum*. Keeping conditions were controlled by the Regional Ethical Committee for Animal Experiments, conforming to the Standards of the European Union.

Body weight of mice was measured twice, prior to the experiment and on the day of their extermination.

4.3. Drug administration

The polyphenolic extract solutions have been administered by gavage orally. Doxorubicin was administered intraperitoneally (i.p.). In both types of administration, drugs were added to the mice in 0.1 ml/10 gbw volume dissolved in vehicles described in the section “Materials”.

4.4. Study design

4.4.1. 1st stage experiments

Number of mice: 30

To find the proper doxorubicin dose to damage the bone marrow first the dose-response curve was determined. 0, 25, 50, 100 and 200 mg/kgbw of doxorubicin was administered to the murine groups intraperitoneally. The following day the mice were exterminated to obtain bone marrow samples. Bone marrow hemopoiesis was characterized by its cellularity and by culturing granulocyte-macrophage progenitor cells. Under sterile condition special soft-gel bone marrow cultures were prepared, in which only the granulocyte-macrophage progenitor cells could grow. During the 7-day culturing period CFU-GM cells formed colonies. These colonies could be counted under stereomicroscope. There were three mice in each group, and the experiment was repeated twice.
A detailed flow chart can be found on page 10 (Table 1).

Table 1. FLOW CHART for 1st type experiments

1st day	**2nd day**	**2-9th days**	**9th day**
1x			
→	**X**	→	**X**
Doxorubicin/vehicle i.p.	**extermination**		**counting colonies**
bone marrow		**culturing time**	**under**
damage **regeneration**			**stereomicroscope**
	Blood samples		
	Femoral bone marrow		
	Soft-gel bone marrow cultures		
End points:			
Body weights on the 0. day and on the	8th day		
	White blood cell count		Colony number
	Bone marrow cell count		(frequency of CFU-GM)
Calculated end points:	Cellularity of bone marrow		**CFU-GM POOL**
	Absolute neutrophil count (ANC)		

4.4.2. 2nd stage experiments

Number of mice: 27

To determine whether the studied fruit extract with high polyphenolic content could influence granulopoiesis in bone marrow murine groups were pretreated with 0, 0.4 and 4 ml/kgbw of the extract, respectively. The mice were treated for 7 consecutive days. Doxorubicin was administered 1 hour after the last dose of the extract on the 7th day. Biological samples were obtained 24 hours later, on the following day. Mature white blood cells were evaluated from the peripheral blood of the mice. Bone marrow hemopoiesis was characterized by its cellularity and by culturing granulocyte-macrophage progenitor cells. Under sterile condition special soft-gel bone marrow cultures were prepared, in which only the granulocyte-macrophage progenitor cells could grow. During the 7- day culturing period CFU-GM cells formed colonies. These colonies could be counted under stereomicroscope. There were three mice in each group, and the experiment was repeated 3 times.

A detailed flow chart can be found on page 12 (Table 2).

Table 2. FLOW CHART for 2nd type experiments

1, 2, 3, 4, 5,6,7th days	**7th day**	**1 day**	**8th day**	**8-15th days**	**15th day**
7x	**1x**				
	X	→	**X**	→	**X**
p.o. treatment	**DOXO/vehicule i.p.**		**extermination**		**counting colonies**
fruit extract/vehicle	**bone marrow**			**culturing time**	**under**
	damage	**regeneration**			**stereomicroscope**

Blood samples

Femoral bone marrow

Soft-gel bone marrow cultures

End points:
body weight on 0th day

8th day

White blood cell count — Colony number
Bone marrow cell count — (frequency of CFU-GM)

Calculated end points:

Cellularity of bone marrow — CFU-GM pool
Absolute neutrophil count (ANC)

4.5. Murine groups and treatment schedule

Tables 3-4. show murine groups and treatment schedule for the different types of experiments. In the 1st type experiments 5 groups were treated with the increment doses of doxorubicin. The 1st, control group was administered with vehicle in the same manner.

Table 3. Murine groups in the 1st type experiments

animals		**treatment**	
		i.p. **1x on 1st day**	
groups	**Number of mice**	**Vehicle 0.9% NaCl**	**doxorubicin mg/kgbw**
1.	6	+	-
2.	6	-	25
3.	6	-	50
4.	6	-	100
5.	6	-	200
	30		

In the 2nd type experiments 3 groups were treated with the increment doses of the studied fruit extract. The 1st, control group was administered with vehicle in the same manner. Each mouse was administered the same doxorubicin dose on the 7th day 1 hour after the last dose of the fruit extract.

Table 4. Murine groups in the 2nd type experiments

animals		**treatment**		
		p.o.		**i.p.**
		7 times on 1-7 days		**1x on 7th day**
groups	**Number of mice**	**Vehicle Acidic water (pH:2-3)**	**Observed fruit extract ml/kgbw**	**doxorubicin mg/kgbw**
1.	9	+	-	50
2.	9	-	0.4	50
3.	9	-	4	50
	27			

4.6. Biological samples

Blood was obtained from the retroorbital plexus of the mice. Bone marrow was obtained from the femora of the mice after their extermination by cervical dislocation. One of the femurs was removed and the bone marrow cells were aseptically washed out. Single cell suspensions were prepared suspending them in serum-free McCoy's 5A medium (GIBCO) through a thin needle by a syringe.

4.7. CFU-GM colony assay

4.7.1. Preparing bone marrow cultures

Soft agar cultures were made by using inocula of 10^5/ml bone marrow cells in petri dishes (Greiner, Nürtingen, Germany). Murine bone marrow cells were grown in McCoy's 5A modified medium (GIBCO Grand Island NY USA) supplemented with

amino acids, Na pyruvate, $NaHCO_3$ and antibiotics (streptomycin, penicillin) according to Pike and Robinson (1970) as well as with 0.3% agar (Ionagar No2, Oxoid, London, Great Britain), as well as with 20-25% horse serum. The best batch of serum was selected in preliminary experiments from several samples supplied by the manufacturer. The source of colony-stimulating factor was the medium conditioned by WEHI-3B cells (WEHI-3B-CM), produced and tested in our laboratory and used at a concentration necessary for the growth of maximum number of colonies. WEHI-3B-CM contains a variety of growth factors. Three parallel petri dishes (Greiner, Nürtingen, Germany) containing the cultures were incubated for 7 days in a humidified atmosphere containing 5% CO_2 in CO_2 incubator (JOUAN Co, France).

4.7.2. Evaluation of bone marrow cultures

Seven days later, at the end of the culturing period, colonies - defined as groups of at least 50 cells - were counted under a dissecting microscope (Olympus SM60, Olympus, Hamburg, Germany). The above cultural conditions are suitable for granulocyte-macrophage progenitor cells for proliferation and differentiation. Only these cells can proliferate and due to soft agar medium descendants will remain together and form colonies containing granulocytes and/or monocytes as we can check it in smears or cytospin preparatums under microscope.

4.8. End points from peripheral blood

After the doxorubicin dose on the following day during the bone marrow regeneration blood samples of mice were obtained from their retroorbital plexus immediately before their extermination for blood smears and for counting white blood cell numbers. Blood smears were stained with May-Grünwald-Giemsa. From the blood samples of the mice white blood cell counts (WBC) and absolute neutrophil counts were determined. The latter data were calculated from WBCs and the frequencies of neutrophil granulocytes in blood smears.

End points are the following:

1. white blood cell count (WBC)
2. absolute neutrophil granulocyte count (ANC) = WBC * frequency of neutrophils

4.9. End points from bone marrow

Femoral bone marrow was obtained after the extermination of the mice. From bone marrow cell suspensions cell numbers (BMC) were counted in a hemocytometer to determine the cellularity of the bone marrow, which was expressed as nucleated cells per femur.

Soft-gel cell cultures were prepared from their bone marrow cell suspensions and after culturing period the number of CFU-GM (granulocyte-macrophage progenitor cells) was determined by counting colonies under stereomicroscope.

Numbers of colonies grown from 10^5 bone marrow cells showed the intensity of regeneration of CFU-GM pool after the bone marrow damage caused by doxorubicin. The CFU-GM content of femur (CFU-GM pool) was calculated with the help of the colony numbers and cellularity and was expressed as CFU-GM per femur.

End points are the following:

1. Bone marrow cell count (BMC)
2. Cellularity = BMC * volume of bone marrow cell suspension means the total nucleated cell number in the femora of mice
3. Frequency of CFU-GM = number of colonies of CFU-GM/ 10^5 bone marrow cells = number of granulocyte-macrophage colony forming units/ 10^5 bone marrow cells
4. CFU-GM content in the whole femoral bone marrow = cellularity * frequency of CFU-GM

4.10. Statistical analysis

Data for individual mice were used for statistical evaluation. Each hematologic variable mentioned above was evaluated at each time with a non-parametric analysis of variance, namely ANOVA. If ANOVA analysis revealed significant inhomogeneity, multiple comparison test according to Bonferroni was used for evaluating statistical differencies. The GraphPadPrism program (version 3.0, GraphPad Software, Inc., San Diego, CA, USA) was used to perform these calculations. Differences were regarded as statistically significant if $p<0.05$.

5. RESULTS

5.1. 1st TYPE EXPERIMENTS

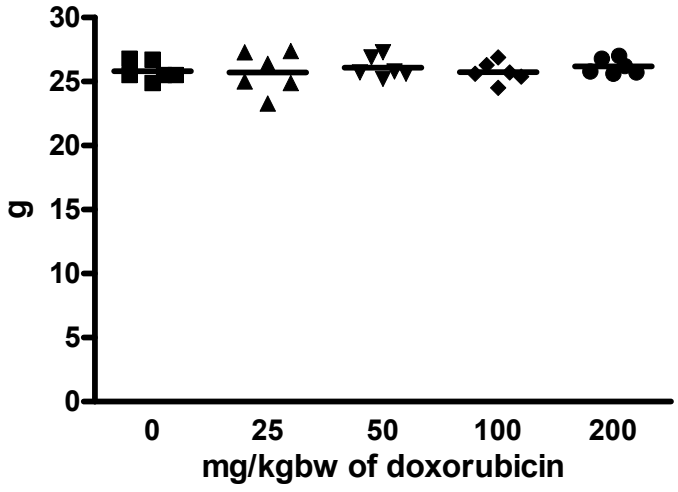
5.1.1. Results from body weights

In these experiments for the 5 murine groups were treated with 0, 25, 50, 100 and 200 mg/kgbw of doxorubicin intraperitoneally. For the control group vehicle administered instead of doxorubicin. Mice were exterminated 24 hours later to study their blood and bone marrow samples.

Neither body weights of mice treated with different doses of doxorubicin i.p. nor body weights of control mice treated with vehicle were significantly different on the day of their extermination (2nd day) compared with the beginning values on the first day (Figures 1, 2). Neither were differencies among body weights of the murine groups. Data and their statistical analysis are shown in the Appendix (Table A1, A2).

Figure 1.

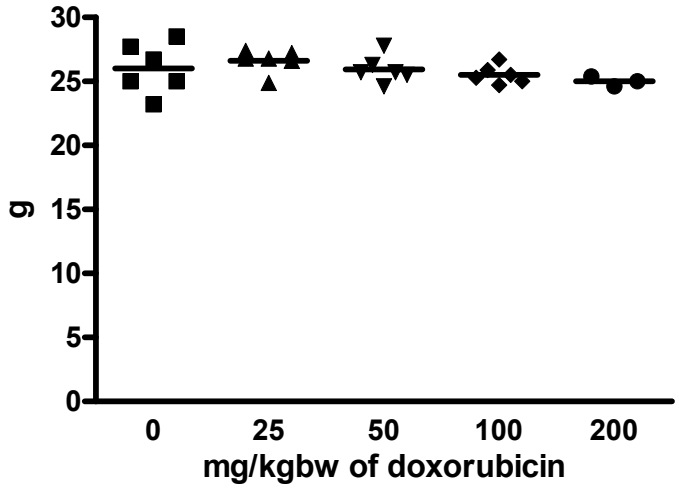
Body weights of mice prior to the experiment



Symbols show the individual data of each mouse.

Figure 2.

Body weights of mice on the 2nd day



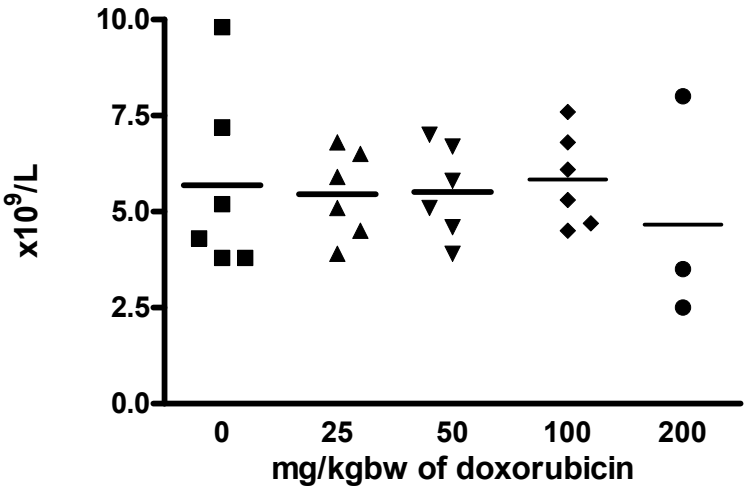
Symbols show the individual data of each mouse.

5.1.2. Results from peripheral blood

5.1.2.1. White blood cell counts on the 1st day of bone marrow regeneration after damage caused by doxorubicin

White blood cell counts (WBC) of the previously described murine groups were not significantly different from each other on the 1st day after bone marrow damage caused by doxorubicin (Figure 3). Three of the six mice treated with 200 mg/kgbw of doxorubicin died. This is the reason why their data are missing. Data and their statistical analysis are shown in the Appendix (Table A3).

Figure 3.
White blood cell counts (WBC) of mice on the 1st day after a single dose of doxorubicin

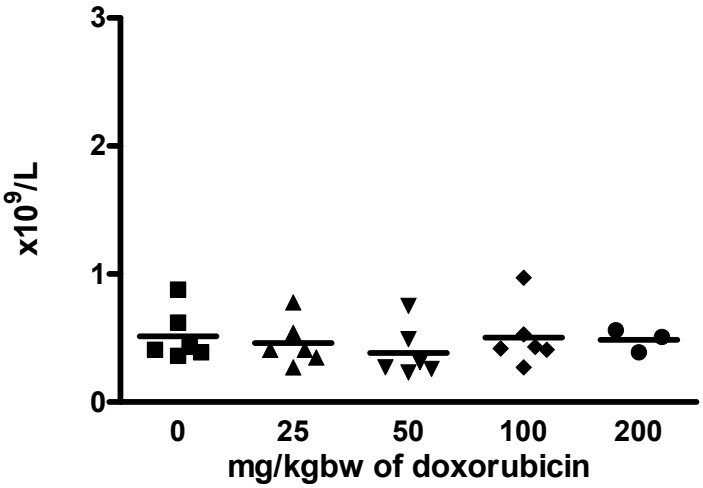


Symbols show the individual data of each mouse.

5.1.2.2. Absolute neutrophil cell counts on the 1st day of bone marrow regeneration after damage caused by doxorubicin

Absolute neutrophil counts (ANC) of the previously described murine groups were not significantly different 1 day after 0-200 mg/kg of doxorubicin dose (Figure 4). Data and their statistical analysis are shown in the Appendix (Table A4).

Figure 4.
Absolute neutrophil cell counts (ANC) of mice 1 day after a single dose of doxorubicin



Symbols show the individual data of each mouse.

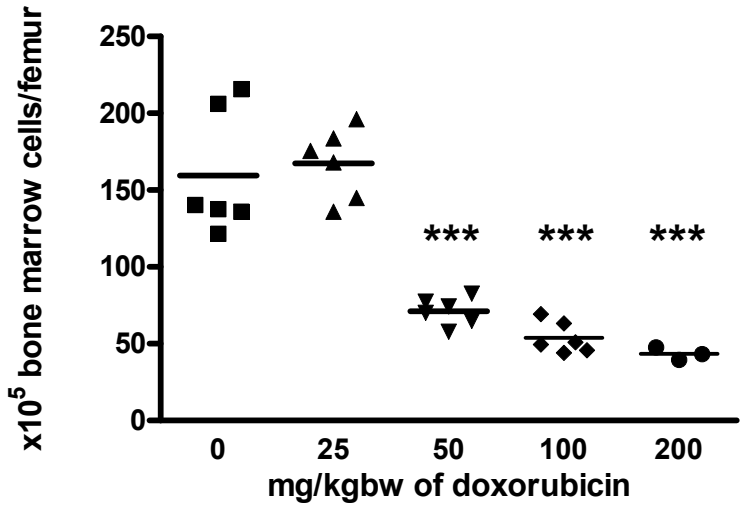
5.1.3. Results from bone marrow evaluation

5.1.3.1. Cellularity of femoral bone marrow of mice

Cellularity of femur shows the whole cell mass of femoral bone marrow of mice. Cellularity of femoral bone marrow of mice was decreased dose-dependently due to the doxorubicin treatment. 25 mg/kgbw dose was no effect on cellularity but 50 mg/kgbw of doxorubicin markedly decreased the cell mass of the bone marrow to about the half of the control value ($P < 0.001$). Cellularity was decreased to the third of the control value by the 100 mg/kgbw dose ($P < 0.001$) (Figure 5). Data and their statistical analysis are shown in the Appendix (Table A5)

Figure 5.

Cellularity of bone marrow 1 day after a single dose of doxorubicin



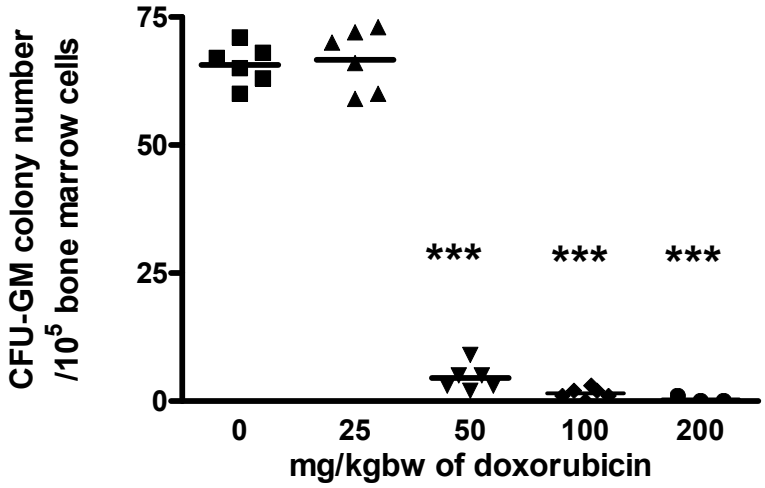
Symbols show the individual data of each mouse. Stars in the figure signe the significant differences from the values of the control group **(P < 0.001).**

5.1.3.2. CFU-GM colonies in femoral bone marrow of mice

Colonies growing in bone marrow cell cultures of 10^5 bone marrow cells show frequency of CFU-GM progenitors in bone marrow. Doxorubicin decreased colony numbers of CFU-GM progenitors dose-dependently. Twenty five mg/kgbw of doxorubicin had no effect on CFU-GM frequency, while the 50 mg/kgbw and the 100 mg/kgbw of doxorubicin decreased it to about 10 % and 2 % of the control value. Three of the six mice treated with 200 mg/kgbw of doxorubicin were died and there were no detectable CFU-GM progenitors in 2 of the 3 left survived mice. Differences from the control group were markedly significant in the case of the 3rd, 4th and 5th groups ($P<0.001$), and they were also significant between the 3rd and the 4th and the 5th groups ($P<0.01$) (Figure 6). Data and their statistical analysis are shown in the Appendix (Table A6).

Figure 6.

Numbers of CFU-GM colonies in bone marrow on the 1st day after a single dose of doxorubicin



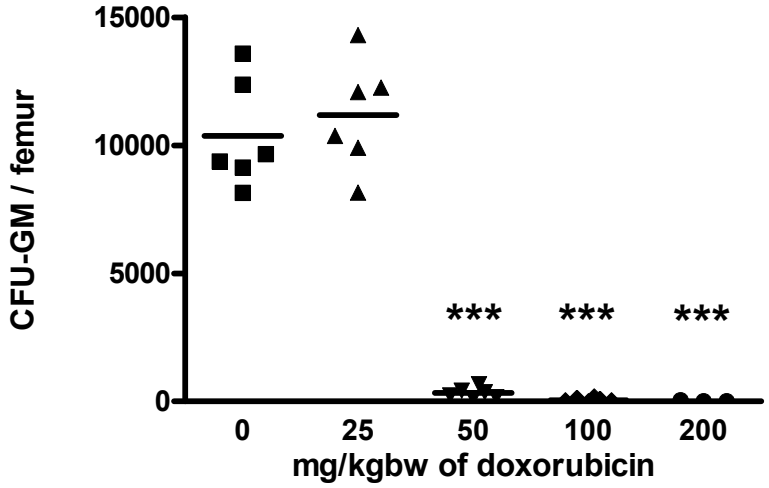
Symbols show the individual data of each mouse. Stars in the figure signe the significant differences from the values of the control group **(P < 0.001).**

5.1.3.3. CFU-GM contents of femora of mice

CFU-GM content of femoral bone marrow damaged markedly and dose-dependently due to the doxorubicin treatment. Twenty five mg/kgbw of doxorubicin had no effect on CFU-GM pool, while the 50 mg/kgbw and the 100 mg/kgbw of doxorubicin decreased it to about 10 % and 2 % of the control value. Three of the six mice treated with 200 mg/kgbw of doxorubicin were died and there were no detectable CFU-GM progenitors in 2 of the 3 left survived mice, thus their CFU-GM pools were empty. They would die on the following days if we did not exterminate them. Differencies from the control group were markedly significant in the case of the 3rd, 4th and 5th groups (P<0.001), and they were also significant between the 3rd and the 4th and the 5th groups (P<0.01) (Figure 7). Data and their statistical analysis are shown in the Appendix (Table A7).

Figure 7.

CFU-GM content of femoral bone marrow on the 1st day after a single dose of doxorubicin



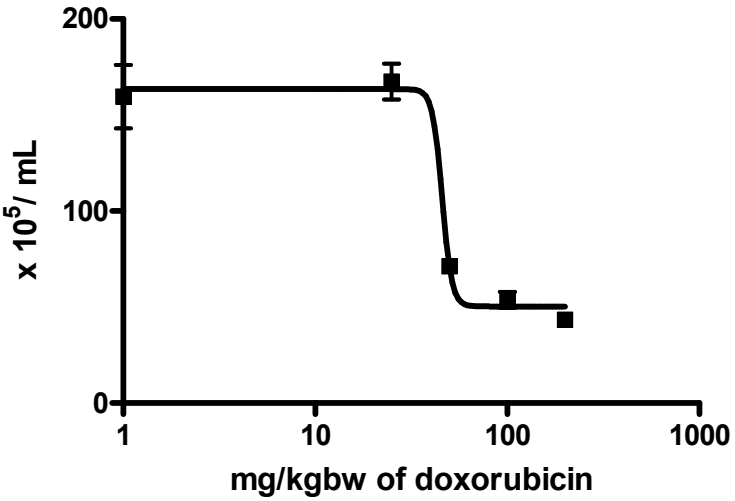
Symbols show the individual data of each mouse. Stars in the figure signe the significant differences from the values of the control group **(P < 0.001).**

5.1.3.4. 50 % inhibitory concentrations (IC50) of doxorubicin

Fifty percent inhibitory concentrations (IC50) of doxorubicin were determined from the dose-effect curves related to the measured parameters. The dose-effect curves namely related to the cellularity, number of CFU-GM colonies as well as the CFU-GM content of femoral bone marrow were similar to each others (Figures 8, 9, 10).

Figure 8.

Cellularity of femoral bone marrow

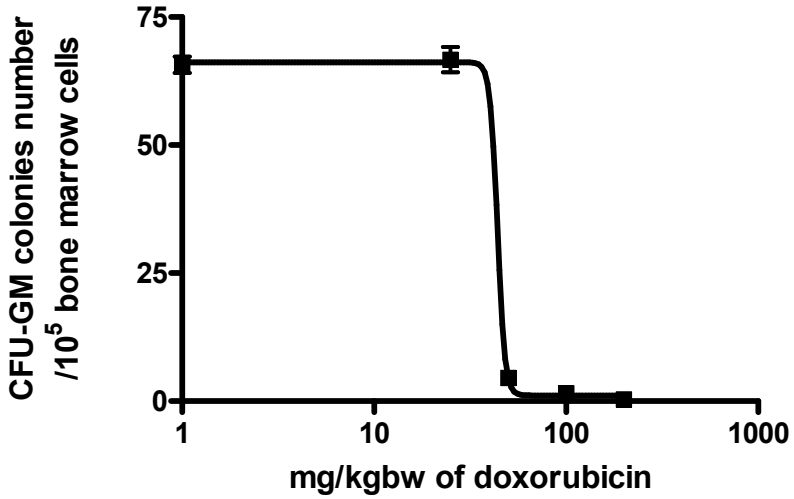


IC50 = 45.85 mg/kgbw

The dose-effect curves were steep, and IC50 values of doxorubicin related to these parameters which characterized bone marrow function were practically same, 44-48 mg/kgbw. We chose the 50 mg/kgbw of doxorubicin dose for the second type of experiments.

Figure 9.

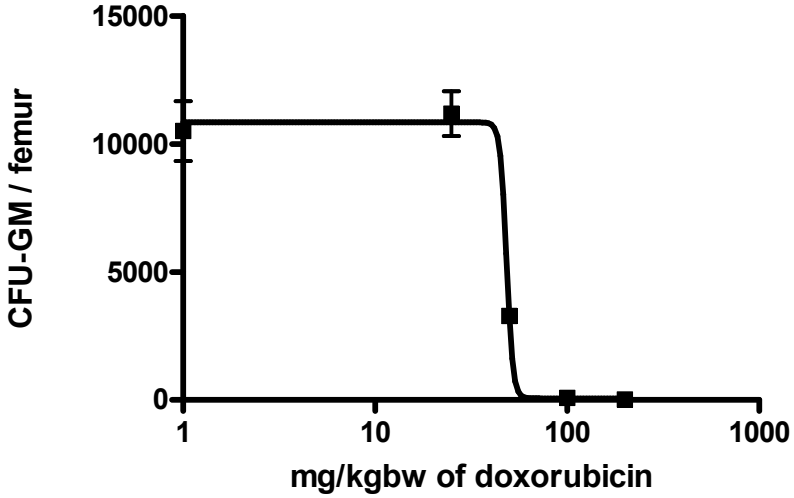
Number of CFU-GM colonies grown from 10^5 bone marrow cells



IC50 = 43.91 mg/kgbw

Figure 10.

CFU-GM content of femoral bone marrow



IC50 = 47.60 mg/kgbw

5.2. 2 ND TYPE EXPERIMENTS

5.2.1. Results from body weights

In these 2nd type experiments 3 murine groups were. The mice of the control group were administered doxorubicin alone and the mice of the 2nd and 3rd groups were pretreated with 0.4 and 4 ml/kgbw of fruit extract respectively for 7 consecutive days before the same doxorubicin dose. The 50 mg/kgbw of doxorubicin i.p. was used one hour after the last p.o. dose of the fruit extract or vehicle.

Neither body weights of mice pretreated with 2 different doses of the fruit extract for 7 consecutive days before the doxorubicin dose nor body weights of control mice treated with doxorubicin alone were significantly different on the day of their extermination (8th day) compared with the beginning values on the first day (Figures 11, 12). Neither were differencies among the body weights of the murine groups. Data and their statistical analysis are shown in the Appendix (Table A8).

Figure 11.

Body weights of mice treated with repeated doses of fruit extract 7 times before a single doxorubicin dose on the 1st day

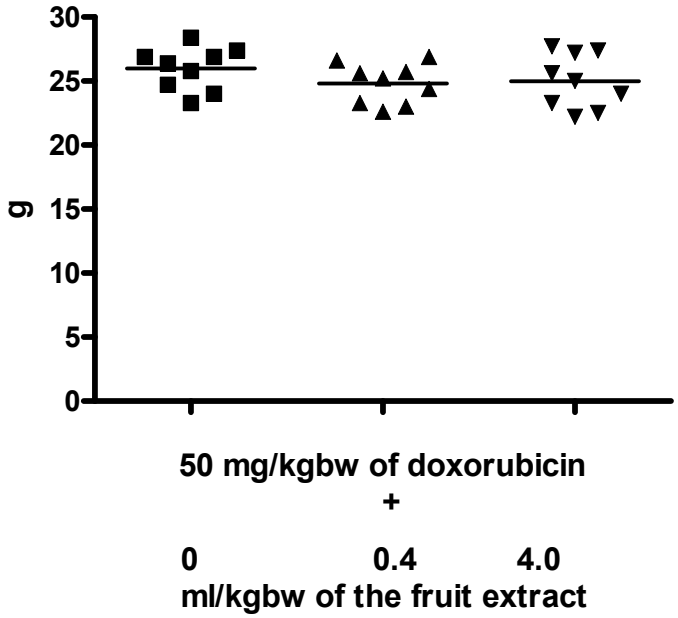
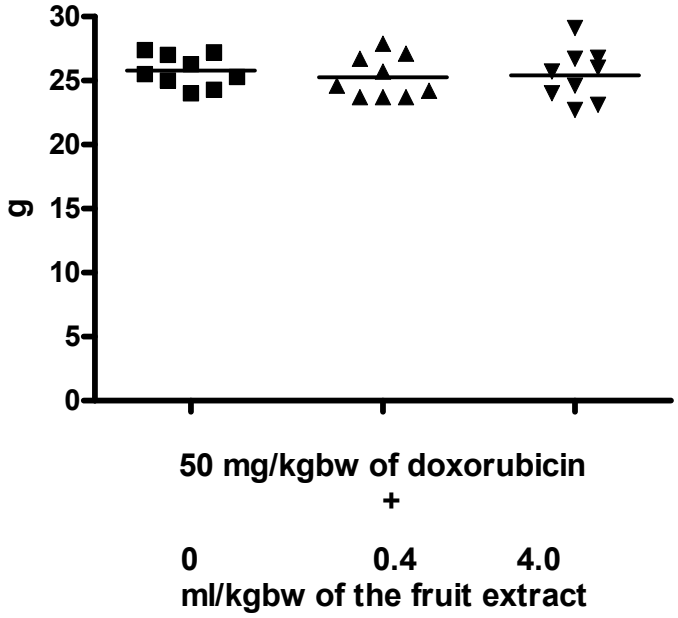


Figure 12.

Body weights of mice treated with repeated doses of fruit extract 7 times before a single doxorubicin dose on the 8th day



Symbols show the individual data of each mouse.

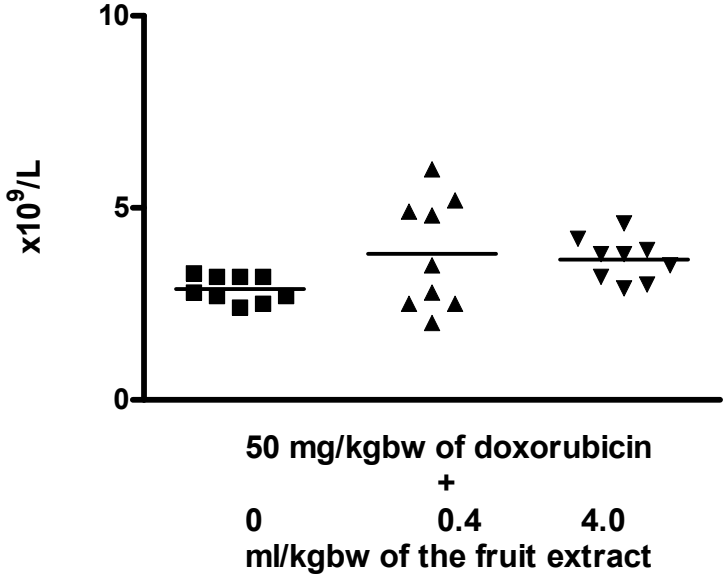
5.2.2. Results from peripheral blood

5.2.2.1. White blood cell counts on 1st day of bone marrow regeneration after doxorubicin damage

White blood cell counts (WBC) of the above murine groups were not significantly different 24 hours after the bone marrow damage caused by doxorubicin (Figure 13). Data and their statistical analysis are shown in the Appendix (Table A9).

Figure 13.

White blood cell counts (WBC) of the pretreated mice on the 1st day after a single dose of doxorubicin



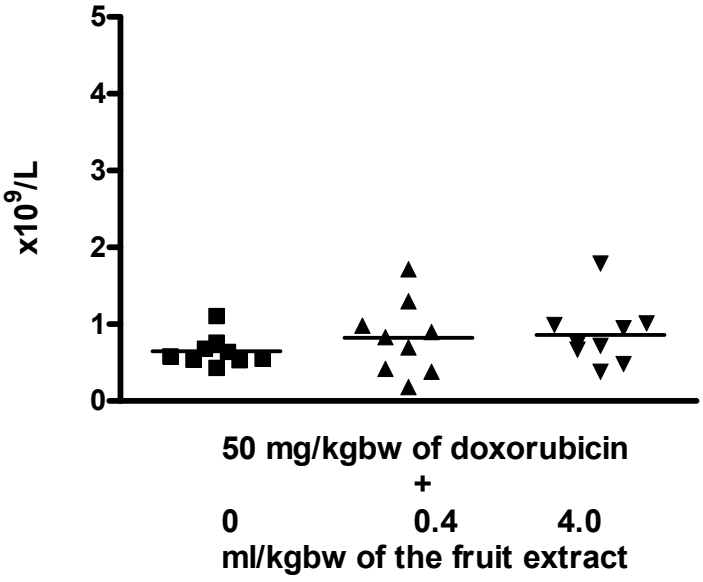
Symbols show the individual data of each mouse.

5.2.2.2. Absolute neutrophil cell counts on 1st day of bone marrow regeneration after doxorubicin dose

Absolute neutrophil counts (ANC) of the previously described murine groups were not significantly different on the following day after bone marrow damage caused by doxorubicin (Figure 14). Pretreatment with 0.4 ml/kgbw of fruit extract for 7 consecutive days did not influence ANCs measured on the following day after the doxorubicin caused bone marrow damage. However 4 ml/kgbw of fruit extract increased absolute neutrophil counts, these changes were not significant. Data and their statistical analysis are shown in the Appendix (Table A10).

Figure 14.

Absolute neutrophil cell counts (ANC) of the pretreated mice on the 1st day after a single dose of doxorubicin



Symbols show the individual data of each mouse.

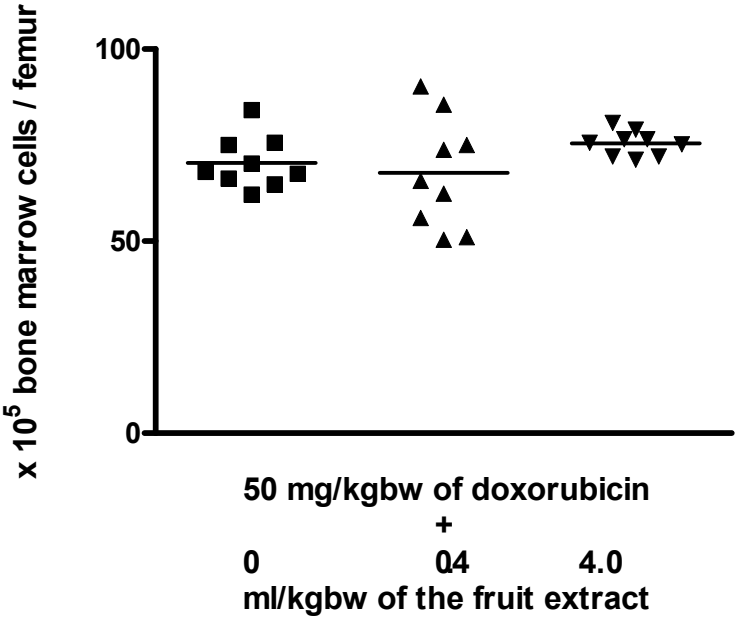
4.1.1. Results from bone marrow evaluation

4.1.1.1. Cellularity of femoral bone marrow of mice

Cellularity of femur shows the whole cell mass of femoral bone marrow of mice. Fruit extract in the used doses for 7 consecutive days preceding doxorubicin dose did not influence cellularity of the femoral bone marrow of mice. Although the mean of cellularity data of the mice pretreated with 4 ml/kgbw of fruit extract was higher than the control values of the mice treated with doxorubicin alone, difference was not significant (Figure 15). Data and their statistical analysis are shown in the Appendix (Table A11).

Figure 15.

Cellularity of bone marrow of the pretreated mice on the 1st day after a single dose of doxorubicin



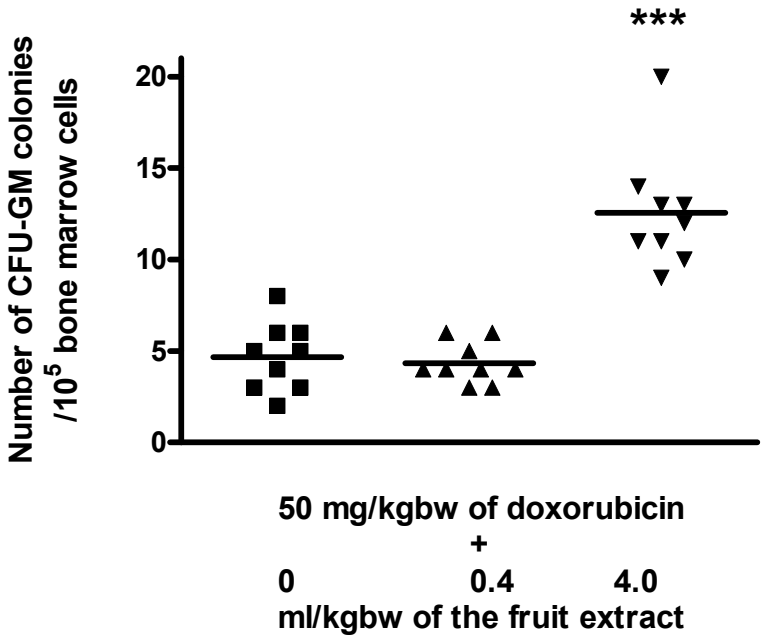
Symbols show the individual data of each mouse.

4.1.1.2. CFU-GM colonies in femoral bone marrow of mice

Colonies growing in bone marrow cell cultures of 10^5 bone marrow cells show frequency of CFU-GM progenitors in bone marrow. The studied fruit extract in 4 ml/kgbw dose increased significantly numbers of colonies of the femoral bone marrow of mice comparing to the numbers of CFU-GM colonies of mice treated with doxorubicin alone ($P < 0.001$). The 7-day long pretreatment protected successfully granulocyte-macrophage progenitor cells from doxorubicin damage. (Figure 16) Data and their statistical analysis are shown in the Appendix (Table A12).

Figure 16.

Numbers of CFU-GM colonies in bone marrow of the pretreated mice on the 1st day after a single dose of doxorubicin



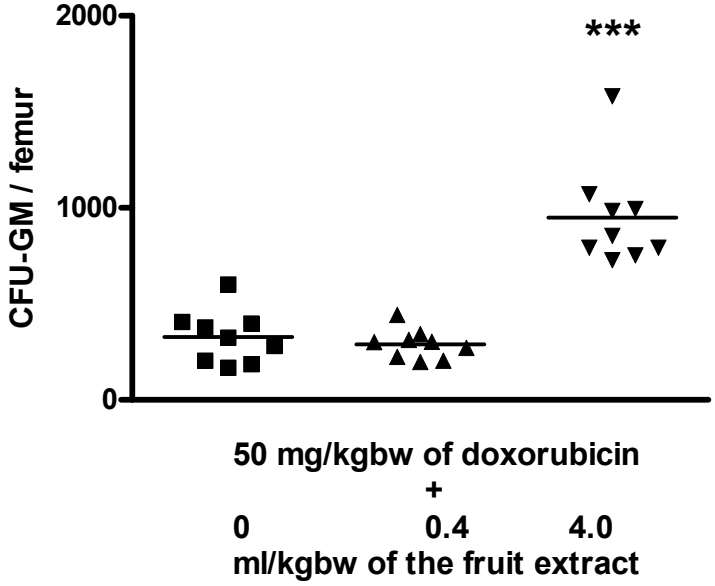
Symbols show the individual data of each mouse. Stars in the figure show the significant differences from the values of doxorubicin treated group ($P < 0.001$).

4.1.1.3. CFU-GM contents of femora of mice

Fruit extract in 4 ml/kgbw dose for 7 consecutive days preceding doxorubicin dose increased CFU-GM pool of the femoral bone marrow of mice significantly, compared with the values of the control mice in group treated with doxorubicin alone ($P < 0.001$). It could successfully protect granulocyte-macrophage progenitor cells from the great damage caused by doxorubicin. (Figure 17). Data and their statistical analysis are shown in the Appendix (Table A13).

Figure 17.

CFU-GM content of femoral bone marrow of the pretreated mice on the 1st day after a single dose of doxorubicin



Symbols show the individual data of each mouse.

Stars in the figure show the significant differences from the values of doxorubicin- treated group ($P < 0.001$).

6. DISCUSSION AND CONCLUSIONS

6.1. EFFECTS OF DOXORUBICIN ON HEMOPOIESIS

The first aim of this study was to investigate damage of hemopoiesis after a single doxorubicin dose in the early period of bone marrow regeneration, namely on the 1st day after the damage.

Myelosuppression is the most common dose-limiting toxicity encountered with cytostatic drugs (Wintrobe 1999). Cytostatic agents inhibit proliferation of hemopoietic stem and progenitor cells in bone marrow. As stem cells have much longer duplicating times than progenitors, progenitor cells are more sensitive to such damage. Due to damage of hemopoietic progenitors they will not be able to compensate for the loss of mature blood cells possessing a short life-span. The major target is the granulocyte-macrophage progenitor cell (CFU-GM), which is responsible for supplying the human body with monocytes and macrophages. If CFU-GM pool of bone marrow decreases, neutropenia can be observed in blood.

A single doxorubicin dose causes damage in hemopoiesis dose-dependently in the studied dose range. In the femoral bone marrow cellularity decreased to about half, due to 50 mg/kbw of doxorubicin. Granulocyte-macrophage colony-forming cells (CFU-GM) were especially sensitive to the damage caused by doxorubicin. Dose-effect curves related to CFU-GM colony numbers and CFU-GM content of femora of the mice were as steep as the dose-effect curve related to the cellularity, but 50 mg/kgbw of doxorubicin caused greater decrease in these values than in the whole cellular content of bone marrow. The fifty percent inhibitory concentrations were 44-48 mg/kgbw. Due to the damage of granulocyte-macrophage progenitor cells the lack of the mature cells in the peripheral blood could be seen only after a latent period, thus the absolute neutrophil counts were normal on the 1st day of the bone marrow regeneration. To evaluate whether the studied fruit extract, FLAVIN7® has any effect on all the previously measured parameters of the bone marrow damage caused by doxorubicin the 50 mg/kgbw of doxorubicin dose was chosen.

6.2. EFFECTS OF FLAVIN7® ON DAMAGE OF HEMOPOIESIS CAUSED BY A SINGLE DOXORUBICIN DOSE

The second aim of this study is to investigate whether

the fruit extract with high polyphenol content (Flavin7®), can protect hemopoiesis of bone marrow from damage caused by doxorubicin *in vivo* in mice.

Flavonoids are very common and widespread secondary plant metabolites. They have a wide range of biological and physiological activities and serve as chemotaxonomic marker compounds. Flavonoids and other polyphenol compounds are present in all green plants. They have a great importance in life of plants. A plant produces more flavonoids as a protective response when it undergoes stress, such as attack by fungi and bacteria or exposure to ultraviolet radiation. They have some protective effects in the cells in partly due to their antioxidant effects. Increased flavonoid levels are thought to enhance certain biological functions in humans. Indeed some flavonoids have antioxidative, cardioprotective, possibly anticarcinogenic and chemopreventive effects. Therefore, they have been extensively investigated both in the past and during recent years.

A number of naturally occurring flavonoids from herbs have shown chemopreventive properties against carcinogenesis using *in vitro* and in animal models (Chen et al. 1998, Fukutake et al. 2000, Lahiri-Chatterjee et al. 1999, Zheng et al. 1997). Chemoprevention refers to the application of compounds to block, reverse or prevent the development of invasive cancers (Young and Wilson 2002). Numerous plant-derived polyphenolic compounds with antioxidant and free radical scavenging properties can prevent tumour formation (Surh 1999). Epidemiological evidence suggests that diets rich in fruit and vegetables decrease the risk of premature mortality from major clinical conditions, including cancer and heart disease (Andlauer et al. 1998, Block et al. 1992, Duthie GG 2003). However, it is not yet clear which components or

combination of components in fruit and vegetables are protective and what is their mechanism of action.

As some chemoprotective agents have also myeloprotective effects we thought that it is worth to investigate whether the FLAVIN7® can protect bone marrow from damage caused by an anticancer drug. Cacao liquid polyphenols inhibited DNA strand cleavage induced by mitomycin C, an antitumour antibiotic *in vitro* (Yamagishi et al. 2001). Doxorubicin, an another antitumour antibiotic, is used much more frequently in many different types of malignant diseases. If the polyphenolic fruit extract we investigated could also decrease DNA damage, it might protect bone marrow from damage caused by doxorubicin.

Bone marrow hemopoiesis of mice was damaged by a single 50 mg/kgbw doxorubicin dose. Effect of the fruit extract, FLAVIN7® on the damage caused in the previously described way was studied. The question was whether it could influence the measured parameters of the hemopoietic damage. Mice were pretreated with the fruit extract for 7 consecutive days before doxorubicin treatment. The fruit extract was administered by gavage per orally. The control mice were given vehicle instead of the fruit extract before the same doxorubicin dose.

Neither cellularity of bone marrow nor white blood cell counts were influenced by the fruit extract. This fruit extract, however, specifically protected granulocyte-macrophage progenitor cells. CFU-GM colony numbers were significantly higher in the group which was treated with 4 ml/kgbw of fruit extract before doxorubicin ($P < 0.01$). It involved more intensive proliferation of the granulocyte-macrophage progenitor cells than in the control group treated with doxorubicin alone. Due to the more intensive proliferation replacing the damaged CFU-GM pool was not only faster but CFU-GM pool expanded. CFU-GM content of femur was significantly greater in mice treated with the fruit extract in 4 ml/kgbw dose than the values of the control group ($P < 0.01$). In the peripheral blood samples absolute neutrophil counts did not significantly differ in the pretreated mice from the control values, however, mean was higher than the control

value, namely it was 0,86 x10^9/L in the pretreated group and 0,67 x10^9/L in the group treated with doxorubicin alone.

From these results we can conclude that FLAVIN7® protects bone marrow's granulocyte-macrophage progenitor cells.

This is of great importance, as during neutropenic period originated from the damage of granulocyte-macrophage progenitor cells severe infections may develop with high mortality. An increased risk of infectious complications occurs directly related to the degree and duration of granulocytopenia (Pizzo 1993). Leading causes of mortality of patients with malignant tumours are infections associated with neutropenia (Harrison 2001). It is a clinical experience that despite modern antibiotic and antifungal therapy these serious infections will not improve until ANC values of patients are normalized (Bodey et al. 1994; Grauer et al. 1994). For these patients bacterio- and fungistatic agents are not good for therapy because macrophages are not able to facilitate their effects (Downhour et al. 2002). High toxic doses of antiinfective drugs are required for patients with bad condition. This increases toxicity and worsens life expectancies.

On the other hand, flavonoids have antiproliferative effects in some tumour and leukemia cell lines (Ito et al. 1999). The previous observations encourage to develop also synthetic flavonoids against malignant diseases. A synthetic flavonoid, flavopiridol is a hopeful anticancer effect in chronic B-cell leukemia (König et al. 1997). Flavonoids may have a very good combinative effects in malignant diseases as they may protect normal bone marrow progenitor cells from damages caused by anticancer drugs together with an antiproliferative effects on the malignant tumour cells (Faderl et Estrov 2003).

By supplying more progenitors for the more intensive granulopoiesis this fruit extract could decrease the immuno- and myelosuppressive effects of cytostatic drugs or other toxic molecules, which can decrease mortality.

7. SUGGESTIONS

On the basis of our results, the fruit extract, FLAVIN7®, seems to be a very promising product. Antracycline cytostatic drugs including doxorubicin are widely used and a n agent or combination of various compounds which can prevent the myelotoxicity and possibly the cardiotoxicity of these cytostatic drugs has a great importance. To determine the effective dose range, of course, repeated experiments using several other doses of the fruit extract are necessary.

It would be desirable to study, whether FLAVIN7® can show similar advantageous effects on bone marrow regeneration after damage caused also by other cytostatic agents, e.g. carboplatin, cyclophosphamide or damage caused by irradiation.

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9. APPENDIX

Table A1. Body weight of the mice treated with a single doxorubicin dose prior to the experiment

mouse No.	Body weights (g) of mice treated with				
	0	25	50	100	200
	mg/kgbw of doxorubicin				
1.	25.5	24.9	27.3	26.3	25.6
2.	26.7	23.3	25.8	25.6	25.7
3.	26.8	27.4	26.9	25.4	262
4.	25.5	26.4	25.7	25.7	27.0
5.	24.9	27.3	25.2	26.9	25.8
6.	25.5	25.0	25.6	24.5	26.8
mean	25.72	26.08	25.73	25.83	26.18
	±	±	±	±	±
SEM	0.6539	0.3361	0.3333	0.1856	0.2428

STATISTICAL ANALYSIS:

One-way analysis of variance

P value 0,8869

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 5

F 0,2819

R squared 0,04316

Bartlett's test for equal variances

Bartlett's statistic (corrected) 5,923

P value 0,2050

P value summary ns

Do the variances differ signif. (P < 0.05) No

ANOVA Table	SS	df	MS
Treatment (between columns)	1,092	4	0,2730
Residual (within columns)	24,21	25	0,9683
Total	25,30	29	

Bonferroni's Multiple Comparison Test

	Mean Diff.	**t**	**P value**
0 vs 25	0,1000	0,1760	P > 0.05
0 vs 50	-0,2667	0,4694	P > 0.05
0 vs 100	0,08333	0,1467	P > 0.05
0 vs 200	-0,3667	0,6454	P > 0.05
25 vs 50	-0,3667	0,6454	P > 0.05
25 vs 100	-0,01666	0,02933	P > 0.05
25 vs 200	-0,4667	0,8214	P > 0.05
50 vs 100	0,3500	0,6161	P > 0.05
50 vs 200	-0,1000	0,1760	P > 0.05
100 vs 200	-0,4500	0,7921	P > 0.05

Table A2. Body weight of the mice treated with a single doxorubicin dose 1 day after the treatment

mouse No.	Body weights (g) of mice treated with				
	0	25	50	100	200
	mg/kgbw of doxorubicin				
1	27.7	26.8	27.8	24.7	25.0
2	28.5	26.8	25.5	25.9	24.6
3	25.0	27.4	26.3	25.5	25.4
4	25.0	26.6	25.7	25.0	-
5	23.2	27.2	25.7	26.7	-
6	26.7	24.9	24.6	25.3	-
mean	26.62	25.93	25.52	25.00	26.62
	±	±	±	±	±
SEM	0.3637	0.4356	0.2903	0.2309	0.3637

STATISTICAL ANALYSIS

One-way analysis of variance

P value 0,3749

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 5

F 1,115

R squared 0,1685

ANOVA Table	SS	df	MS
Treatment (between columns)	6,480	4	1,620
Residual (within columns)	31,98	22	1,454
Total	38,46	26	

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value
0 vs 25	-0,6000	0,8620	P > 0.05
0 vs 50	0,08333	0,1197	P > 0.05
0 vs 100	0,5000	0,7183	P > 0.05
0 vs 200	1,017	1,193	P > 0.05
25 vs 50	0,6833	0,9817	P > 0.05
25 vs 100	1,100	1,580	P > 0.05
25 vs 200	1,617	1,896	P > 0.05
50 vs 100	0,4167	0,5986	P > 0.05
50 vs 200	0,9333	1,095	P > 0.05
100 vs 200	0,5167	0,6061	P > 0.05

STATISTICAL ANALYSIS

AMONG BODY WEIGHTS ON THE 0. AND THE 1ST DAY

One-way analysis of variance

P value 0,7022

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 10

F 0,7038

R squared 0,1188

ANOVA Table	SS	df	MS
Treatment (between columns)	7,572	9	0,8414
Residual (within columns)	56,18	47	1,195
Total	63,76	56	

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value
0 vs 1.DAY 0	-0,2000	0,3168	P > 0.05
0 vs 1. DAY 25	-0,8000	1,267	P > 0.05
0 vs 1.DAY 50	-0,1167	0,1848	P > 0.05
0 vs 1.DAY 100	0,3000	0,4752	P > 0.05
0 vs 1.DAY 200	0,8167	1,056	P > 0.05

Table A3. White blood cell (WBC) of the mice treated with a single doxorubicin dose 1 day after the treatment

mouse No.	WBC (x 10^9 / L) of mice treated with				
	0	25	50	100	200
	mg/kgbw of doxorubicin				
1.	3.8	5.9	7.0	7.6	2.5
2.	4.3	6.8	5.1	4.7	8.0
3.	9.8	4.5	4.6	5.3	3.5
4.	7.2	6.5	3.9	6.1	-
5.	5.2	3.9	6.7	6.8	-
6.	3.8	5.1	5.8	4.5	-
mean	5.683	5.450	5.517	5.833	4.667
	±	±	±	±	±
SEM	0.9745	0.4674	0.4936	0.4991	1.691

STATISTICAL ANALYSIS

One-way analysis of variance

P value 0,9107

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 5

F 0,2432

R squared 0,04235

ANOVA Table	SS	df	MS
Treatment (between columns)	2,962	4	0,7406
Residual (within columns)	66,99	22	3,045
Total	69,95	26	

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value
0 vs 25	0,2333	0,2316	P > 0.05
0 vs 50	0,1667	0,1654	P > 0.05
0 vs 100	-0,1500	0,1489	P > 0.05
0 vs 200	1,017	0,8239	P > 0.05
25 vs 50	-0,06667	0,06617	P > 0.05
25 vs 100	-0,3833	0,3805	P > 0.05
25 vs 200	0,7833	0,6348	P > 0.05
50 vs 100	-0,3167	0,3143	P > 0.05
50 vs 200	0,8500	0,6889	P > 0.05
100 vs 200	1,167	0,9455	P > 0.05

Table A4. Absolute neutrophil cell count (ANC) of the mice treated with a single doxorubicin dose 1 day after the treatment

mouse No.	ANC (x 10^9 / L) of mice treated with				
	0	25	50	100	200
	mg/kgbw of doxorubicin				
1.	0.41	0.35	0.49	0.97	0.51
2.	0.39	0.54	0.26	0.53	0.56
3.	0.88	0.41	0.23	0.42	0.39
4.	0.43	0.78	0.31	0.43	-
5.	0.36	0.27	0.27	0.27	-
6.	0.62	0.41	0.75	0.41	-
mean	5.683	5.450	5.517	5.833	4.667
	±	±	±	±	±
SEM	0.9745	0.4674	0.4936	0.4991	1.691

STATISTICAL ANALYSIS

One-way analysis of variance

P value 0,8043

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 5

F 0,4032

R squared 0,06830

ANOVA Table	SS	df	MS
Treatment (between columns)	0,06422	4	0,01606
Residual (within columns)	0,8761 22		0,03982
Total	0,9403 26		

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value
0 vs 25	0,05500	0,4774	P > 0.05
0 vs 50	0,1300	1,128	P > 0.05
0 vs 100	0,01000	0,08679	P > 0.05
0 vs 200	0,02833	0,2008	P > 0.05
25 vs 50	0,07500	0,6510	P > 0.05
25 vs 100	-0,04500	0,3906	P > 0.05
25 vs 200	-0,02667	0,1890	P > 0.05
50 vs 100	-0,1200	1,042	P > 0.05
50 vs 200	-0,1017	0,7205	P > 0.05
100 vs 200	0,01833	0,1299	P > 0.05

Table A5. Cellularity of femoral bone marrow of the mice treated with a single doxorubicin dose 1 day after the treatment

mouse No.	Cellularity (x 10^5 / femur) of mice treated with				
	0	25	50	100	200
	mg/kgbw of doxorubicin				
1.	215.6	183.6	70.1	50.9	39.5
2.	137.7	196.2	82.6	45.8	43.2
3.	206.1	144.9	64.8	63.2	47.6
4.	121.6	175.5	57.9	44.0	-
5.	140.4	136.0	77.3	69.2	-
6.	136.0	168.0	74.3	49.5	-
mean	159.6	167.4	71.17	53.77	43.43
	±	±	±	±	±
SEM	16.49	9.399	3.632	4.133	2.341

STATISTICAL ANALYSIS

One-way analysis of variance

P value P<0.0001

P value summary ***

Are means signif. different? (P < 0.05) Yes

Number of groups 5

F 35,21

R squared 0,8649

ANOVA Table	SS	df	MS
Treatment (between columns)	75200	4	18800
Residual (within columns)	11740	22	533,8
Total	86940	26	

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value
0 vs 25	-7,792	0,5841	P > 0.05
0 vs 50	88,41	6,628	P < 0.001
0 vs 100	105,8	7,932	P < 0.001
0 vs 200	116,1	7,109	P < 0.001
25 vs 50	96,20	7,212	P < 0.001
25 vs 100	113,6	8,516	P < 0.001
25 vs 200	123,9	7,586	P < 0.001
50 vs 100	17,40	1,304	P > 0.05
50 vs 200	27,73	1,698	P > 0.05
100 vs 200	10,33	0,6325	P > 0.05

Table A6. CFU-GM colony formation from femoral bone marrow of the mice treated with a single doxorubicin dose 1 day after the treatment

mouse No.	Numbers of CFU-GM colonies / 10^5 bone marrow cells of mice treated with				
	0	25	50	100	200
	mg/kgbw of doxorubicin				
1.	63	66	5	2	1
2.	68	73	5	1	0
3.	60	72	2	3	0
4.	67	70	3	1	
5.	65	60	3	2	
6.	71	59	9	0	
mean	65.67	66.67	4.500	1.500	0.3333
	±	±	±	±	±
SEM	1.585	2.472	1.025	0.4282	0.3333

STATISTICAL ANALYSIS

One-way analysis of variance

P value P<0.0001

P value summary ***

Are means signif. different? (P < 0.05) Yes

Number of groups 5

F 502,9

R squared 0,9892

ANOVA Table	SS	df	MS
Treatment (between columns)	27100	4	6775
Residual (within columns)	296,3	22	13,47
Total	27390	26	

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value
0 vs 25	-1,000	0,4719	P > 0.05
0 vs 50	61,17	28,87	P < 0.001
0 vs 100	64,17	30,28	P < 0.001
0 vs 200	65,33	25,18	P < 0.001
25 vs 50	62,17	29,34	P < 0.001
25 vs 100	65,17	30,75	P < 0.001
25 vs 200	66,33	25,56	P < 0.001
50 vs 100	3,000	1,416	P > 0.05
50 vs 200	4,167	1,606	P > 0.05
100 vs 200	1,167	0,4496	P > 0.05

Table A7. CFU-GM progenitor cell content of the femoral bone marrow of the mice treated with a single doxorubicin dose 1 day after the treatment

mouse No.	CFU-GM / femur of mice treated with				
	0	25	50	100	200
	mg/kgbw of doxorubicin				
1.	13583	12078	351	102	39.5
2.	9364	14308	413	46	0
3.	12366	10368	130	190	0
4.	8147	12250	174	44	-
5.	9126	8160	232	138	-
6.	9656	9912	669	0	-
mean	10374	11179	328.2	86.67	13.17
	±	±	±	±	±
SEM	862.5	877.9	80.86	28.60	13.17

STATISTICAL ANALYSIS

One-way analysis of variance

P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	39,04
R squared	0,7649

Bartlett's test for equal variances

Bartlett's statistic (corrected)	10,63
P value	0,0049
P value summary	**
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	2479000	2	1240000
Residual (within columns)	762100	24	31750
Total	3241000	26	

Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**P value**
Column A vs Column B	38,71	0,4608	P > 0.05
Column A vs Column C	-622,6	7,412	P < 0.001
Column B vs Column C	-661,3	7,872	P < 0.001

Table A8. Body weight of the mice pretreated with FLAVIN7® before a single doxorubicin dose

mouse No.	Body weights (g) of mice treated with 50 mg/kgbw of doxorubicin and pretreated with					
	0	0.4	4.0	0	0.4	4.0
	ml/kgbw of FLAVIN7®					
	prior to the experiment			1 day after doxorubicin		
1.	23.3	22.6	27.7	25.3	24.2	29.1
2.	27.4	23.0	22.2	27.4	23.7	22.7
3.	26.4	24.4	22.5	25.0	25.7	24.0
4.	24.0	25.6	25.6	24.3	23.7	24.6
5.	26.9	25.7	27.4	26.3	27.9	26.0
6.	26.9	25.2	24.0	27.0	27.1	26.7
7.	25.8	26.6	25.0	25.5	26.7	23.1
8.	28.4	26.9	23.3	27.2	23.7	25.7
9.	24.7	23.3	27.2	24.0	24.6	26.8
mean	25.98	24.81	24.99	25.78	25.26	25.41
	±	±	±	±	±	±
SEM	0.5592	0.5237	0.7094	0.4196	0.5462	0.6767

STATISTICAL ANALYSIS

On the 0th day:

One-way analysis of variance

P value 0,3532

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 1,087

R squared 0,08308

Bartlett's test for equal variances

Bartlett's statistic (corrected)	0,8037
P value	0,6691
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	7,112	2	3,556
Residual (within columns)	78,49	24	3,271
Total	85,61	26	

Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**P value**
Column B vs Column C	1,167	1,368	P > 0.05
Column B vs Column D	0,9889	1,160	P > 0.05
Column C vs Column D	-0,1778	0,2085	P > 0.05

On the 1st day:

One-way analysis of variance

P value	0,7952
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	3
F	0,2313
R squared	0,01891

Bartlett's test for equal variances

Bartlett's statistic (corrected)	1,678
P value	0,4321
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

ANOVA Table	SS	df	MS
Treatment (between columns)	1,294	2	0,6470
Residual (within columns)	67,13	24	2,797
Total	68,42	26	

Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**P value**
Column B vs Column C	0,5222	0,6624	P > 0.05
Column B vs Column D	0,3667	0,4651	P > 0.05
Column C vs Column D	-0,1556	0,1973	P > 0.05

Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**P value**
0. day 0 vs 1.day 0	0,2000	0,2436	P > 0.05
0.day 0.4 vs 1.day 4,0	-0,4444	0,5413	P > 0.05
0.day 0,4 vs 1.day 4,0	-0,2667	0,3248	P > 0.05

Table A9. White blood cell (WBC) of the mice pretreated with FLAVIN7® before a single 50 mg/kgbw of doxorubicin dose 1 day after the treatment

Mouse No.	WBC (x 10^9 / L) of mice pretreted with		
	0	0.4	4.0
	mL/kgbw of FLAVIN7®		
1.	2.4	2.0	30
2.	3.2	4.9	4.6
3.	2.7	4.8	3.8
4.	3.2	2.5	3.9
5.	2.5	3.5	3.2
6.	2.7	2.8	3.5
7.	32	2.5	2.9
8.	3.3	5.2	3.8
9.	2.8	6.0	4.2
mean	2.889	3.800	3.656
	±	±	±
SEM	0.1136	0.4819	0.1872

STATISTICAL ANALYSIS

One-way analysis of variance

P value 0,0976

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 2,568

R squared 0,1763

Bartlett's test for equal variances

Bartlett's statistic (corrected) 15,53

P value 0,0004

P value summary ***

Do the variances differ signif. (P < 0.05) Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	4,316	2	2,158
Residual (within columns)	20,17	24	0,8405
Total	24,49	26	

Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**P value**
Column A vs Column B	-0,9111	2,108	P > 0.05
Column A vs Column C	-0,7667	1,774	P > 0.05
Column B vs Column C	0,1444	0,3342	P > 0.05

Table A10. Absolute neutrophil count (ANC) of the mice pretreated with FLAVIN7® before a single 50 mg/kgbw of doxorubicin dose 1 day after the treatment

Mouse No.	ANC (x 10^9 / L) of the mice pretreted with		
	0	0.4	4.0
	mL/kgbw of FLAVIN7®		
1.	0.430	0.180	0.48
2.	0.640	1.715	1.79
3.	1.107	0.380	0.38
4.	0.580	0.700	0.74
5.	0.550	0.420	0.99
6.	0.680	0.980	0.95
7.	0.540	1.300	0.67
8.	0.760	0.830	0.72
9.	0.530	0.900	1.01
mean	0.6463	0.8228	0.8589
	±	±	±
SEM	0.06579	0.1599	0.1373

STATISTICAL ANALYSIS

One-way analysis of variance

P value 0,4627

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 0,7961

R squared 0,06221

Bartlett's test for equal variances

Bartlett's statistic (corrected) 5,465

P value 0,0650

P value summary ns

Do the variances differ signif. (P < 0.05) No

ANOVA Table	SS	df	MS
Treatment (between columns)	0,2328	2	0,1164
Residual (within columns)	3,510	24	0,1462
Total	3,743	26	

Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**P value**
Column A vs Column B	-0,1764	0,9787	P > 0.05
Column A vs Column C	-0,2126	1,179	P > 0.05
Column B vs Column C	-0,03611	0,2003	P > 0.05

Table A11. Cellularity, total cell content of femoral bone marrow of the mice pretreated with FLAVIN7® before a single 50 mg/kgbw of doxorubicin dose 1 day after the treatment

Mouse No.	Cellularity (x 10^5 / femur) of the mice pretreted with		
	0	0.4	4.0
	mL/kgbw of FLAVIN7®		
1.	2.4	2.0	30
2.	3.2	4.9	4.6
3.	2.7	4.8	3.8
4.	3.2	2.5	3.9
5.	2.5	3.5	3.2
6.	2.7	2.8	3.5
7.	32	2.5	2.9
8.	3.3	5.2	3.8
9.	2.8	6.0	4.2
mean	2.889	3.800	3.656
	±	±	±
SEM	0.1136	0.4819	0.1872

STATISTICAL ANALYSIS

One-way analysis of variance

P value 0,2358

P value summary ns

Are means signif. different? (P < 0.05) No

Number of groups 3

F 1,535

R squared 0,1134

Bartlett's test for equal variances

Bartlett's statistic (corrected)	14,44
P value	0,0007
P value summary	***
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	270,8	2	135,4
Residual (within columns)	2117	24	88,20
Total	2388	26	

Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**P value**
Column A vs Column B	2,617	0,5911	P > 0.05
Column A vs Column C	-5,017	1,133	P > 0.05
Column B vs Column C	-7,633	1,724	P > 0.05

Table A12. CFU-GM colony number of femoral bone marrow of the mice pretreated with FLAVIN7® before a single 50 mg/kgbw of doxorubicin dose 1 day after the treatment

Mouse No.	CFU-GM colonies (x / 10^5 bone marrow cells) of the mice pretreted with		
	0	0.4	4.0
	mL/kgbw of FLAVIN7®		
1.	3	3	10
2.	4	5	9
3.	5	4	11
4.	2	4	13
5.	3	6	11
6.	8	4	20
7.	6	3	14
8.	5	4	13
9.	6	6	12
mean	4.667	4.333	12.56
	±	±	±
SEM	0.6236	0.3727	1.069

STATISTICAL ANALYSIS

One-way analysis of variance

P value P<0.0001

P value summary ***

Are means signif. different? (P < 0.05) Yes

Number of groups 3

F 38,91

R squared 0,7643

Bartlett's test for equal variances

Bartlett's statistic (corrected)	7,791
P value	0,0203
P value summary	*
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	389,9	2	194,9
Residual (within columns)	120,2	24	5,009
Total	510,1	26	

Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**P value**
Column A vs Column B	0,3333	0,3159	P > 0.05
Column A vs Column C	-7,889	7,477	P < 0.001
Column B vs Column C	-8,222	7,793	P < 0.001

Table A13. CFU-GM content of femoral bone marrow of the mice pretreated with FLAVIN7® before a single 50 mg/kgbw of doxorubicin dose 1 day after the treatment

Mouse No.	CFU-GM / femur of the mice pretreted with		
	0	0.4	4.0
	mL/kgbw of FLAVIN7®		
1.	186.3	197.10	752.00
2.	280.8	312.00	726.75
3.	378.0	224.20	792.00
4.	168.3	300.20	983.45
5.	204.0	442.80	792.00
6.	600.4	342.00	1581.00
7.	405.0	270.75	1071.00
8.	324.0	204.00	994.50
9.	397.0	302.40	854.40
mean	327.1	288.4	949.7
	±	±	±
SEM	45.73	25.68	88.51

STATISTICAL ANALYSIS

One-way analysis of variance

P value P<0.0001

P value summary ***

Are means signif. different? (P < 0.05) Yes

Number of groups 3

F 39,04

R squared 0,7649

Bartlett's test for equal variances

Bartlett's statistic (corrected)	10,63
P value	0,0049
P value summary	**
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	2479000	2	1240000
Residual (within columns)	762100	24	31750
Total	3241000	26	

Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**P value**
Column A vs Column B	38,71	0,4608	P > 0.05
Column A vs Column C	-622,6	7,412	P < 0.001
Column B vs Column C	-661,3	7,872	P < 0.001