



## Antioxidant and immuno-enhancing effects of *Echinacea purpurea* (American herb) *in vivo*

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### SUMMARY

We studied the protective effect of *Echinacea purpurea* against radiation by evaluating changes in the peripheral blood cell count and peripheral blood antioxidant activity. *Echinacea purpurea* administration had a suppressive effect on radiation-induced leukopenia, especially on lymphocytes and monocytes and resulted in a faster recovery of blood cell counts. Mouse peripheral blood antioxidant activity was increased by *Echinacea purpurea*, and a relationship between the suppressive effect on radiation-induced leukopenia and the antioxidant effect was suggested.

**Key words:** *Echinacea purpurea*; Immunology; Radiation protection; Antioxidant

### INTRODUCTION

Radiation can have tremendous therapeutic benefits for humans; however, it is also associated with the risk of serious adverse effects. Examples of radiation-protective agents that have been clinically include: SH compounds, such as cysteine and WR-2721 (amifostine), which remove radicals produced by radiation and thereby protect the body from the indirect effects of radiation (Georgieva *et al.*, 2002; Andreassen *et al.*, 2003); granulocyte colony-stimulating factor (G-CSF), which prevents immunosuppression from radiation exposure; and anti-immunosuppressives, such as OK-432 (Jorgensen *et al.*, 2003; Yang *et al.*, 2003).

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However, these medications have the potential to cause serious adverse effects, particularly when combined with other medications. With this limitation in mind, new medications derived from naturally occurring materials, which have fewer side effects and greater radiation protective potential, have been studied and developed.

Recently, many traditional medicinal herbs have been re-evaluated as therapeutic agents. Several medicinal herbs have been shown to have beneficial pharmacological and physiological effects, and since they may have less harmful direct effects and side effects than other medicines, there has been increasing interest in and demand for such products.

We took note of *Echinacea* (*Echinacea* genus, *Chrysanthemum* family), which is one of the most widely used medical herbs. *Echinacea* is a perennial plant native to limited areas of the western and central desert regions of North America, and is now grown throughout the world for decoration,

food, and medical use. Echinacea is considered to be a food by the U.S. Food and Drug Administration and as a medical ingredient by Commission E (Task Force E of the Federal Bureau of Health of Germany). It is used as an ingredient in over 280 types of plant-based medicines. In Japan in March 1998, it was added to the class 2 food category.

Echinacea has been used as a medicinal herb by Native Americans since ancient times. They chewed dried Echinacea, roots and all, using it as a panacea for various ailments such as infection, injury, inflammation, and fever (Moerman, 1998). In the early 1970's, the German doctor H.C.F. Meyer introduced Echinacea to the world. Since then its pharmacological effects have been widely studied. Antiviral (Beuscher *et al.*, 1995; Kwarek *et al.*, 1996), antibacterial (Brown, 1986), and immuno-enhancing (Bauer *et al.*, 1988; Bukovsky *et al.*, 1993) effects have been reported.

The main components of Echinacea are: polysaccharides (Agnier *et al.*, 1985), caffeic acid derivatives, flavonoids, polyacetylene, and glycoproteins (Jacobson, 1967; Becker *et al.*, 1982; Bauer *et al.*, 1989). Among these components, Echinacin, a peculiar glucose chain derived from Echinacea, has been reported to attach to the surface of T cells and macrophages, which protect the body from viral attack, and to activate them (Goel *et al.*, 2002a; 2002b). Echinacea also contains many types of polyphenols - antioxidants that are also found in red wine and which eliminate reactive oxygen species known to cause aging and cancer. The polyphenols present in large amounts in Echinacea are caffeic acid and Echinacoside. The immune system effects are greatly increased due to the combination of these various components. In addition, it has been reported that Echinacea has an interferon-like effect, activating macrophages and inducing production of interleukin-1 and interferon (Rininger *et al.*, 2000).

Recently the ability of Echinacea to alleviate allergies and AIDS has been studied. See *et al.* (1997) reported that Echinacea enhanced some immune functions in both healthy people and

AIDS patients. There are nine known species of Echinacea. Therapeutic effects have been reported in three species, *Echinacea purpurea*, *Echinacea pallida*, and *Echinacea angustifolia*, which are all used as medical herbs (Bauer, 2002). In this study, we used *Echinacea purpurea* (*Echinacea purpurea*) which has been studied the longest among these three medical herbs and which has greater physiological activity compared to the other two (Luettig *et al.*, 1989).

An important marker in the body for assessing the protective effect of chemical agents against radiation is the blood cell count, since hematopoietic tissue and lymphocytes in peripheral blood are highly radiosensitive. Immune function declines severely as a result of decreases in leukocytes and myeloid cells due to radiation exposure (Hall, 2000).

We studied the antioxidant and immuno-stimulating effects of *Echinacea purpurea* and evaluated changes in the peripheral blood cell count and peripheral blood antioxidant activity due to radiation exposure.

## MATERIALS AND METHODS

### Experimental animals

Five week old male ICR [Crj: CD-1 (Swiss Hanchka)] mice with an average weight of 18-20 g were purchased from Japan SLC Inc. and kept under standard conditions (room temperature 22 +/- 3°C, humidity 60%) and consistent feeding (CA-1, Japan Clare, Inc.) and drinking water (Top-water). The mice were acclimated to the breeding and experimental environment for one week prior to experimentation.

### *Echinacea purpurea*

Whole plants of *Echinacea purpurea* in the flowering season (July) were pressed, and then the extracted juice was dried and refined to a powder, which was used for this study. *Echinacea purpurea* dried powder was suspended in saline and the suspension was then administered into the abdominal cavity

of mice at a dose of 360 mg/kg every other day. Mice were used for experiments after at least three weeks of *Echinacea purpurea* administration and *Echinacea purpurea* administration was continued until the end of the experiment.

#### Application of radiation

Two Gy of x-ray whole body irradiation was applied to each mouse using an x-ray generator designed for animal use (Phillips, Inc.). The table was rotated at a constant speed so that the mouse, restrained in a plastic jig, was irradiated evenly. The conditions for irradiation were: source voltage, 200 kV; rate of radiation, 0.35 Gy/min; and supplemental filter, 0.1 mm Cu + 1 mm Al.

#### Measurement of peripheral blood cell counts in mice

Changes in peripheral blood cell counts were studied in 4 groups of mice: a control group, which was administered saline; an *Echinacea purpurea* only group, which was administered *Echinacea purpurea*; an irradiation only group, which was irradiated with 2 Gy of x-ray (2 Gy); and an *Echinacea purpurea* and irradiation group, which was irradiated with 2 Gy of x-ray after administration of *Echinacea purpurea* (*Echinacea purpurea* + 2 Gy). Ten mice for each group were used in the experiment.

The tail vein of each mouse was cut with a Spitz knife and 10  $\mu$ l of peripheral blood was collected with a capillary tube. The blood cell count was performed with an automated blood cell counter (Celltac-a MEK-6318, Nippon Koden Inc.). The number of peripheral leukocytes, lymphocytes, granulocytes, and monocytes, which all have relatively high sensitivity to radiation, and primary cells of the immune system were counted. In order to observe changes in the peripheral blood cell counts, measurements were done on the day preceding irradiation and at 3 hours, 12 hours, 24 hours, 3 days, 7 days, 15 days, and 30 days after irradiation. Statistical analysis was performed by a parametric ANOVA test among the groups to determine significant differences in blood cell counts for each group.

#### Measurement of serum SOD activity in mouse peripheral blood

Antioxidant activity was studied in two groups of mice: a control group that was administered saline and an *Echinacea purpurea* group that was administered *Echinacea purpurea* (n = 10 per group).

Serum SOD activity was measured by the nitroblue tetrazolium (NBT) reduction method using an SOD Activity Detection Kit (Wako Junyaku Industry, Inc.). The NBT reduction method measures SOD activity using NBT as a detector for  $O_2^-$ , coupling an  $O_2^-$  production reaction (xanthine - xanthine oxidase) with the disparity reaction by SOD, and measuring the rate of decline in reduction coloration by  $O_2^-$  as the rate of inhibition. This method is suitable for the quantitative measurement of antioxidant activity (Sloley *et al.*, 2001). The basic measurement protocol is as follows. In anesthetized mice, whole blood was collected from the heart using a syringe (Terumo Company) with a 23 gauge needle, heparin (5 units/ml) was added, and the serum was separated from whole blood by centrifugation at 1,500 rpm for 15 minutes. Next, 10  $\mu$ l/well of sample was added to a 96-well microplate. The samples were specimen (S), blind (Bl), specimen-blind (S-Bl), and reagent-blind (Bl-Bl). The serum for specimen (S) and specimen-blind (S-Bl) and distilled water for blind (Bl) and reagent-blind (Bl-Bl) were added. After the addition of the samples, 100  $\mu$ l/well of the coloration reagent [0.1 M phosphate buffer, pH 8.0; 0.4 mM xanthine; and 0.24 mM NBT] was added and the plates were stirred for 1 minute. After stirring, 100  $\mu$ l/well of the enzyme solution (0.1 M phosphate buffer, pH 8.0; xanthine oxidase, 0.049 units/ml) for the specimen (S) and blind (Bl) samples, and 100  $\mu$ l/well of the blank solution (0.1 M phosphate buffer, pH 8.0) for the specimen-blind (S-Bl) and reagent-blind (Bl-Bl) samples, were added and stirred for 1 minute, followed by incubation at 37°C for 28 minutes. After the incubation, 20  $\mu$ l/well of the reaction-quenching reagent (69 mM dodecyl sodium sulfate) was added to each sample, stirred for 5

minutes, and the absorbance was measured using a microplate reader MPR A4 (Toyo Sotatsu Inc.) at a wavelength of 560 nm. SOD activity was determined from the absorbance according to the formula (1)

SOD activity (inhibition rate %) =

$$\frac{(E_{BI} - E_{BI-BI}) - (E_S - E_{S-BI})}{(E_{BI} - E_{BI-BI})} \times 100 \quad (1)$$

$E_S$ : absorbance of specimen

$E_{BI}$ : absorbance of blind

$E_{S-BI}$ : absorbance of specimen-blind

$E_{BI-BI}$ : absorbance of reagent-blind

We did blood coagulation prevention sake heparin processing (five unit/ml) that collected blood of whole blood and divided only serum. Therefore, serum concentration is about 20%.

Significant differences in SOD activity between group pairs were determined by using a non-parametric Wilcoxon test.

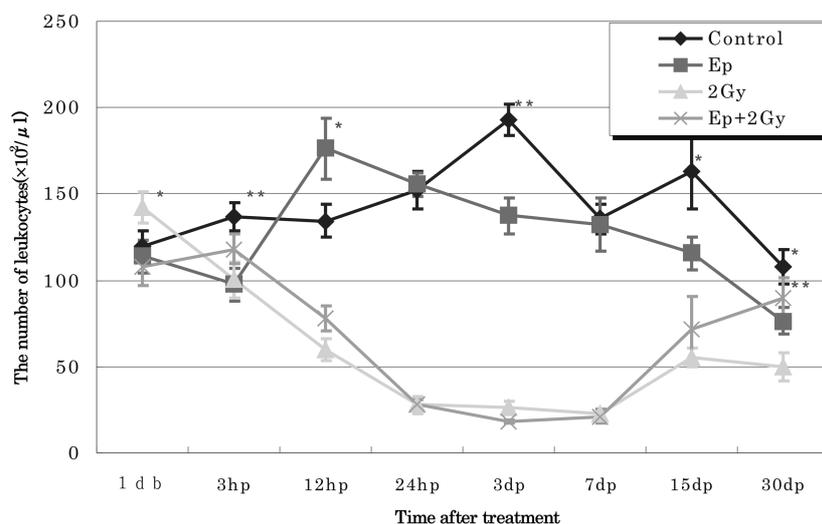
## RESULTS

### Changes in mouse peripheral blood cell counts

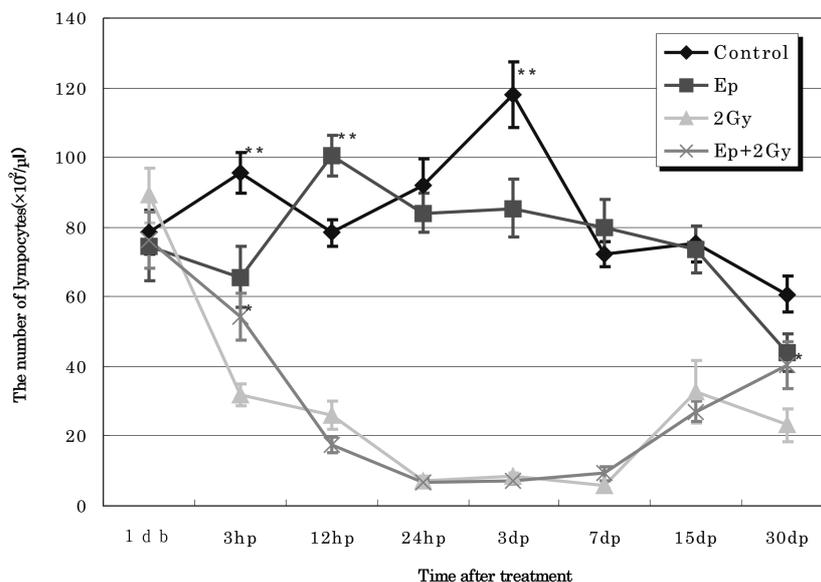
The study of cytopenia following irradiation (Fig.

1-4) showed that the number of leukocytes in the irradiation only group (2 Gy group) declined markedly, while the decline was suppressed in the *Echinacea purpurea* and irradiation group (*Echinacea purpurea* + 2 Gy group), although the suppression was not statistically significant ( $P=0.06$ ). *Echinacea purpurea* administration also suppressed irradiation-induced cytopenia of leukocytes. However, there was a slight difference in the influence due to differences in radio-sensitivity and the life span of the cells. In contrast to the effects observed for other blood cells, granulocyte counts were transiently increased by irradiation, due to an influx from the reserve blood pool associated in response to cytopenia. The degree of this increase was proportional to the degree of cytopenia.

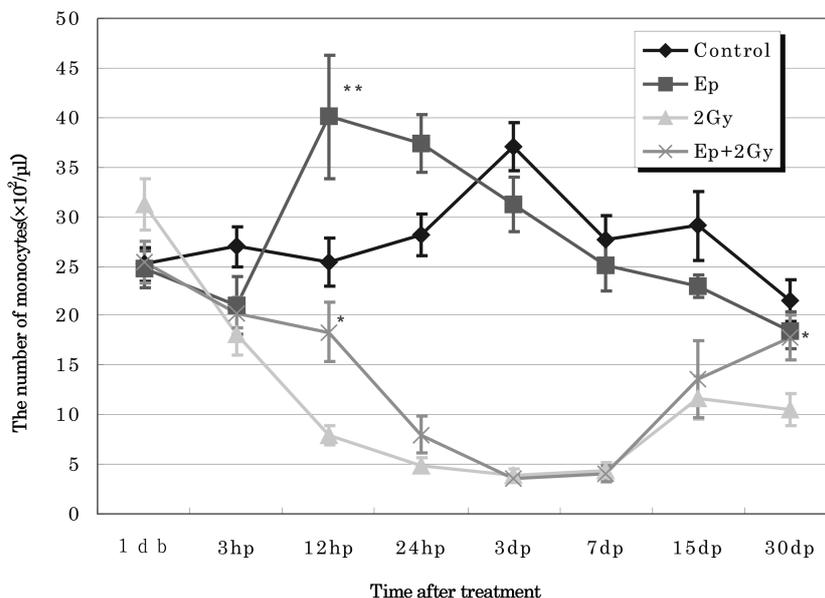
The study of blood cell count recovery after irradiation showed that leukocyte count recovery after irradiation occurred significantly faster in the *Echinacea purpurea* and irradiation group (*Echinacea purpurea* + 2 Gy group) than in the irradiation only group (2 Gy group) ( $P<0.01$ ). Although there is little difference in radio-sensitivity and lifespan of among lymphocytes, granulocytes, and monocytes, the recovery in the number of these cell types after



**Fig. 1.** The change in the number of leukocytes in the blood taken from the tail vein of whole body irradiated mice. Each lineargram represents the mean value  $\pm$  SE leukocytes from 10 mice (M). Significantly different \* $P<0.05$ , \*\* $P<0.01$  Control vs Ep, 2Gy vs Ep+2Gy (1dp-1 day point,3hp-3 hour point).



**Fig. 2.** The change in the number of lymphocytes in the blood taken from the tail vein of whole body irradiated mice. Each line graph represents the mean value  $\pm$  SE lymphocytes from 10 mice (M). Significantly different \* $P < 0.05$ , \*\* $P < 0.01$  Control vs Ep, 2Gy vs Ep+2Gy (1dp-1 day point, 3hp-3 hour point).

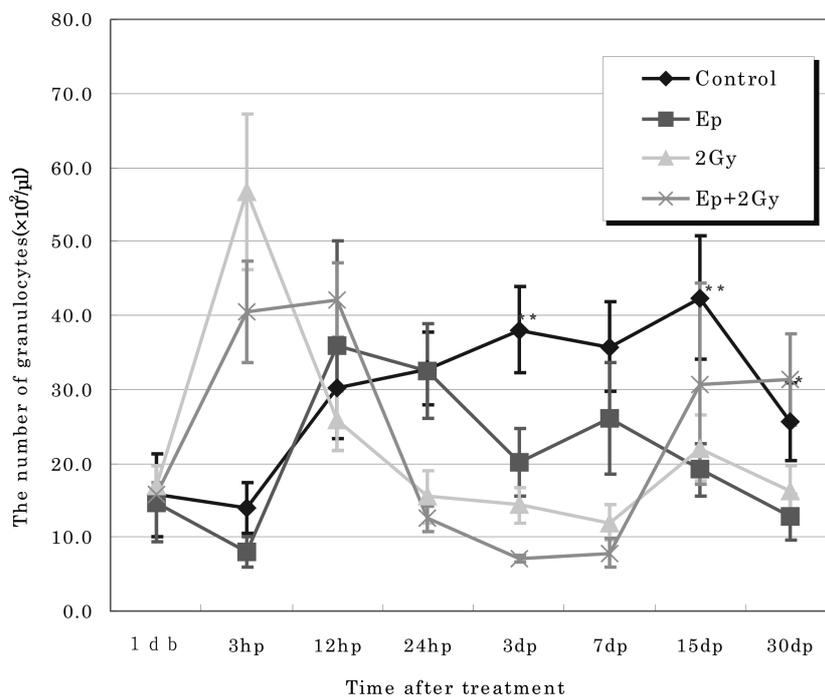


**Fig. 3.** The change in the number of monocytes in the blood taken from the tail vein of whole body irradiated mice. Each line graph represents the mean value  $\pm$  SE monocytes from 10 mice (M). Significantly different \* $P < 0.05$ , \*\* $P < 0.01$  Control vs Ep, 2Gy vs Ep+Ep (1dp-1 day point, 3hp-3 hour point).

irradiation tended to significantly faster with *Echinacea purpurea* administration ( $P < 0.05$ ).

#### SOD activity in mouse peripheral blood

The measurement of mouse serum SOD activity by



**Fig. 4.** The change in the number of granulocytes in the blood taken from the tail vein of whole body irradiated mice. Each line graph represents the mean value  $\pm$  SE Granulocytes from 10 mice (M). Significantly different \* $P < 0.05$ , \*\* $P < 0.01$  Control vs Ep, 2Gy vs Ep+2Gy (1dp-1 day point, 3hp-3 hour point).

**Table 1.** Effect of *Echinacea purpurea* on SOD activity in mice serum

Groups	SOD activity (%)
	Mean $\pm$ SEM
Control	27.4 $\pm$ 2.2
<i>Echinacea purpurea</i>	35.8 $\pm$ 2.3*

\*Significant difference ( $P < 0.05$ ) between Control group and *Echinacea purpurea* group by Wilcoxon test.

the NBT reduction method (Table 1) revealed that SOD activity in the *Echinacea purpurea* group was higher than in the control group and a significant increase in SOD activity by *Echinacea purpurea* administration was noted ( $P < 0.05$ ).

## DISCUSSION

### Changes in mouse peripheral blood cell counts

Among the consequences of the early response to irradiation, the most problematic is blood cell injury. Usually mature functional cells, such as

peripheral blood cells, have low radio-sensitivity. However, lymphocytes, although they are mature functional cells, have relatively high radio-sensitivity in comparison with other leukocyte types (Hall, 2000). Leukocytes play an important immunological role in protecting the body from toxic foreign substances. Therefore, leukopenia due to irradiation lowers immune function and can cause infectious diseases. Because of this, the direct cause of death after irradiation is often due to infection. Therefore, in this study, we evaluated the radio-protective effect of *Echinacea purpurea* on blood cells. The study of leukopenia due to irradiation suggested a suppressive effect of *Echinacea purpurea* administration on irradiation-induced leukopenia, especially on lymphocytopenia and monocytopenia. The cause of this effect is likely due to antioxidants in *Echinacea purpurea* such as echinacoside and caffeic acid (Xiong *et al.*, 1996; Sloley *et al.*, 2001). Hu *et al.* studied the antioxidant effects of *Echinacea purpurea*'s

using the DPPH method and reported that Echinacocide and caffeic acid in *Echinacea purpurea* were potent scavengers of free radicals such as hydroxyl radicals (-OH) and superoxide ( $O_2^-$ ) (Hu and Kitts, 2000) Freeman and Crapo reported that oxidizing-reducing agents reduced the cellular injury caused by  $O_2^-$  generated by ionized radiation (Freeman and Crapo, 1982). The suppressive effect on leukopenia due to radiation in our study also seems to be due to antioxidant substances in *Echinacea purpurea*, such as echinacocide and caffeic acid, which eliminate free radicals generated by irradiation and prevent cellular membrane destruction of blood cells by oxidization.

We studied the leukocyte count recovery after irradiation and found that the number of leukocytes, especially lymphocytes, recovered earlier in mice treated with *Echinacea purpurea*. Wagner *et al.* examined blood cell counts in mice treated with *Echinacea purpurea* and found that polysaccharides and echinacocide in *Echinacea purpurea* increased the number of leukocytes (Wagner *et al.*, 1991). Vinti Goel *et al.* (2001a; 2001b) reported that cichoric acid and echinacin in *Echinacea purpurea* activate macrophages. The early recovery in the leukocyte count in our study appears to be due to the ability of polysaccharides and echinacocide to increase the number of leukocytes, and the ability of cichoric acid and echinacin to activate macrophages and to stimulate bone marrow and the reformation of hematopoietic stem cells.

#### **Antioxidant effect in mouse peripheral blood**

It was suggested from the study of the radioprotective effect of *Echinacea purpurea* on blood cells that the suppressive effect on radiation-induced leukopenia is due to a free radical scavenging effect by antioxidant substances in *Echinacea purpurea*, such as Echinacocide and caffeic acid. To confirm whether antioxidant activity in the peripheral blood is in fact increased by *Echinacea purpurea* administration, we evaluated antioxidant activity in the peripheral blood following *Echinacea purpurea*

administration by measuring serum SOD activity using the NBT reduction method. We found a significant increase in SOD activity, which based on previous reports (Hu and Kitts, 2000), is likely due to antioxidants in *Echinacea purpurea*, such as echinacocide and caffeic acid. Administration of extracts of Bunashimeji and Propolis, which contain large amounts of antioxidants similar to those in *Echinacea purpurea*, such as polysaccharides and flavonoids, has been shown to protect against oxidative modification of serum lipids (Isla *et al.*, 2001).

In our study, we assumed that SOD activity in peripheral blood was increased because of antioxidants such as echinacocide and caffeine acid in *Echinacea purpurea* which eliminate superoxide ( $O_2^-$ ) by a free radical scavenging effect. Our results indicate that the suppressive effect of *Echinacea purpurea* on leukopenia due to irradiation in mice is due to an increase in blood antioxidant activity.

## **CONCLUSIONS**

*Echinacea purpurea* has been used by Native Americans as a medicinal herb since ancient times. The medicinal effects of this plant have attracted the attention of modern researchers and many studies evaluating these effects are now in progress. We investigated the immunostimulatory and radioprotective effect of *Echinacea purpurea* in irradiated mice treated with *Echinacea purpurea*.

Our results suggested that the radio-protective effect of *Echinacea purpurea* was due to its ability to minimize the irradiation-induced decrease in the number of leukocytes, lymphocytes, and monocytes and that this effect was due to the free radical scavenging properties of its constituents, such as echinacocide and caffeic acid. Our results also suggested the ability of polysaccharides and echinacocide in *Echinacea purpurea* to increase the number of leukocytes, and of cichoric acid and echinacin to promote the early recovery of leukocyte, lymphocyte, and monocyte counts by bone marrow

stimulation associated with macrophage activation. We also observed an increase in the antioxidant activity of peripheral blood in mice, presumably due to antioxidants in *Echinacea purpurea*, such as Echinacocide and caffeic acid.

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