CHAPTER III

RESULTS

3.1 Mutagenicity study of pinocembrin in rat liver

Table 3-1 presents micronucleated hepatocyte and mitotic index in the experiment aimed to investigate the mutagenic effect of pinocembrin. The micronucleated hepatocytes frequencies after administration of 1, 10 and 100 mg/kg bw of pinocmbrin found that pinocembrin did not induce the number of micronucleated hepatocytes and mitotic index when compared to a control group. These results suggested that pinocembrin did not present mutagenicity in rat liver.

3.2 Effect of pinocembrin on lipid peroxidation in rat liver

Figiure 3-1 shows the concentration-response curves for the inhibitory effects of pinocembrin on lipid peroxidation in rat liver. In the present study, no difference was observed in TBARS level in the pinocembrin treated groups when compared with control. It is clearly shown that pinocembrim did not induce lipid peroxidation in rat liver.

3.3 Effect of pinocembrin on xenobiotic-metabolizing enzymes in rat liver

The immunodetection data showed that pinocembrin did not affect the expression of CYP1A1, CYP2B1, CYP2C11, CYP2E1, CYP3A1, and NADPH: cytochrome P450 reductase protein as compared to the control group as shown in Figure 3-2 and Table 3-2. The effects of pinocembrin on NADPH: cytochrome P450 reductase, heme oxygenase, NADPH: quinone reductase, UDP-glucuronyltranferase and glutathione-*S*-transferase activities are shown in Table 3-3. The 10 and 100 mg/kg bw pinocembrin treatments significantly increased heme oxygenase activity *($p<0.05$)* as compared with the control group. However, there were no significant differences in the activities of NADPH: cytochrome P450 reductase, NADPH: quinone oxidoreductase, UDP-glucuronyltranferase and glutathione-*S*-transferase in the pinocembrin treated groups.

Table 3-1 Mutagenicity of pinocembrin in rat liver

Pinocembrin (mg/kg bw)	Initial body weight (g)	Final body weight (g)	MNHEPs/1,000 hepatocytes	Mitotic index (%)
0	190 ± 9	226 ± 13	1.28 ± 1.11	1.03 ± 0.28
1	191 ± 8	238 ± 14	2.32 ± 1.74	0.99 ± 0.43
10	192± 8	212 ± 25	1 11 ± 1.44	0.74 ± 0.25
100	188 ± 4	229 ± 16	0.70 ± 0.80	1.36 ± 0.24

Values are mean ± SD

MNHEPs /1,000 hepatocytes

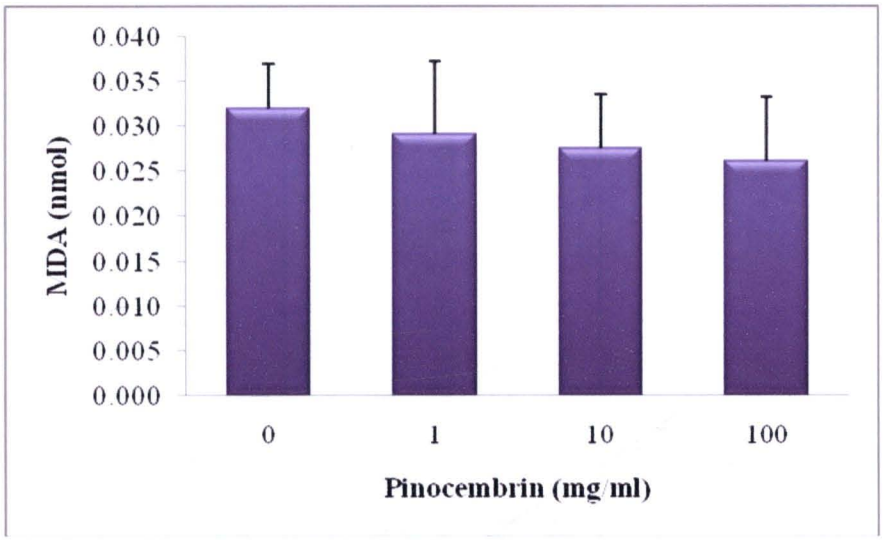

Figure 3-1 Effect of pinocembrin on lipid peroxidation

Bar are expressed as mean ± SD

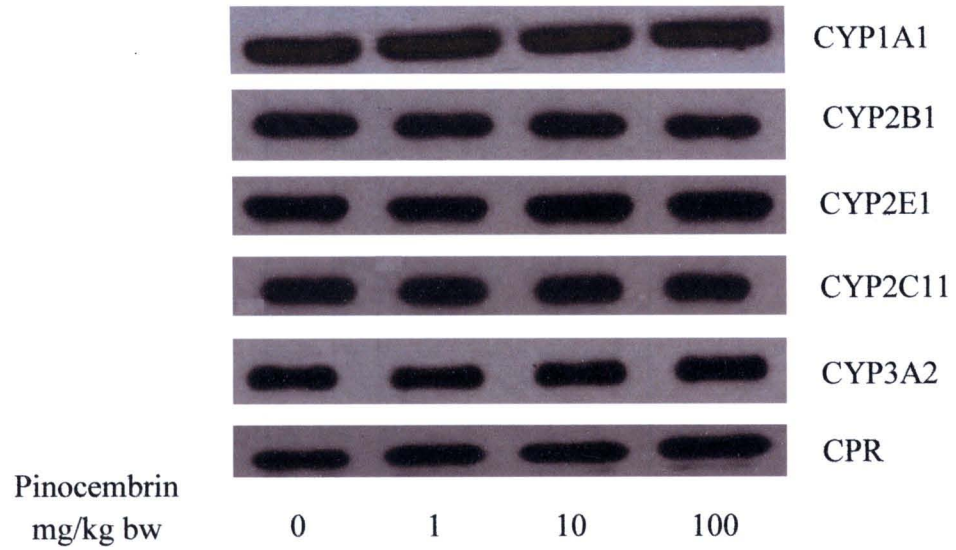

Figure 3-2 Western blot analysis of liver microsomes from rat treated with various doses of pinocembrin.

Table 3-2 Effect of pinocembrin on the expression of cytochrome P450 isoenzymes and cytochrome P450 reductase in rat liver.

Pinocembrin (mg/kg bw)	Fold change					
	CYP1A1	CYP2B1	CYP2C11	CYP2E1	CYP3A2	CPR
0	1.00±0.25	1.00±0.09	1.00±0.14	1.00±0.08	1.00±0.28	1.00±0.13
1	1.09±0.41	0.89±0.14	1.11±0.23	1.02±0.08	1.08±0.39	0.91±0.11
10	1.09±0.32	1.01±0.22	1.13±0.18	1.12±0.14	1.22±0.38	0.93±0.11
100	1.08±0.21	0.91±0.22	1.05±0.25	1.14±0.12	1.24±0.31	1.03±0.22

Values expressed as mean ± SD

Table 3-3 Effect of pinocembrin on the activities of some phase I and phase II enzymes in rat liver

Pinocembrin (mg/kg bw)	Phase I and Phase II enzyme activities				
	CPR ($x10^{-3}$U/mg protein)	HO (nmol /min/mg protein)	NQO1 (nmol/min/mg protein)	UGT (nmol/min/mg protein)	GST ($x10^{-2}$U/mg protein)
0	3.16±1.14	19.29±0.88	2704.10±585.06	22.18±1.87	91.78±18.26
1	2.48±0.93	20.54±0.59	2061.99±510.33	24.22±4.78	87.71±18.20
10	3.31±1.37	22.02±1.62*	2268.68±255.67	20.74±6.15	90.20±11.79
100	3.23±1.99	27.22±1.76*	2199.32±217.45	20.99±5.59	108.05±14.63

CPR: Cytochrome P450 reductase; HO: Heme oxygenase; NQO: NADPH quinone reductase;

UGT: UDP-glucuronyltransferase; GST: Glutathione-*S*-transferase

Values expressed as mean ± SD

*significantly different from control group, $p<0.05$

3.4 Inhibitory effect of pinocembrin on diethylnitrosamine-induced micronucleated hepatocyte formation in rat

To investigate the inhibitory effect of pinocembrin on diethylnitrosamine-induced micronucleated hepatocyte formation, rats were given a double intraperitoneally injected with 30 mg/kg bw diethylnitrosamine and orally fed with various doses of pinocembrin, 2, 10 and 50 mg/kg bw, for 6 days from the first day of injection. The number of micronucleated hepatocytes was examined from 2000 hepatocytes. Rats treated with 2, 10 and 50 mg/kg bw of pinocembrin showed no significant effect on the number of micronucleus formation induced by diethylnitrosamine, as shown in Table 3-4. From the results indicated that pinocembrin did not inhibit the micronucleus formation induced by diethylnitrosamine in rat liver.

3.5 Preventive effect of pinocembrin on diethylnitrosamine- induced micronucleus formation in rat liver

Due to pinocembrin lacked of inhibitory effect, the next study was designed to increase the concentration of pinocembrin and duration of treatment. Rats were orally administered with 10, 25 and 50 mg/kg bw of pinocembrin before 6 days of the first injection of 30 mg/kg bw of diethylnitrosamine. The number of micronucleated hepatocytes and mitotic index are summarized in Table 3-5. The treatment of 10 mg/kg bw of pinocembrin showed a slightly decrease micronucleated hepatocytes.

Since, the last result has revealed that 10 mg/kg bw of pinocembrin tend to prevent inhibitory effect on diethylnitrosamine-induced micronucleus formation than 25 and 50 mg/kg bw, the reduction of mutagenic potency of diethylnitrosamine and prolong administration of pinocembrin exposure were performed. Rats were orally fed with 10 mg/kg bw of pinocembrin 14 days before 20 mg/ kg bw of dietylnitrosamine injection for 21 days. When compared the number of micronucleus formation between 2 diethylnitrosamine treated groups, 20 mg/ kg bw treated rats were induced micronucleated hepatocytes less than 30 mg/ kg bw treated rats. Oral administration of 10 mg/kg bw of pinocembrin reduced 30% of the micronucleus frequency in rat liver when compared to positive control but have no significant difference, as shown

in Table 3-6. The results of the present investigation clearly showed that pinocembrin did not prevent the micronucleus formation induced by diethylnitrosamine in rat liver.

Table 3-4 Inhibitory effect of pinocembrin on diethylnitrosamine-induced micronucleus formation in rat liver

Treatment	Initial body weight (g)	Final body weight(g)	MNHEPs/1,000 hepatocytes	Mitotic index (%)
DEN	197 ± 5.4	216 ± 7.5	31.84 ± 8.98	3.38 ± 1.12
DEN+PC2 mg/kg bw	206 ± 11.1	228 ± 8.5	27.90 ± 12.40	3.73 ± 1.50
DEN+PC10 mg/kg bw	200 ± 7.9	226 ± 13.9	28.50 ± 4.82	2.94 ± 0.62
DEN+PC50 mg/kg bw	201 ± 8.2	218 ± 12.5	27.59 ± 11.91	2.81 ± 0.99

Values expressed as mean ± SD

MNHEPs /1,000 hepatocytes

DEN = diethylnitrosamine, 30 mg/kg bw; i.p.

Table 3-5 Preventive effect of pinocembrin on diethylnitrosamine-induced micronucleated hepatocyte formation in rat

Treatment	Initial body weight(g)	Final body weight(g)	MNHEPs/1,000 hepatocytes	% Inhibition	Mitotic index (%)
DEN	176± 8	248± 18	26.80 ± 5.30	-	2.10 ± 0.40
DEN+PC10 mg/kg bw	168± 6	235± 7	20.10 ± 3.10	25.10	1.90 ± 0.30
DEN+PC 25 mg/kg bw	171 ± 9	243± 10	26.50 ± 7.30	1.25	2.30 ± 0.70
DEN+PC 50 mg/kg bw	173± 5	235 ± 12	23.20 ± 4.20	13.59	2.10 ± 0.20

Values expressed as mean ± SD

MNHEPs /1,000 hepatocytes

DEN = diethylnitrosamine, 30 mg/kg bw; i.p.

Table 3-6 Protective effect of pinocembrin on diethylnitrosamine-induced micronucleated hepatocyte formation in rat

Treatment	Initial body weight(g)	Final body weight(g)	MNHEPs/1,000 hepatocytes	% Inhibition	Mitotic index (%)
NSS	97 ± 3	257 ± 24	2.75 ± 1.06	-	2.55 ± 0.57
NSS+PC 10 mg/kg bw	100 ± 4	240 ± 17	2.12 ± 1.80	-	1.50 ± 0.22
DEN	97 ± 6	233 ± 23	14.58 ± 2.13*	-	1.88 ± 0.48
DEN + PC10 mg/kg bw	94± 3	223 ± 24	11.70 ± 3.90	30	1.63 ± 0.23

Values expressed as mean ±SD

MNHEPs /1,000 hepatocytes

NSS=0.9% Normal saline solution (i.p.)

DEN = diethylnitrosamine, 20 mg/kg bw ; i.p.

*significantly different from control group (NSS), $p<0.05$

3.6 Effect of pinocembrin on promotion stage in diethylnitrosamine-induced rat hepatocarcinogenesis

To evaluate the effect of pinocembrin on promotion stage in diethylnitrosamine-induced rat hepatocarcinogenesis, glutathione-*S*-transferase placental from formation in rat liver was used. The modified method of the medium term bioassay system of Ito based on the two-step model of hepatocarcinogenesis was developed in our laboratory for the rapid detection of carcinogenic/anticarcinogenic agents by measuring levels of positive and negative biomarkers of carcinogenicity in wistar rats. Diethylnitrosamine was used as a carcinogen to initiate hepatocarcinogenesis because it is a proven and specific carcinogen for hepatocarcinogenesis. Throughout the experimental period, body weight of animals was no significant differences between the control and treated groups, as shown in Figure 3-3. Data for average water intake and diet consumption during the experiment period revealed no apparent change in any groups, as shown in Table 3-7. Relative organ weights data are summarized in Table 3-8. No significant differences were observed in the relative organ weights between each of the vehicle control groups and the pinocembrin treated groups. There were no significant differences in the serum AST ALT and ALP activities among the groups, as shown in Table 3-9. Lipid peroxidation of rats is shown in Table 3-10. From the results, TBARS contents did not show significant differences among DEN treated groups or among NSS treated groups. The quantitative data for GST-P positive foci are summarized in Table 3-11. From the results, it is evident that the rats treated with the 2 mg/kg bw of pinocembrin tended to prevent GST-P positive foci formation when compared to group 1 (positive control), while, 10 mg/kg bw of pinocembrin were not significantly different from group 1. On the other hand, when rats treated with pinocembrin after diethylnitrosamine injection, total numbers of GST-P positive foci in the group receiving 10 mg/kg bw of pinocembrin showed a slightly decrease, whereas 2 mg/kg bw of pinocembrin did not decreased the numbers of GST-P positive foci. These results indicated that pinocembrin did not protect against diethylnitrosamine-induced hepatocarcinogenesis of Wistar rat.

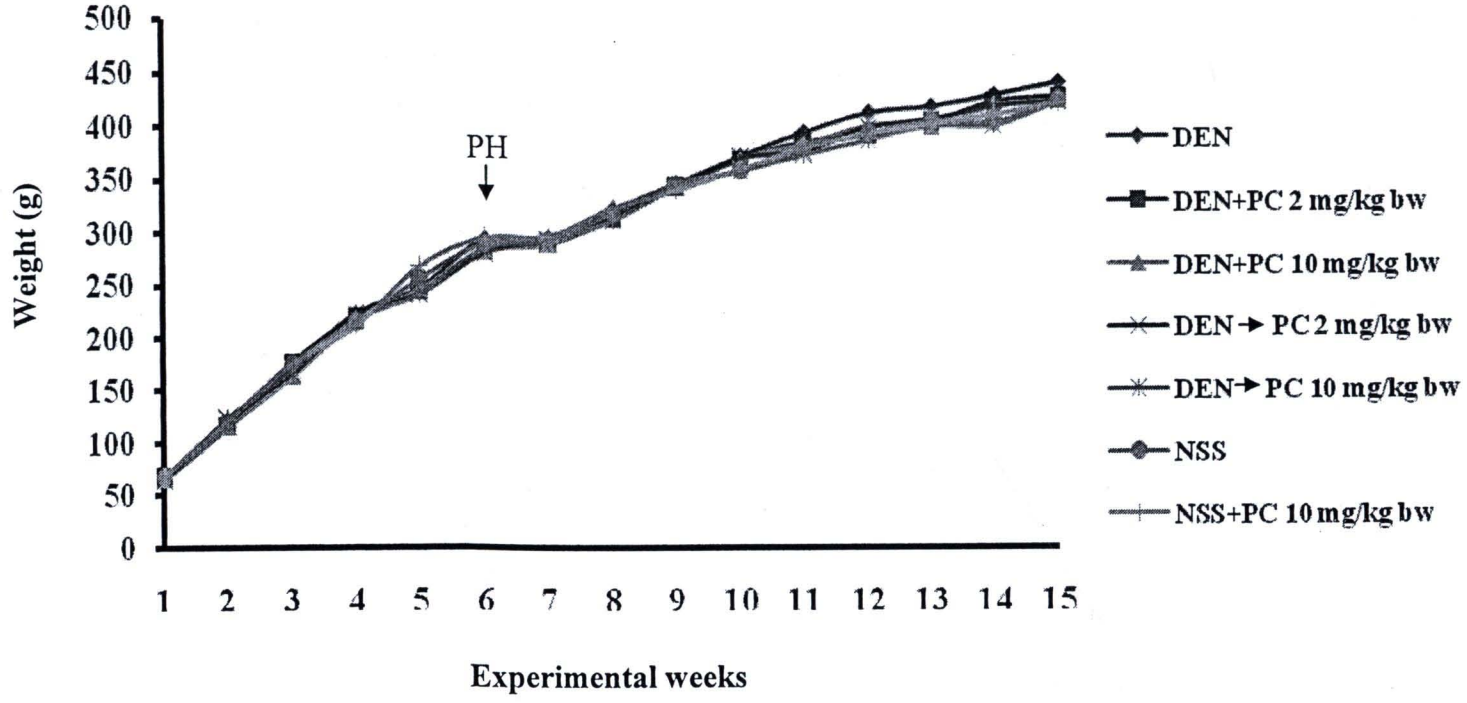

Figure 3-3 Growth curve of rats in medium-term carcinogenicity experiment

Table 3-7 General appearance of rats in medium-term carcinogenicity experiment

Treatment	Exposure period of pinocembrin	Parameters				
		Initial body weight (g)	Final body weight (g)	% Body weight change	Water intake (ml/rat/day)	Food intake (g/rat/day)
DEN	-	65±1	436±32	84.99	30±8	21±3
DEN+PC 2 mg/kg bw	week 1-15	67±2	429± 46	84.28	30±7	20±3
DEN+ PC 10 mg/kg bw	week 1-15	67±3	427±35	84.33	34±10	21±4
DEN→PC 2 mg/kg bw	week 5-15	65±4	432± 28	84.95	32±6	19±3
DEN→PC10 mg/kg bw	week 5-15	68±2	422±20	83.89	35±8	20±4
NSS	-	68±2	429±35	84.05	39±8	21±4
NSS+ PC10 mg/kg bw	week 1-15	69±7	421±41	83.53	35±12	20±3

Values expressed as mean ± SD

DEN = diethylnitrosamine, 100 mg/kg bw, 2 times; i.p.; NSS= 0.9% Normal saline solution, 2 times; i.p.

\+ = Pinocembrin treatment before 2 weeks of the first DEN injection (week 1-15 of experiment)

→ = Pinocembrin treatment after 1 week of DEN injections (week 5-15 of experiment)

Table 3-8 Relative organ weight of rats in medium-term carcinogenicity experiment

Treatment	Exposure period of pinocembrin	Relative organ weight (%)		
		Liver	Spleen	Kidney
DEN	-	2.75±0.14	0.20±0.02	0.55±0.02
DEN+PC 2 mg/kg bw	week 1-15	2.95±0.21	0.19±0.02	0.56±0.06
DEN+ PC 10 mg/kg bw	week 1-15	2.84±0.19	0.20±0.02	0.56±0.04
DEN→PC 2 mg/kg bw	week 5-15	2.85±0.27	0.19±0.01	0.54±0.10
DEN→PC10 mg/kg bw	week 5-15	2.78±0.23	0.19±0.02	0.52±0.04
NSS	-	2.68±0.37	0.20±0.04	0.51±0.04
NSS+ PC10 mg/kg bw	week 1-15	2.78±0.34	0.20±0.03	0.52±0.04

Values expressed as mean ± SD

DEN = diethylnitrosamine, 100 mg/kg bw, 2 times; i.p.; NSS= 0.9% Normal saline solution, 2 times; i.p.

+ = Pinocembrin treatment before 2 weeks of the first DEN injection (week 1-15 of experiment)

→ = Pinocembrin treatment after 1 week of DEN injections (week 5-15 of experiment)

Table 3-9 Blood biochemicals analysis of rats in medium-term carcinogenicity experiment

Treatment	Exposure period of pinocembrin	Enzyme activity (IU/L)		
		AST	ALT	ALP
DEN	-	101.4±18.6	53.1±11.1	122.0±20.6
DEN+PC 2 mg/kg bw	week 1-15	81.4±14.8	42.6±8.7	133.3±26.7
DEN+ PC 10 mg/kg bw	week 1-15	108.1±15.9	73.0±20.2	137.7±34.3
DEN→PC 2 mg/kg bw	week 5-15	96.8±11.5	57.4±20.5	152.3±55.8
DEN→PC10 mg/kg bw	week 5-15	92.2±13.1	41.8±10.2	139.3±32.4
NSS	-	103.0±16.0	47.6±13.2	140.4±61.32
NSS+ PC10 mg/kg bw	week 1-15	99.6±24.3	42.2±9.7	124.0±43.2

AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase

Values expressed as mean ± SD

DEN = diethylnitrosamine, 100 mg/kg bw, 2 times; i.p.; NSS= 0.9% Normal saline solution, 2 times; i.p.

\+ = Pinocembrin treatment before 2 weeks of the first DEN injection (week 1-15 of experiment)

→ = Pinocembrin treatment after 1 week of DEN injections (week 5-15 of experiment)

Table 3-10 Lipid peroxidation of rats in medium-term carcinogenicity experiment

Treatment	Exposure period of pinocembrin	MDA (nmol/mg protein)
DEN	-	0.043±0.009
DEN+PC 2 mg/kg bw	week 1-15	0.050±0.012
DEN+ PC 10 mg/kg bw	week 1-15	0.056±0.015
DEN→PC 2 mg/kg bw	week 5-15	0.048±0.012
DEN→PC10 mg/kg bw	week 5-15	0.043±0.009
NSS	-	0.044±0.006
NSS+ PC10 mg/kg bw	week 1-15	0.052±0.015

Values expressed as mean ± SD

DEN = diethylnitrosamine, 100 mg/kg bw, 2 times; i.p.; NSS= 0.9% Normal saline solution, 2 times; i.p.

+ = Pinocembrin treatment before 2 weeks of the first DEN injection (week 1-15 of experiment)

→ = Pinocembrin treatment after 1 week of DEN injections (week 5-15 of experiment)

Table 3-11 Number and the distribution of size of GST-P positive foci of rats in medium-term carcinogenicity experiment

Treatment	Exposure period of pinocembrin	No. foci containing hepatocytes>20 cells/cm^2	The distribution of size of GST-P positive foci			
			20-30$^{\#}$	31-50$^{\#}$	51-100$^{\#}$	>100$^{\#}$
DEN	-	5.33±1.12	1.76±0.57	1.96±0.79	1.06±0.81	0.55±0.64
DEN+PC 2 mg/kg bw	week 1-15	3.43±1.81	0.75±0.87	0.68±0.26	1.33±1.06	0.67±0.87
DEN+ PC 10 mg/kg bw	week 1-15	5.40±4.09	1.10±0.90	1.22±1.05	1.62±1.12	1.47±1.43
DEN→PC 2 mg/kg bw	week 5-15	7.27±2.91	2.27±1.36	1.96±1.28	1.76±1.16	1.22±0.92
DEN→PC10 mg/kg bw	week 5-15	3.47±1.29	0.81±0.81	0.95±0.79	0.61±0.19	1.10±0.41
NSS	-	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
NSS+ PC10 mg/kg bw	week 1-15	0.06±0.12	0.06±0.14	0.00±0.00	0.00±0.00	0.00±0.00

Values expressed as mean ± SD

DEN = diethylnitrosamine, 100 mg/kg bw, 2 times; i.p.; NSS= 0.9% Normal saline solution, 2 times; i.p.

$^{\#}$ The number of positive hepatocytes per each focus. *significantly different from negative control group, $p<0.05$

\+ = Pinocembrin treatment before 2 weeks of the first DEN injection (week 1-15 of experiment)

→ = Pinocembrin treatment after 1 week of DEN injections (week 5-15 of experiment)