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# Hepatoprotective Effect of C-Phycocyanin: Protection for Carbon Tetrachloride and *R*-(+)-Pulegone-Mediated Hepatotoxicty in Rats

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**Effect of C-phycocyanin (from *Spirulina platensis*) pretreatment on carbontetrachloride and *R*-(+)-pulegone-induced hepatotoxicity in rats was studied. Intraperitoneal (i.p.) administration (200 mg/kg) of a single dose of phycocyanin to rats, one or three hours prior to *R*-(+)-pulegone (250 mg/kg) or carbontetrachloride (0.6 ml/kg) challenge, significantly reduced the hepatotoxicity caused by these chemicals. For instance, serum glutamate pyruvate transaminase (SGPT) activity was almost equal to control values. The losses of microsomal cytochrome P450, glucose-6-phosphatase and aminopyrine-*N*-demethylase were significantly reduced, suggesting that phycocyanin provides protection to liver enzymes. It was noticed that the level of menthofuran, the proximate toxin of *R*-(+)-pulegone was nearly 70% more in the urine samples collected from rats treated with *R*-(+)-pulegone alone than rats treated with the combination of phycocyanin and *R*-(+)-pulegone. The possible mechanism involved in the hepatoprotection is discussed.** © 1998 Academic Press

***Key Words: Spirulina platensis;* C-phycocyanin; carbon tetrachloride; *R*-(+)-pulegone, a monoterpene ketone; hepatotoxicity; hepatoprotective effect.**

*Spirulina platensis*, a unicellular filamentous blue-green algae is gaining more attention these days because of its nutritional and various medicinal properties(1,2). *Spirulina maxima* has preventive effect on the fatty liver induced by a fructose-rich diet in the rat (2) suggesting that this algae contains a factor or factors which affect the fructose-induced alterations of triglyceride metabolism. It has also been shown that C. phycocyanin, one of the major biliproteins of *Spirulina platensis* has antioxidant properties (3). These studies have prompted us to evaluate the potential of C. phycocyanin in the prevention of chemical-induced liver injury.

Carbon tetrachloride ($CCl_4$) is one of the compounds that is most studied with respect to the manifestation of toxic injury in liver (4,5). The hepatotoxicity of carbontetrachloride has been shown to be the consequence of the formation of haloalkane free radicals which are formed through catalysis by the liver microsomal cytochrome P450 enzyme system (6). Like carbon tetrachloride, R-(+)-pulegone, a monoterpene ketone is also a potent hepatotoxin (7,8) and liver microsomal cytochrome P450 system is involved in its bioactivation to reactive metabolites responsible for the toxicity (9,10).

In the present communication, we report the effect of *C. phycocyanin* on carbontetrachloride and R-(+)-pulegone -induced hepatotoxicity in rats.

## MATERIALS AND METHODS

*C. phycocyanin* (isolated from *Spirulina platensis*) was a generous gift from Cyanotec Bio-products (p) ltd. Bangalore, India.

*Animals and treatment.* Male albino rats (2-3 months old) weighing 160-180 g were used throughout the course of this investigation. Rats were housed in groups and were fed *ad libitum*. Six groups (A, B, C, D, E, and F) of rats, each group with 6 animals were used in the following way. Unless otherwise mentioned, all treatments were carried out intraperitoneally (i.p.). Group A and B (control rats) received coconut oil (0.3 ml); group C and E received R-(+)-pulegone at a dosage of 250 mg/kg as a suspension in 0.3 ml of coconut oil; group D and F received carbontetrachloride at a dosage of 0.6 ml/kg as a suspension in coconut oil (0.3 ml). Rats from group B, E and F were pretreated with phycocyanin (200 mg/kg) dissolved in water (0.5 ml) one hour prior to the administration of coconut oil (control rats), R-(+)-pulegone and carbontetrachloride, respectively, 24 h After the administration of R-(+)-pulegone and carbontetrachloride, the animals were sacrificed by cervical dislocation and blood was collected by cardiac puncture for SGPT determinations. The above experiment was repeated by pretreating the animals with phycocyanin three hours prior to the administration of R-(+)- pulegone

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**TABLE 1**

Effect of Phycocyanin Pretreatment[+] on $CCl_4$ (0.6 ml/kg) and *R*-(+)-Pulegone (250 mg/kg)-Induced Hepatotoxicity in Rats

| Enzyme activity | Control† | Phycocyanin | R(+)Pulegone | $CCl_4$ | Phycocyanin + R(+)Pulegone | Phycocyanin + $CCl_4$ |
|---|---|---|---|---|---|---|
| Cytochrome P450 (nmol/mg) | 0.78 ± 0.01 | 0.72 ± 0.03 | 0.38 ± 0.01 | 0.34 ± 0.05 | 0.63 ± 0.04 | 0.52 ± 0.02 |
| % change | — | −7.7 | −50.3 | −55.8 | −18.7 | −33.0 |
| SGPT (units/ml) | 32.2 ± 3.6 | 32.2 ± 3.1 | 490.0 ± 39.2 | 469.0 ± 21 | 36.2 ± 3 | 34.6 ± 1.4 |
| Fold change | — | 0.0 | +15.2 | +14.5 | +0.12 | +0.07 |
| G-6-Phosphatase (nmol/min/mg) | 191.5 ± 4 | 180.0 ± 2 | 131.2 ± 2.3 | 97.5 ± 3.7 | 172.5 ± 3.75 | 142.5 ± 7.5 |
| % change | — | −6.0 | −31.5 | −49.0 | −10.0 | −25.5 |
| Aminopyrine-N-demethylase (nmol/min/mg) | 7.0 ± 0.3 | 6.34 ± 0.45 | 3.0 ± 0.33 | 3.5 ± 0.16 | 5.67 ± 0.19 | 5.33 ± 0.33 |
| % change | — | −9.4 | −57.0 | −50.0 | −19.0 | −23.8 |

*Note.* [+] Rats were pretreated with C. phycocyanin (200 mg/kg) 1h prior to the administration of $CCl_4$ and R-(+)-pulegone. †Animals treated with vehicle alone. Values represent mean ± S. D. of 3 independent experiments, each consisting of tissues pooled from 6 rats. Details are mentioned in "Methods".

and carbontetrachloride. Urine samples were collected in bottles at 0-4°C from rats belonging to the groups C and E.

*Enzyme assays.* Microsomes were prepared from liver by a differential centrifugation method (11). The microsomes were suspended in Tris-HCl buffer (0.05 M, pH 7.8) containing 0.25M sucrose and 20% glycerol. Cytochrome P450 (12), serum glutamate pyruvate transaminase (SGPT) (13), glucose-6-phosphatase (14), aminopyrine-N-demethylase (15) and protein (16) were determined according to published methods. All statistical analyses were performed using Student's t-test and levels of significance determined at $p < 0.05$.

*Extraction of urinary metabolites.* Urine samples collected from animals belonging to groups C and E were separately extracted with diethylether as reported earlier (17). The ether extracts were concentrated and subjected to GC analyses.

*Gas chromatography.* Analyses were carried out on a Shimadzu GC model 14 A instrument equipped with a hydrogen flame ionization detector. The instrument was fitted with a Shimadzu HR-1 wide bore capillary column (15 m × 0.5 mm diameter). $N_2$ At a flow rate of 30 ml/min was used as the carrier gas. The temperature of the column was held at 80°C for 10 min, and then it was raised to 120°C at the rate of 5°C/min.

## RESULTS AND DISCUSSIONS

Effect of C. phycocyanin pretreatment on $CCl_4$ and R-(+)-pulegone-induced hepatotoxicity in rats is shown in Tables 1 and 2. Consistent with earlier investigations (4-8), it was noticed that i.p. administration of a single dose of R-(+)-pulegone (250 mg/kg) and $CCl_4$ (0.6 ml/kg) to rats caused marked decrease in microsomal cytochrome P450, aminopyrine-N-demethylase and glucose-6-phosphatase activities and a significant increase in SGPT level. Phycocyanin (200 mg/kg) when administered alone did not alter liver function. The levels of all the activities tested were similar to those of control values (Tables 1 and 2). However, administration of Phycocyanin one or three hours prior to R-(+)-pulegone or $CCl_4$ challenge, the response to these hepatotoxins were significantly reduced (Tables 1 and 2). In fact SGPT activities were approximately 15 fold greater in rats receiving R-(+)-pulegone or $CCl_4$ alone than in rats treated with the combination of phycocyanin and R-(+)-pulegone or phycocyanin and $CCl_4$ (Tables 1 and 2). When the rats were pretreated with phycocyanin one hour before the administration of R-(+)-pulegone , the losses of cytochrome P450, glucose-6-phosphatase and aminopyrine-N-demethylase activities were only 18, 10, 19%, respectively as compared to 50, 32 and 57% in animals treated with only R-(+)-pulegone (Table 1). Likewise, when $CCl_4$ was administered to phycocyanin pretreated rats, there was a 33, 26 and 27% decrease in cytochrome P450, glucose-6-phosphatase and aminopyrine-N-demethylase activities as against 56, 49 and 50% in rats treated with only $CCl_4$ (Table 1). The response to R-(+)-pulegone and $CCl_4$ was also significantly reduced when phycocyanin pretreatment was made three hours prior to the administration of R-(+)-pulegone and $CCl_4$ (Table 2). These studies have indicated that pretreatment of rats with phycocyanin prior to the administration of R-(+)-pulegone and $CCl_4$ resulted in the protection of liver functions by nearly 65 and 50%, respectively. In fact the levels of SGPT were almost equal to control values in rats treated with phycocyanin, one or three hours prior to the administration of R-(+)-pulegone and $CCl_4$ (Tables 1 and 2). It appears that phycocyanin pretreatment has a greater effect on R-(+)-pulegone than $CCl_4$ induced hepatotoxicity.

In order to find out the effect of phycocyanin on the mode of metabolism of R-(+)-pulegone, experiments were carried out *in vivo* where the urine samples collected from rats treated with R-(+)-pulegone and rats treated with the combination of phycocyanin and R-(+)-pulegone were analyzed by GC and the levels of various major metabolites present in the urine extracts from these two groups were compared (conditions for GC analyses were as mentioned under "methods"). The

**TABLE 2**

Effect of Phycocyanin Pretreatment[+] on $CCl_4$ (0.6 ml/kg) and *R*-(+)-Pulegone (250 mg/kg)-Induced Hepatotoxicity in Rats

| Enzyme activity | Control† | Phycocyanin | R(+)Pulegone | $CCl_4$ | Phycocyanin + R(+)Pulegone | Phycocyanin + $CCl_4$ |
|---|---|---|---|---|---|---|
| Cytochrome P450 (nmol/mg) | 0.71 ± 0.02 | 0.61 ± 0.01 | 0.27 ± 0.01 | 0.26 ± 0.01 | 0.58 ± 0.01 | 0.51 ± 0.01 |
| % change | — | −15.0 | −61 | −64 | −17 | −28.0 |
| SGPT (units/ml) | 33.25 ± 1.1 | 28.0 ± 2.0 | 504.0 ± 36.0 | 485.3 ± 18.7 | 31.27 ± 1.0 | 30.34 ± 1.8 |
| Fold change | — | −0.15 | +15.0 | +14.6 | −0.06 | −0.09 |
| G-6-Phosphatase (nmol/min/mg) | 225.1 ± 7.6 | 228.5 ± 7.5 | 152.2 ± 7.9 | 126.6 ± 4.6 | 193.3 ± 7.3 | 147.5 ± 1.2 |
| % change | — | +1.5 | −32.3 | −43.7 | −14.0 | −34.5 |
| Aminopyrine-N-demethylase (nmol/min/mg) | 7.33 ± 0.33 | 6.0 ± 0.33 | 3.24 ± 0.45 | 3.58 ± 0.24 | 6.16 ± 0.32 | 5.83 ± 0.2 |
| % change | — | −18.0 | −55.7 | −51.0 | −16.0 | −20.3 |

*Note.* [+] Rats were pretreated with C. phycocyanin (200 mg/kg) 3h prior to the administration of $CCl_4$ and R-(+)-pulegone. Other details are as in Table 1.

typical gas chromatogram shows (Fig 1A and B) that the level of menthofuran was significantly higher (nearly 70% more) in the urine of rats treated with R-(+)-pulegone alone than in the urine of rats treated with the combination of phycocyanin and R-(+)-pulegone. However, there was only marginal changes in the levels of other major metabolites (Fig. 1). This is a significant observation since menthofuran is considered as the proximate toxin of R-(+)-pulegone and is responsible for at least half of the hepatocellular necrosis caused by R-(+)-pulegone (18). It is known that microsomal cytochrome P450 system carries out the regiospecific oxidation of R-(+)-pulegone to its allylic alcohol (9-hydroxypulegone)which upon cyclization followed by dehydration yields menthofuran (9,10). The cytochrome P-450 system further converts menthofuran to its epoxide which could easily give rise to an $\alpha$, $\beta$-unsaturated- $\gamma$-ketoaldehyde, a highly reactive metabolite known to covalently interact with tissue macromolecules generating toxicity (19,20). So it is quite possible that phycocyanin could interact preferentially with individual species of cytochrome P-450 and thus could affect the formation of 9-hydroxypulegone which is the precursor of menthofuran. It is also possible that the cytochrome P450 mediated reaction involved in the conversion of menthofuran to its epoxide may be inhibited so that the reactive metabolite viz. $\alpha$, $\beta$-unsaturated-$\gamma$-ketoaldehyde may not be formed in sufficient quantities to elicit toxicity. So it appears that prior administration of phycocyanin protects against $CCl_4$ and R-(+)-pulegone mediated toxicity by lowering the biotransformation of these hepatotoxins into toxic intermediates. This assumption is supported by the fact that higher levels of menthofuran was shown to be present in the urine of rats treated with R-(+)-pulegone alone than in the urine of rats treated with the combination of phycocyanin and R-(+)-pulegone (Fig 1). It is also possible that the haloalkane free radicals produced from $CCl_4$ and reactive metabolites formed from R-(+)-pulegone by the liver microsomal cytochrome P450 systems are being scavenged by phycocyanin. In fact recently it has been reported that phycocyanin has the ability to scavenge alkoxy and hydroxyl radicals (3).

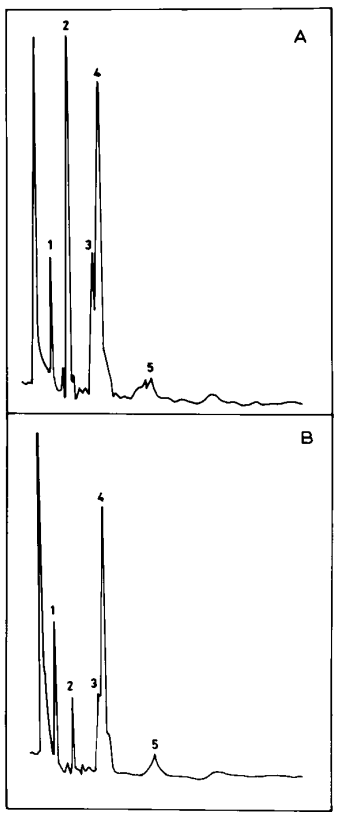


**FIG. 1.** GC separation of major urinary metabolites from (A) *R*-(+)-pulegone treated rats, (B) *R*-(+)-pulegone was administered to *C. phycocyanin* pretreated rats. (1) *p*-Cresol, (2) *R*-(+)-menthofuran, (3) *R*-(+)-pulegone, (4) piperitone, and (5) piperitenone. Experimental conditions are as reported under "Methods."

More experiments have to be carried out to establish the mechanism involved in the hepatoprotection by phycocyanin.

## CONCLUSIONS

The results presented here demonstrate that *C. phycocyanin*, one of the major biliproteins of *Spirulina platensis* can significantly reduce R-(+)-pulegone and $CCl_4$ induced liver injury in rats. The responses to both of these hepatotoxins are significantly reduced in the presence of phycocyanin possibly due to lower levels of reactive metabolites formed. Phycocyanin may inhibit some of the cytochrome P450 mediated reactions involved in the formation of reactive metabolites. It is also possible that phycocyanin may act as an efficient radical scavenger.

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