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Protection by *Osbeckia aspera* Against Carbon Tetrachloride-mediated Alterations in Microsomal Drug Metabolizing Enzyme Activity

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Abstract

Previous investigations have confirmed the protective effect of *Osbeckia aspera* leaf extract on carbon tetrachloride-mediated liver injury in rat models. It is well known that the earliest alterations in liver cell structure and function following carbon tetrachloride poisoning involve the endoplasmic reticulum and its drug metabolizing enzymes. Therefore, we investigated whether an aqueous leaf extract of *O. aspera* could offer protection against carbon tetrachloride-induced changes in the microsomal drug metabolizing enzymes aniline hydroxylase and *p*-aminopyrine *N*-demethylase. This enzyme activity was compared with phenobarbital-induced righting reflex and lipid peroxidation.

Treatment of rats with the aqueous leaf extract of *O. aspera* (before or after the administration of carbon tetrachloride) resulted in a marked decrease in carbon tetrachloride-mediated alterations in aniline hydroxylase and *p*-aminopyrine *N*-demethylase activity, phenobarbital-induced loss of righting reflex and malondialdehyde formation due to lipid peroxidation. The K_m value of these enzymes in control and *Osbeckia*-treated rats were the same.

These results show that the plant extract can markedly decrease the carbon tetrachloride-mediated reduction in aniline hydroxylase and *p*-aminopyrine *N*-demethylase activity and inhibit peroxidative damage to the cell membrane. Phenobarbital-induced sleeping time in rats and kinetic enzyme studies suggested that the effects of the plant extract was neither due to an induction of the drug-metabolizing enzymes under investigation, nor due to an alteration in the K_m values of these enzymes.

Osbeckia aspera and *Osbeckia octandra* (Melastomaceae) are two very closely related species of plants recommended by traditional medical practitioners in Sri Lanka for the alleviation of various forms of liver disease. Previous in-vivo and in-vitro investigations (Thabrew et al 1987, 1995a, b, c; Jayatilaka et al 1990; Houghton et al 1993) have confirmed that crude extracts of *O. octandra* and *O. aspera* can protect against liver injury mediated by various toxins with different modes of actions. For example, Thabrew et al (1987) have shown that significant protection against carbon tetrachloride-mediated liver injury in rats (as assessed by improvements in serum enzyme levels and liver histopathology) could be achieved by pre-or post-treatment of the rats with an aqueous extract of *O. octandra* leaves. Similar observations have been made with *O. aspera* leaf extract (Jayatilaka 1991).

The earliest alterations in liver cell structure and function following carbon tetrachloride poisoning involve the endoplasmic reticulum (Smuckler 1976). The mixed function oxidase enzymes involved in the metabolism of drugs and other foreign compounds are an integral part of the endoplasmic reticulum (Brodie et al 1958) and carbon tetrachloride is known to affect significantly the activity of these enzymes (Conney 1972). An investigation has therefore been carried out to determine whether aqueous extracts of *O. aspera* leaf extract could also offer protection against carbon tetrachloride-induced changes in the activity of liver microsomal drug metabolizing enzymes. All experiments were carried out with the leaf extract of *O. aspera*.

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Materials and Methods

Experimental animals
Male Wistar rats, 150–200 g, were used in all experiments. The rats were maintained on a standard laboratory diet of rat pellets purchased from Moosajee & Co. Ltd, Sri Lanka, and had free access to food and water.

Preparation of plant extract
The fresh leaves of *O. aspera* were collected from Wanduramba in Galle district, Sri Lanka. The plant identity was confirmed by comparing with the authentic samples from the Royal Botanical Garden (Peredeniya, Sri Lanka). The leaves (200 g) were cut into small pieces and homogenized with 1·5 L of distilled water and refluxed for 4 h. The mixture was strained through muslin and the final volume of extract was reduced to 100 mL in-vacuo. For each experiment, the extract was prepared from freshly collected plant material.

Treatment of animals
The rats were divided into six groups of 10 each. Group 1 served as controls and were dosed orally with distilled water (25 mL kg^{-1} per day). Group 2 was administered a single intraperitoneal dose of carbon tetrachloride (carbon tetrachloride–olive oil, 1:1; 0·2 mL carbon tetrachloride/100 g per day) and killed 24 h later. Group 3 was administered a single dose of carbon tetrachloride and killed after 4 days. Group 4 was treated with a single dose of carbon tetrachloride and after 24 h were dosed with the plant extract for a further 3 days. Group 5 was dosed with *Osbeckia aspera* extract for seven days and on the seventh day after administering the plant extract, a single intraperitoneal dose of carbon tetrachloride was given and killed after 24 h. Group 6 was dosed for 7 days with *Osbeckia* extract (25 mL kg^{-1} per day). In a previous study (Jayatilaka 1991) this dose of *Osbeckia aspera* was found to give maximum protection against carbon tetrachloride-mediated liver injury. The animals were dosed with the plant extact for only 3 days in the post-treatment experiments and 7 days in the pretreatment experiments because in previous pharmacological investigations (Thabrew et al 1987; Jayatilaka 1991) it was found that 3 days post-treatment and 7 days pretreatment were sufficient for *Osbeckia* extracts to offer maximum protection against carbon tetrachloride-mediated liver damage in rats. At the end of each experimental period, rats were anaesthetized with diethyl ether and blood was collected by cardiac puncture and transferred to EDTA bottles.

Livers of rats from all groups were excised and portions fixed in buffered formalin for histopathological assessment of damage. The remaining portions of livers were used to prepare microsomes (Kamath et al 1971) for enzyme determinations.

Enzyme assays
Aniline hydroxylase activity was determined by measuring the amount of *p*-aminophenol formed from aniline hydrochloride (Shenkman et al 1967). The *p*-aminophenol formed is coupled with phenol in an alkaline medium to form an indophenol dye. *p*-Aminopyrine *N*-demethylase activity was assessed by the method of LaDu et al (1955). Briefly, it involves the *N*-demethylation of *p*-aminopyrine to 4-aminoantipyrine with a stepwise formation of formaldehyde. Estimation of the amount of formaldehyde formed during *N*-demethylation of *p*-aminopyrine was carried out by the method of Nash (1953).

Protein estimation
Microsomal protein was determined by the method of Lowry et al (1951).

Assessment of lipid peroxidation
The formation of malondialdehyde (Alleman et al 1985) was used as an index of lipid peroxidation in the liver.

Method of phenobarbital-induced loss of righting reflex
This method was based on that reported by Frasier et al (1957). Loss of righting reflex was induced by administration of sodium phenylbarbital (75 mg kg^{-1}, i.p.). The sleeping time was taken as the time between the loss and the recovery of the righting reflex.

Statistical analysis
The mean values ± s.e.m. in each group were calculated by using Student's *t*-test. Statistical difference was verified by one-way analysis of variance.

Results

Table 1 summarizes the effect of carbon tetrachloride and *Osbeckia aspera* extract on the activity of the hepatic microsomal enzymes aniline hydroxylase and *p*-aminopyrine *N*-demethylase. There was a significant reduction in the activity of

Table 1. Effect of *Osbeckia aspera* on carbon tetrachloride-induced alterations in the activity of rat liver microsomal aniline hydroxylase and *p*-aminopyrine *N*-demethylase.

Group	Enzyme activity	
	Aniline hydroxylase[a]	*p*-Aminopyrine *N*-demethylase[b]
1	355·00 ± 4·8	134·50 ± 10·7
2	192·20 ± 22·5**	64·41 ± 10·8*
3	210·66 ± 10·6**	69·82 ± 1·0*
4	313·70 ± 10·7*††	128·90 ± 7·8†
5	330·81 ± 6·7††	112·71 ± 8·9†
6	405·60 ± 10·1*	149·98 ± 11·0

[a]Aniline hydroxylase activity is expressed as nmol *p*-aminophenol formed (mg protein)$^{-1}$ mL^{-1}. [b]*p*-Aminopyrine *N*-demethylase activity is expressed as nmol formaldehyde formed (mg protein)$^{-1}$ mL^{-1}. Group 1 were controls (25 mL kg^{-1} distilled water per day); group 2 received a single intraperitoneal dose of carbon tetrachloride (carbon tetrachloride: olive oil, 1 : 1 : 0·2 mL/100 g per day, i.p.) and killed 24 h later, group 3 was administered a single dose of carbon tetrachloride and killed after 4 days, group 4 was treated with a single dose of carbon tetrachloride and after 24 h were dosed with the plant extract for further 3 days, group 5 pre-treated for 7 days with the plant extract and then given a single intraperitoneal dose of carbon tetrachloride and killed 24 h later and group 6 received only the plant extract for 7 days (25 mL kg^{-1} per day). Results are the mean of 10 values ± s.e.m. The significance between the groups were verified by one-way analysis of variance. $^{**}P < 0{\cdot}001$, $^{*}P < 0{\cdot}01$, compared with controls. $\dagger P < 0{\cdot}001$, $\dagger\dagger P < 0{\cdot}01$ compared with the corresponding CCl_4-treated groups.

aniline hydroxylase and *p*-aminopyrine *N*-demethylase within 24 h of exposure of rats to a single intraperitoneal sub-lethal dose of carbon tetrachloride (Group 2 compared with Group 1).

When the rates of recovery of enzyme activity in rats treated with carbon tetrachloride and killed after four days (Group 3) and the rats treated with plant extract subsequent to carbon tetrachloride treatment (Group 4) were compared, the activity of aniline hydroxylase and *p*-aminopyrine *N*-demethylase in the plant extract-treated group (Group 4) were found to be only 11·63 ± 1·2% and 4·16 ± 2·8% less than the respective enzyme activity in the control group (Group 1) while enzyme activity in the group untreated with plant extract (Group 3) were markedly depressed (aniline hydroxylase and *p*-aminopyrine *N*-demethylase activity were, respectively, 40·6 ± 6·8% and 48·1 ± 5·4% less than control values). Pretreatment with *O. aspera* extract for 7 days (Group 5) before challenge with carbon tetrachloride also moderated the reduction in the microsomal enzyme activity resulting from the administration of carbon tetrachloride only. The percentage reduction in aminopyrine *N*-demethylase and aniline hydroxylase activity by carbon tetrachloride (Group 2) compared with control rats, was 45·8 ± 4·2% and 52·1 ± 5·4%, respectively, while the reduction was only 6·8 ± 1·4% and 16·22 ± 2·1%, respectively, in the *O. aspera*-pretreated group (Group 5). The *Osbeckia* leaf extract by itself (Group 6) significantly increased the activity of the microsomal enzyme aniline hydroxylase by 14·8 ± 2·1% but had no significant effect on basal activity of *p*-aminopyrine *N*-demethylase, when compared with enzyme activity in control rats.

The above results were supported by the evidence obtained from phenobarbital-induced loss of righting reflex (Table 2). Pre- or post-treatment of rats with the plant extract resulted in a significant decrease in the phenobarbital-induced loss of righting reflex, which indicated that the plant extract brought about an improvement in the activity of the microsomal enzymes.

The effect of the plant extract on the catalytic properties of the microsomal enzymes aniline hydroxylase and *p*aminopyrine *N*-demethylase are shown in Figure 1. K_m values for these enzymes in the controls and in the *Osbeckia*-treated rats were the same.

As observed in previous investigations (Thabrew et al 1987; Jayatilaka 1991), histopathological examination of livers of rats treated with carbon tetrachloride showed centrilobular necrosis with mononuclear infiltration in the portal area, fatty deposition and loss of cell boundaries. In our experiment, rats pretreated with *Osbeckia* extract and subsequently given carbon tetrachloride showed no noticeable hepatocellular necrosis or

Table 2. Effect of *Osbeckia aspera* extract on carbon tetrachloride-mediated alterations of phenobarbital-induced loss of righting reflex in rats.

Group	Phenobarbital-induced loss of righting reflex (min)
1	35·0 ± 1·5
2	173·2 ± 1·7*
3	123·0 ± 2·1**
4	80·2 ± 3·0**
5	77·5 ± 2·5**
6	38·0 ± 3·2

Group 1 were controls (25 mL kg^{-1} distilled water per day); group 2 received a single intraperitoneal dose of carbon tetrachloride (carbon tetrachloride: olive oil, 1 : 1 : 0·2 mL/100 g per day, i.p.) and killed 24 h later; group 3 was administered a single dose of carbon tetrachloride and killed after 4 days; group 4 was treated with a single dose of carbon tetrachloride and after 24 h were dosed with the plant extract for further 3 days; group 5 pre-treated for 7 days with the plant extract and then given a single intraperitoneal dose of carbon tetrachloride and killed 24 h later; group 6 received only the plant extract for 7 days (25 mL kg^{-1} per day). Results are the mean of 6 values ± s.e.m. The significance between the groups were verified by one-way analysis of variance. $^{*}P < 0{\cdot}001$, $^{**}P < 0{\cdot}001$ compared with the control group and the respective CCl_4 control groups.

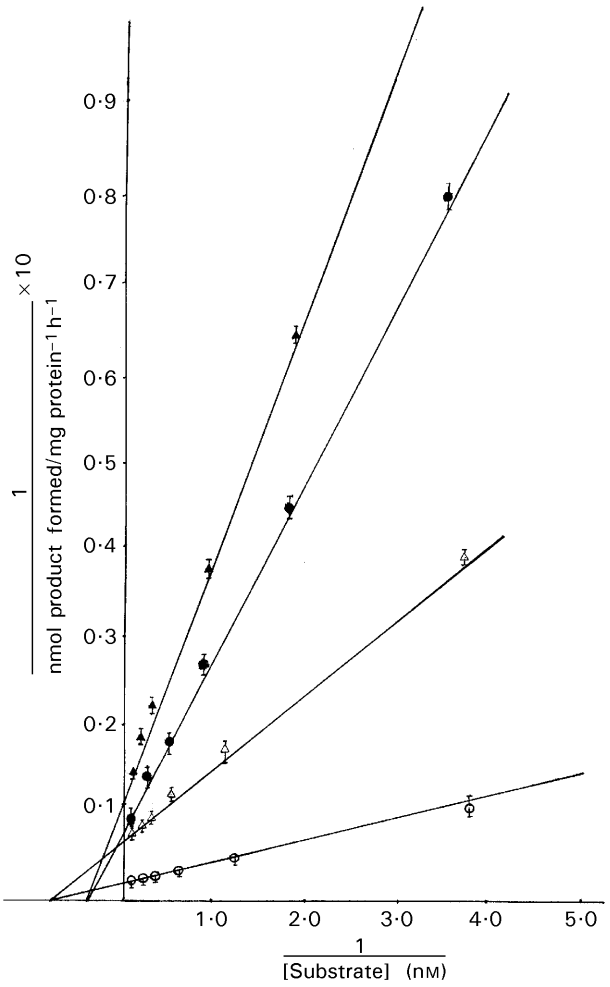

Figure 1. Lineweaver-Burk plots of microsomal aniline hydroxylase and *p*-aminopyrine *N*-demethylase activity in the livers of controls rats and rats pretreated with *Osbeckia* extract for 7 days. Enzyme activity was measured as nmol formed of *p*-aminophenol or formaldehyde, respectively, $(\text{mg protein})^{-1}\,\text{h}^{-1}$. Each point represents the mean of 10 values ± s.e.m. Microsomal aniline hydroxylase activity: ○, control; ▽, *Osbeckia*-treated. *p*-Aminopyrine *N*-demethylase activity: ●, control; ▲, *Osbeckia*-treated.

mononuclear infiltration, and the reticulum framework was well retained. Livers of rats treated with the plant extract for 3 days subsequent to treatment with carbon tetrachloride (Group 4) also showed a well-preserved architecture when compared with livers from rats killed 4 days after exposure to a single dose of carbon tetrachloride (Group 3).

One of the principal mechanisms of carbon tetrachloride-induced liver injury is lipid peroxidation by free-radical derivatives of carbon tetrachloride (Recknagel et al 1974). In the process of lipid peroxidation, malondialdehyde is formed as an end product of the breakdown of peroxidized polyunsaturated fatty acid located in the phospholipid fraction of biomolecules. As shown in Table 3, within 24 h of carbon tetrachloride treatment of rats

Table 3. Effect of *Osbeckia aspera* extract on carbon tetrachloride-induced malondiadehyde formation in rats.

Group	Malondialdehyde (nmol (mg protein)$^{-1}$)	Increase relative to control (%)
1	0·035 ± 0·008	–
2	0·058 ± 0·012*	69·8
3	0·047 ± 0·006*	34·5
4	0·032 ± 0·0012†	8·6
5	0·029 ± 0·0016†	16·6

Group 1 were controls (25 mL kg^{-1} distilled water per day); group 2 received a single intraperitoneal dose of carbon tetrachloride (carbon tetrachloride: olive oil, 1 : 1 : 0·2 mL/100 g per day, i.p.) and killed 24 h later; group 3 was administered a single dose of carbon tetrachloride and killed after 4 days; group 4 was treated with a single dose of carbon tetrachloride and after 24 h were dosed with the plant extract for further 3 days; group 5 pre-treated for 7 days with the plant extract and then given a single intraperitoneal dose of carbon tetrachloride and killed 24 h later. Results are the mean of 6 values ± s.e.m. *$P < 0.001$, compared with control. †$P < 0.001$, compared with the corresponding CCl_4-treated group.

there was a marked increase in malondialdehyde formation. The rate of reduction of malondialdehyde generation was significantly faster in *Osbeckia* post-treated rats (Group 4) than in rats that were treated only with carbon tetrachloride and left to recover for 4 days without any treatment (Group 3). Treatment with *Osbeckia* extract before carbon tetrachloride administration (Group 5) also significantly inhibited the carbon tetrachloride-mediated increase in malondialdehyde formation.

Discussion

In agreement with results of previous investigations (Conney 1972; Thabrew et al 1982), the present study demonstrated that a substantial decrease in the activity of microsomal aniline hydroxylase and *p*-aminopyrine *N*-demethylase occurs within 24 h of exposure of rats to a single, sublethal dose of carbon tetrachloride. By pre- or post-treatment of rats with *O. aspera* leaf extract significant protection can be achieved against the carbon tetrachloride-mediated alterations in enzyme activity, a result supported by the alterations in phenobarbital-induced sleeping times of the rats. Although the plant extract by itself appeared to be able to increase the basal aniline hydroxylase activity by 14·8%, this increase alone cannot account for the improvement in the enzyme activity observed when administered to rats treated with carbon tetrachloride. Further, the extract by itself had no significant effect on the basal activity of *p*-aminopyrine *N*-demethylase.

The fact that the K_m values of aniline hydroxylase or *p*-aminopyrine *N*-demethylase were not altered by administration of the plant extract suggests that the *Osbeckia*-mediated improvement in the enzymes' activity is not due to any alterations in the affinities of the enzymes for their respective substrates.

Many components cited in the literature as being beneficial against carbon tetrachloride-mediated liver injury exert their protective action by toxin-mediated lipid peroxidation, either via a decreased production of carbon tetrachloride-derived free radicals (Castro et al 1974; Mailing et al 1974) or through the antioxidant activity of the protective agents themselves (Yasuda et al 1980; Koul & Kapil 1992; Kapil et al 1995). The ability of the *Osbeckia* extract to decrease the amount of malondialdehyde generated by carbon tetrachloride, as evident in the present study, indicates the ability of the plant extract to inhibit peroxidative damage to cell membrane. At present the mechanism by which the plant extract exerts its anti-lipoperoxidative effect is not clear.

Recent investigations (Thabrew et al 1998) have demonstrated that *O. aspera* leaf extract possesses strong antioxidant activity. Thus, though the *Osbeckia* extract may be unable to prevent the generation of toxic free radicals from carbon tetrachloride, because of the strong antioxidant properties it possesses the extent of free radical-mediated damage may be reduced. This would enhance the resistance of hepatocyte membranes, including the endoplasmic reticulum, to carbon tetrachloride-mediated injury.

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