[*Indian Journal of Medical Research* (1985): (82), October, 359]

In vitro Effect of an Ayurvedic Liver Remedy on Hepatic Enzymes in Carbon Tetrachloride Treated Rats

Piyush Bardhan, Sharma, S.K. and **Garg, N.K.**
Central Drug Research Institute, Lucknow, India.

INTRODUCTION

An Ayurvedic compound formulation of *Capparis spinosa, Cichorium intybus, Solanum nigrum, Cassia occidentalis, Terminalia arjuna, Achillea millefolium* and *Tamarix gallica* is being marketed under the trade name Liv.52 (The Himalaya Drug Co., Bombay) for various liver disorders. Oral feeding of this multi-herbal drug preparation to mice, rats and rabbits[2], has been reported to lead to a reduction in mortality and appreciable protection against liver damage due to carbon tetrachloride. It was later reported that during CCl_4 toxicity, oral feeding of this drug to rats protects them against decrease in activity of succinate dehydrogenase, cytochrome c oxidase, total ATPase and aniline hydroxylase in the liver.

We have fractionated Liv.52 powder by extraction with petroleum ether, chloroform, butanol and water (in order) and studied the effect of *in vitro* addition of these extracts on the activities of aminopyrine N-demethylase, aniline hydroxylase, cytochrome c oxidase, total ATPase, succinate dehydrogenase, acid phosphatase, acid ribonuclease and cathepsin B in livers of normal and CCl_4-treated rats.

MATERIALS AND METHODS

Male weanling rats (Druckrey strain, 25-30 g body weight) obtained from the CDRI Animal House, Lucknow, were fed *ad lib* standard pellet diet (Hindustan Lever, Bombay) and allowed free access to water. The rats were divided into two groups of six rats each (3 rats being kept in one cage). The rats of group I were administered carbon tetrachloride (0.7 ml/kg body wt) for two successive days, while the rats of group II were given ip, an equal volume of normal saline. The animals were made to fast overnight and killed 48 hours after the first injection of carbon tetrachloride.

Fractionation of Liv.52: Liv.52 powder was extracted (50 g) with petroleum-ether (60-80°C) in Soxhlet for two hours (about 20 cycles). The extract was concentrated *in vacuo* to dryness (0.622 g). The petroleum-ether extracted residue was re-extracted with chloroform in the same manner. The chloroform extract was concentrated *in vacuo* to dryness (0.672 g). The residue after chloroform extraction was extracted thrice with 100 ml aliquots of butanol by shaking for 30 min. and filtering through Whatman No. 42 paper. These filtrates were combined and concentrated *in vacuo* (0.692 g). Butanol extracted residue was air-dried and mixed with 200 ml of double distilled water, shaken for 30 min. and centrifuged for 10 min. at 12,000 rpm. Three such extractions were carried out. The supernatants were combined, filtered through Whatman No.42 paper and lyophilized (6.82 g). Petroleum-ether chloroform and butanol extractables were re-dissolved in the same solvent to give a final concentration of 1 mg/ml. Aqueous-extractable were re-dissolved in water to give a final concentration of 10 mg/ml 0.1 ml of these extracts were evaporated to dryness (except in case of water extract which was used as such) and suspended in 1.0 ml of appropriate

buffers as used for respective enzyme assays. Homogenous sonicated dispersions of the non-polar extractables were prepared by sonicating them with buffers for 20 min. at 20 kc/sec, energy output 1.5 amp (cell Disruptor, W 220 F, Heat system, ultrasonic, New York). In this manner almost clear translucent micellar dispersions were obtained.

Fractionation of liver homogenate: Excised livers were homogenized with 9 volumes of ice cold KCI (1.15%, w/v) in a Potter-Elvejhem homogeniser fitted with a Teflon pestle. The homogenate was centrifuged at 2000 rpm for 10 min. to remove cell debris. Mitochondrial fraction and post-mitochondrial supernatant were prepared from this supernatant according to the method of Schneider and Hogeboom[4].

In vitro addition of extracts of Liv.52: 100 µg of petroleum-ether, chloroform and butanol-extractables suspended in buffer, or 1 mg of water-extractables, were added per 0.2 ml of liver homogenates (3.2 ± 0.31 mg protein), or 0.1 ml of mitochondrial suspension (3.4 ± 0.41 mg protein) or 1.0 ml of post-mitochondrial supernatants (2.9 ± 0.35 mg protein) to study the effect of *in vitro* additions on various enzymes in the livers of normal and carbon tetrachloride-treated rats.

Enzyme assay: Succinate dehydrogenase[5], cytochrome c oxidase[6] and total ATPase[7] were assayed in mitochondrial fractions of liver according to standard methods. The drug-metabolizing enzymes, aniline hydroxylase and aminopyrine N-demethylase were assayed in post-mitochondrial fractions according to Kato and Gillette[8] and cochin and Axelrod[9] respectively. The lysosomal enzymes acid phosphatase[10], acid ribonuclease[11] and cathepsin B[12] were assayed in the liver homogenate without freezing and thawing. This was done with a view to assessing the release of these enzymes from lysosomes due to their partial rupture *in situ*. The extracts obtained were slightly coloured and were used in very small quantities as to give no visible colour. Simultaneous blank samples were also run. There was no interference of the extracts in colorimetric quantitation of enzymes.

RESULTS AND DISCUSSION

In the present work only 8.8 g of the total extractables were recovered by extraction of 50 g drug powder with petroleum ether, chloroform, butanol and water. The drug, as marketed, contains a large amount of excipients and non-extractable residue. The results given in Table 1 show that the activities of the drug metabolizing enzymes aniline hydroxylase and aminopyrine N-demethylase decreased significantly in carbon tetrachloride-treated rats. This is in agreement with the earlier observations of Fujimoto and Plea[13], and Recknagel[14]. *In vitro* addition of butanol and aqueous extract of Liv.52 to post-mitochondrial supernatants of livers of normal as well as of carbon tetrachloride-treated rats stimulated the activity of both the enzymes. These extracts also raised the level of enzymes in normal animals not exposed to carbon tetrachloride. The degree of stimulation was more in rats given carbon tetrachloride. Chloroform and petroleum-ether extracts had no effect. The active principle(s) of Liv.52 which afford protection against a decrease in the activity of hepatic drug-metabolizing enzymes in CCl_4-toxicity in rats appear to be, as reported by earlier workers, essentially water-soluble as they could not be extracted by petroleum-ether or chloroform.

Activities of succinate dehydroenase, cytochrome c oxidase and total ATPase in mitochondrial fractions of liver were lower in rats given CCl_4 than in normal rats (Table 2). Additional of petroleum-ether extract stimulated the activity of succinate deydrogenase, cytochrome c oxidase

and total ATPase by about 29, 38 and 33 per cent respectively in normal rats and about 36, 51 and 71 per cent in CCl_4-treated rats respectively. The percent stimulation was more in carbon tetrachloride-treated rats. Chloroform, butanol and aqueous extracts had no effect on the activities of these enzymes. These extracts perhaps contain certain activators of these enzymes. One of the mechanisms by which Liv.52 stimulates the activity of hepatic enzymes in normal and carbon-tetrachloride-treated rats appears to be a *per se* activation of these enzymes.

Table 1: Effect of *in vitro* addition of Liv.52 extracts on hepatic drug-metabolizing enzymes (Data are mean ± SD of three rats in each group)

In vitro treatment		Enzymes (Units/g liver)			
		Aminopyrine N-demethylase●		Aniline hydroxylase x 103■	
		Normal	CCl_4-treated	Normal	CCl_4-treated
No treatment (control)		1.72 ± 0.131	0.666 ± 0.54*	0.068 ± 0.006	0.024 ± 0.0036*
Treated group	Petroleum ether-extract	1.80 ± 0.172	0.754 ± 0.071	0.070 ± 0.006	0.077 ± 0.0028
	Chloroform-extract	1.75 ± 0.152	0.684 ± 0.094	0.070 ± 0.006	0.022 ± 0.0013
	Butanol-extract	2.16 ± 0.143** (+25)	1.03 ± 0.102# (+55)	0.087 ± 0.009** (+36)	0.038 ± 0.0035# (+57)
	Aqueous extract	2.54 ± 0.135** (+47)	1.38 ± 0.117# (+107)	0.096 ± 0.006* (+40)	0.049 ± 0.0042# (+100)

Figures in parentheses indicate the percentage stimulation or inhibition as compared to the control. Enzyme units expressed as ●n moles formaldehyde formed/min/mg protein; ■p moles p-aminophenol formed/min/mg protein.
**p* values <0.001 as compared to normal;
**as compared to normal (*p* value <0.001 to 0.05);
as compared to CCl_4-treated (*p* value <0.001 to 0.05)

Table 2: Effect of *in vitro* addition of Liv.52 on hepatic mitochondrial enzymes

In vitro treatment		Enzymes (Units/g liver)					
		Succinate dehydrogenase#		Cytochrome C oxidase$		Total ATPase●	
		Normal	CCl_4-treated	Normal	CCl_4-treated	Normal	CCl_4-treated
No treatment (Control)		0.883 ± 0.064	0.811 ± 0.051	0.019 ± 0.0028	0.015 ± 0.0018	0.350 ± 0.0018	0.350 ± 0.014
Treated group	Petroleum ether-extract	1.14 ± 0.084* (+29)	1.11 ± 0.099* (+36)	0.027 ± 0.016* (+38)	0.024 + ± 0.0018** (+51)	0.467 ± 0.033* (33)	0.339 ± 0.028** (+71)
	Chloroform extract	0.903 ± 0.096	0.886 ±0.075	0.022 ± 0.0020	0.018 ± 0.0009	0.370 ± 0.037	0.221 ± 0.015
	Butanol extract	0.822 ± 0.048	0.877 ± 0.075	0.020 ± 0.0012	0.016 ± 0.0018	0.354 ± 0.025	0.220 ± 0.013
	Aqueous extract	0.754 ± 0.048	0.716 ± 0.056	0.017 ± 0.0012	0.012 ± 0.0012	0.318 ± 0.025	0.160 ± 0.015

Enzymes units expressed at #µ moles potassium ferricyanide reduced/min/mg protein; $2.303 log OD at 0 min/OD at one min. X conc. of cytochrome c X concentration of protein; ●µ moles Pi liberated/min/mg protein. Figures in parentheses indicate the percentage stimulation or inhibition as compared to the control; *p* values, *as compared to normal ($p<0.01$); **as compared to CCl_4-treated ($p<0.01$).

Table 3 shows that the lysosomal enzymes acid phosphatase, acid ribonuclease and cathepsin B increased in the liver after carbon tetrachloride treatment. Since these enzymes were assayed in liver homogenate without freezing and thawing, their increase appears to be due to a possible leakage through lysosomal membranes or their partial rupture. Addition of chloroform extract of the

drug caused a decrease in the activities of the released lysosomal enzymes. Addition of butanol extract gave protection against carbon tetrachloride-induced increase in the activity of cathepsin B. Other extracts had no effect indicating that Liv.52 contains some lipid soluble factor, which stabilizes lysosomal membranes. The *in vitro* effects of the various extracts of Liv.52 on the microsomal, mitochondrial and lysosomal enzymes are in agreement with the earlier reports of Saxena and Garg[8] on the *in vivo* effect of this drug in carbon tetrachloride-toxicity in rats.

Table 3: Effect of *in vitro* addition of extracts of Liv.52 on hepatic lysosomal enzymes

In vitro treatment		Enzymes (Units/g liver)					
		Acid phosphatase[#]		Acid ribonuclease[$]		Cathepsin B[•]	
		Normal	CCl_4-treated	Normal	CCl_4-treated	Normal	CCl_4-treated
No treatment (Control)		0.144 ± 0.0086	0.231 ± 0.018*	0.020 ± 0.0023	0.046 ± 0.0047*	0.016 ± 0.0015	0.022 ± 0.0009**
Treated group	Petroleum ether-extract	0.148 ±0.011	0.231 ± 0.16	0.018 ±0.0017	0.045 ± 0.0048	0.016 ± 0.0009	0.022 ± 0.001
	Chloroform extract	0.111 ± 0.059** (–23%)	0.137 ± 0.012 (–40%)	0.019 ± 0.0007** (-41%)	0.020 ± 0.029*** (--56%)	0.011 ± 0.0009** (-28%)	0.012 ± 0.0016*** (-31%)
	Butanol extract	0.135 ± 0.013	0.218 ±0.018	0.019 ± 0.0019	0.047 ± 0.0055	0.017 ± 0.0015	0.019 ± 0.0018
	Aqueous extract	0.119 ± 0.0065	0.184 ± 0.015	0.016 ± 0.0011	0.039 ± 0.0036	0.017 ± 0.0016	0.022 ± 0.0016

Enzymes units expressed at [#]μ moles potassium ferricyanide reduced/min/mg protein; [$] ΔOD/min/mg protein; [•]μ moles of tyrosine released/min/mg protein. *p* values, **p* value <0.05; **Statistically significant as compared to normal (*p* values <0.001); *** Statistically significant as compared to CCl_4-treated (*p* value <0.001).

SUMMARY

In vitro addition of aqueous or butanol extracts of Liv.52 (a compound Ayurvedic formulation used in liver disorders) to post-mitochondrial fraction of livers of normal and CCl_4-treated rats stimulated the activity of aminopyrine N-demethylase and aniline hydroxylase. Addition of petroleum-ether extract stimulated the activity of cytorome c oxidase, succinate dehydrogenase and total ATPase in the mitochondrial fraction. Chloroform extract of the drug when added to the post-mitochondrial fraction of the liver inhibited the activities of lysosomal acid phosphatase, acid ribonuclease and cathepsin B in total liver homogenate.

ACKNOWLEDGEMENT

One the of the authors (PB) received a Senior Research Fellowship of the Council of Scientific and Industrial Research, New Delhi. Liv.52 was obtained from The Himalaya Drug Co., Bombay as a gift from Dr. R.M. Captain. Excellent technical assistance by Shriyuts N.K. Verma and S. Yadav is gratefully acknowledged.

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