Issue of testicular toxicity of hydroxycitric acid lactone

Fruits of *Garcinia* species of which (–)hydroxycitric acid (HCA) is a constituent, are generally used in Indian culinary preparations as a souring agent and are known to suppress appetite[1–3]. Dried fruits of *Garcinia cambogia* are popularly known as Malabar tamarind or kokum. It has been implicated that HCA inhibits ATP-citrate lyase, a key enzyme responsible for promoting fat synthesis. As a result of this inhibition, energy is diverted for production of glycogen in the liver and muscles. Since higher glycogen levels signal satiety to the brain, HCA suppresses appetite[4–6]. HCA does not act on the central nervous system (CNS) like its FDA-approved counterparts, such as amphetamine, methamphetamine, diethylpropion, phenmetrazine and fenfluramine. Interestingly, several animal studies have suggested that HCA may have hepatoprotective[8–10] and chemoprotective[11] properties. In the early nineties, (–)HCA was sold in the market as a crude extract (dark-brown viscous liquid) and as a calcium salt (brownish-yellow powder) for its anti-obesity formulations. However, recently Saito *et al.*[12] reported that a high dose of HCA though effective in suppressing fat accumulation in male Zucker obese rats, was highly toxic to the testis. HCA products contained only 50–55% of active principle, the rest being calcium and other unaccounted material.

All HCA products available in the market contain varied quantities of HCA, metals and small amounts of other impurities. In the process of further refining the product, we have isolated highly pure HCA lactone (HCAL), a natural form of HCA from which various salts of HCA containing Group IA and Group IIA metals were prepared. Our initial studies showed that HCAL has better anti-obesity activity than double salt (sodium and calcium salt)[13].

Researchers at InterHealth Nutraceuticals, USA have reported that the findings of toxicity with a (–)-HCA product tested in Japan are due to the high amount of HCAL found in the Japanese item[14], produced for Nippon Shinyaku by Renaissance Herbs, Inc., Chatsworth, California. At high dosage, the Renaissance herbs HCA in long-term animal experiments led to a failure of the testicles to develop properly. Trials with fully reacted HCA salts produced by other companies have not shown any significant side effects or toxicity, a point made by the InterHealth scientists. Similar results of stunted testicular development were reported by Roche Pharmaceuticals[15] approximately 35 years ago in tests of the ethylenediamine salt of HCA. Researchers at Roche concluded that the ethylenediamine HCA salt chelates the mineral zinc from the system. Zinc is required for proper testicular development and health.

The negative health findings implicating HCA free acid and HCAL pose problems for manufacturers of beverages intended to include HCA as a functional ingredient. Even fully reacted HCA salts slowly lactonize over a period of time when included in beverages, especially low pH (acid) beverages and any beverage that has been pasteurized. Although there may be little free acid or lactone present at the time of manufacture, under normal conditions the HCAL content of beverages will usually increase significantly within a short period. A detailed study conducted by Rao *et al.*[13] clearly brings out the point that when HCAL is dissolved in water it slowly gets converted into HCA, and this conversion stops after attaining the equilibrium of 60% HCA to 40% HCAL. The equilibrium is reached within 4 h if the temperature is maintained at 80°C, than at room temperature. Acidification also results in the formation of an equilibrium mixture of HCA and HCAL.

In order to dispel negative health postulations made by InterHealth Nutraceuticals that HCAL present in high concentrations in HCA formulations caused the problem of testicular toxicity, we have done *in vivo* toxicity studies by preparing 98% pure HCAL and evaluated acute and chronic oral toxicity in Wistar rats by administering it as a single oral dose.

About 100 g of pure HCAL (Figure 1) was prepared and subjected to median lethal dose using acute toxic class method according to the OECD guidelines for testing of chemicals (Section 4, No. 423, Adopted 17 December 2001).

A sighting study was conducted in animals dosed with 5, 50, 300 and 2000 mg/kg and they were observed for any toxicity signs and mortality. Further, a main study was performed using upper dose limit of 2000 mg/kg body wt and observations made for a period of 14 days. The animals dosed with 5, 50, 300 and 2000 mg/kg did not show any drug-related mortality and toxicity signs for a period of 24 h. A sighting study was performed using 5000 mg/kg body wt, and observations made for a period of 14 days revealed no toxicity signs and mortality (Tables 1 and 2). Necropsy findings showed no pathological lesions.

At the end of the 14-day observation period, no mortality was observed in any of the animals administered with the HCAL at a dose of 2000 mg/kg body wt, both in the sighting study as well as the main study. No HCAL-related toxicity signs were observed in rats for the entire observation period. All the animals showed an increasing trend in body wt on day-14.

Gross pathology conducted on day-14 in the animals revealed few lesions that were not related to test substances and histopathologically, no lesions were found. Hence on the basis of the above observations the LD50 of HCAL has been found to be > 5000 mg/kg body wt.

Figure 2 indicates that there are no histoarchitectural changes in the kidney with respect to glomorulus, epithelium, tubules and blood vessels, and in the testis with respect to seminiferous tubules, germ cells, Sertoli cells and Leydig cells at the dose of 5000 mg/kg body wt.

To assess the chronic toxicity of HCAL, a comparative study was carried out with three different concentrations of HCA and HCAL on food intake (Figure 3 *a*), body weight (Figure 3 *b*), and toxicity profile which includes levels of plasma cholesterol (Figure 4 *a*), triglycerides (Figure 4 *b*) and glucose levels (Figure 4 *c*) in adult male rats.

The experiment consisted of seven groups with six male rats in each group. Group 1: Normal control fed with water; Group 2: HCA @ 300 mg/kg orally

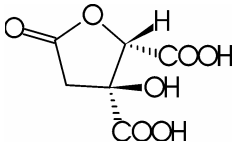

Figure 1. Hydroxycitric acid lactone (HCAL) structure.

Table 1. Histopathological studies of kidney of rats treated with various doses of hydroxycitric acid lactone (HCAL)

Dose (mg/kg)	Congestion	Glomerular hemorrage	Epethelial proliferation	Tubular atropy	Tubular dilation	Denudation and necrosis	Castes in tubules	Hemosidirin in tubules	Epithelial regeneration	Interstetial inflammation	Blood vessels
Control	N	N	N	N	N	N	N	N	N	N	N
300	N	N	N	N	N	N	N	N	N	N	N
1000	N	N	N	N	N	N	N	N	N	N	N
1500	N	N	N	N	N	N	N	N	N	N	N
2000	P	N	N	N	N	N	N	N	N	N	N
2500	P	N	N	N	N	N	N	N	N	N	N
3000	P	N	N	N	N	N	N	N	N	N	N
3500	P	N	N	N	N	N	N	N	N	N	N
5000	P	N	N	N	N	N	N	N	N	N	N

*Each group contains five animals. P, Present. N, Normal.

Table 2. Histopathological studies of testis of rats treated with various doses of HCAL

	Seminiferous tubules			Germ cells				
Dose (mg/kg)	BM	Size	Disarray	Primary	Sec	Sperms	Sertoli cells	Leydig cells
Control	N	N	NIL	N	N	N	N	N
300	N	N	NIL	N	N	N	N	N
1000	N	N	NIL	N	N	N	N	N
1500	N	N	NIL	N	M	N	N	N
2000	N	N	NIL	N	M	N	N	N
2500	N	N	NIL	N	M	N	N	N
3000	N	N	NIL	N	M	N	N	N
3500	N	N	NIL	N	M	N	N	N
5000	N	N	NIL	N	M	N	N	N

*Each group contains five animals. BM, Basement membrane; *Primary, Primary spermatocytes; Sec, Secondary spermatocytes; N, Normal; M, Medium.

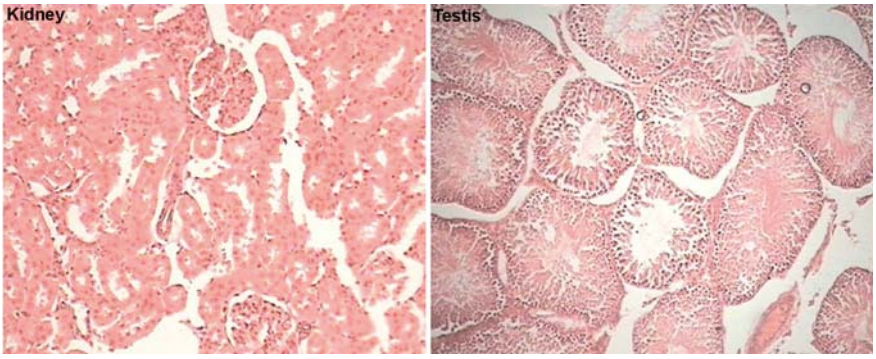

Figure 2. Histopathological slides of rats treated with HCAL (5000 mg/kg).

(1.1 mmol/kg); Group 3: HCA @ 1000 mg/kg orally (3.7 mmol/kg); Group 4: HCA @ 1500 mg/kg orally (5.5 mmol/kg); Group 5: HCAL @ 212 mg/kg orally (1.1 mmol/kg); Group 6: HCAL @ 708 mg/kg orally (3.7 mmol/kg); Group 7: HCAL @ 1063 mg/kg orally (5.5 mmol/kg).

The HCAL dose was based on molar concentration equivalent to that of HCA. The experiment was conducted for 8 weeks. All the animals had free access to food and water throughout the experimental period. A standard environmental condition was maintained in the laboratory animal house with 12 : 12 h dark and light cycle, temperature ranging from 21°C to 22°C and humidity from 60% to 65%.

It can be observed from Figure 3 *a* that there is a gradual decrease in food intake in both HCA and HCAL treated groups as dose increases. Though it is seen that both HCA and HCAL have beneficial effect, HCAL has comparatively more beneficial effect than HCA in terms of reduction of food intake.

Figure 3 *b* shows a general reduction in body weight in treated groups. However HCAL has better beneficial effect in terms of weight reduction compared to HCA.

In the case of animals treated with HCAL, more reduction of food intake and body weight was observed compared to HCA.

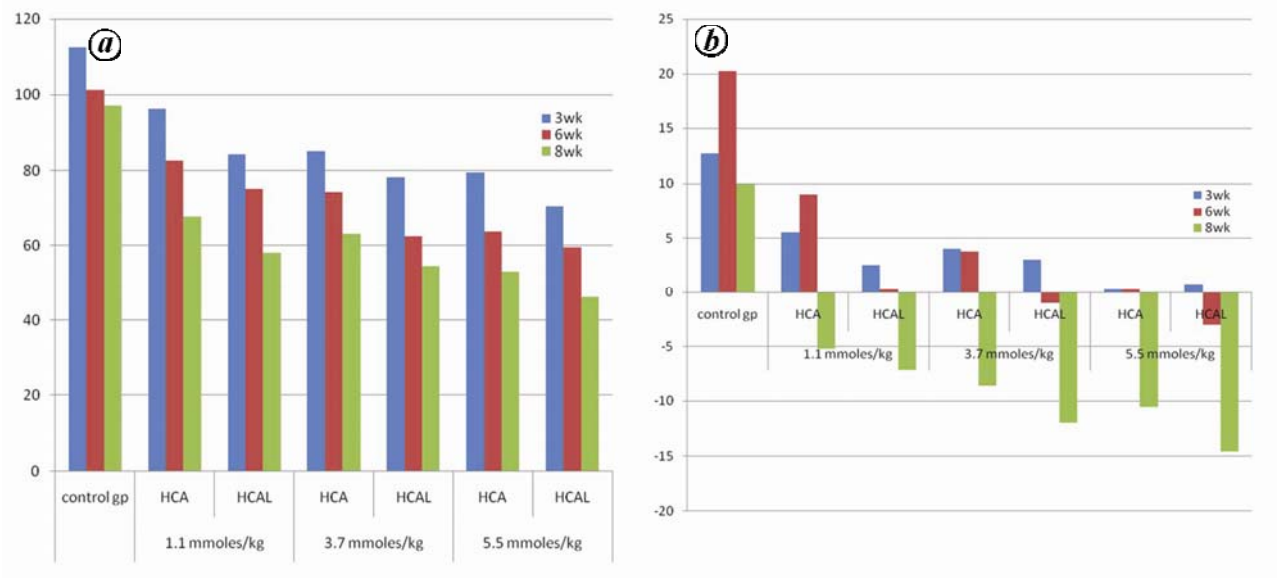

Figure 3. Comparison of (***a***) average food intake (g/rat) weekly up to 8 weeks and (***b***) average body weight gain/loss per rat in total 8 weeks.

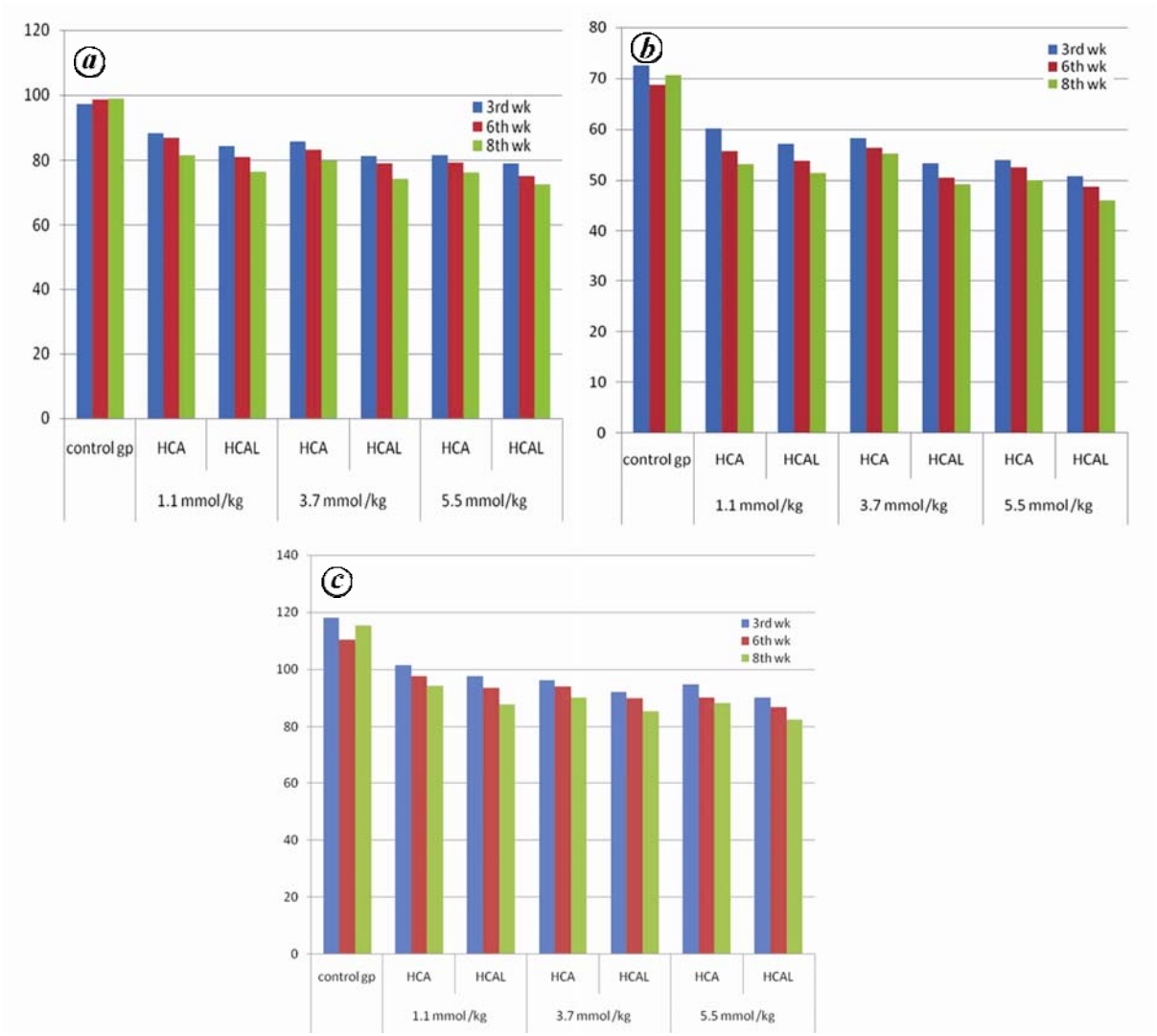

Figure 4. Average (***a***) plasma cholesterol level (mg/dl), (***b***) plasma triglycerides (mg/dl) and (***c***) plasma glucose levels (mg/dl).

Screening of histopathological slides of visceral organs, viz. heart, kidney, spleen, liver and testis showed normal histological architecture without any significant pathological changes in all animals treated with HCA and HCAL.

Thus, both HCA and HCAL significantly reduced food intake and body weight; however, HCAL showed better effect compared to HCA. Both these compounds were proven to be safe based on biochemical and histopathological analysis. In order to dispel the literature report[14], which stated that HCAL present in HCA salts led to a failure of testicular development, we prepared pure HCAL and studied its effects on the testis and its further impact on their development both with acute and chronic treatment. We have concluded that LD50 of HCAL can be considered as > 5000 mg/kg body wt and classified according to the Globally Harmonized System (GHS) category as either 5 or unclassified, which indicates its non-toxic nature.

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Zinc oxide–neem oil conditioning for improving the quality of the micronutrient fertilizer zinc sulphate heptahydrate

In India, zinc (Zn) deficiency was first reported in rice by Nene[1]; it is now widespread all over the country. The latest report[2] indicates that 49% of Indian soils are deficient in Zn. But good responses of several field and fruit crops to Zn fertilization have been reported from different parts of the country[3,4]. The main causes for the wide-spread emergence of Zn deficiency after the Green Revolution are high crop harvests in intensive crop rotations such as rice–wheat[5] accompanied by imbalanced fertilization with high doses of nitrogen, removal of both grain and straw from the field and little to virtually nil application of organic manures.

Soil application of 10–50 kg ha^{-1} yr^{-1} of zinc sulphate heptahydrate (ZSHH) is the most common method of zinc fertilization. According to the Fertilizer Control Order of 1985 (ref. 6), Government of India, ZSHH should be free flowing, contain 21% Zn and should not have a pH less than 4.0. However, as marketed in India, ZSHH suffers from the following drawbacks in quality: (i) Lump formation during storage; (ii) Lack of free flow; (iii) Free acidity that can be injurious to seeds; (iv) Lesser Zn content due to adulteration.

The present study was undertaken to study some of the above properties in commercially marketed ZSHH in India. For this purpose, samples of ZSHH (fertilizer grade), in 1 kg polypropylene packs, were obtained from three manufacturers.

Conditioning with zinc oxide–neem oil (ZONO) was done by taking 1 kg ZSHH in plastic containers and adding different amounts of neem oil and zinc oxide (ZnO) and thoroughly mixing the contents of the container. A number of batch studies suggested that the best ratio was 970 g ZSHH, 20 g ZnO and 10 g neem oil. The technique finally adopted was as follows: 970 g of ZSHH was taken in a 5 l plastic container, 10 g neem oil was added and the contents were thoroughly mixed by applying the lid on the container and manually shaking it for 15 min. The container lid was removed and 20 g ZnO (99% purity and 90% particles of 32 micron size) was added. The contents were again thoroughly mixed by shaking for 15 min after applying the lid. For larger amounts of ZSHH, seed treating drums can be used – these are easily available at a low cost. Neem oil was used because of its low cost and also because it controls many insect pests; it has chemicals that have nitrification inhibiting properties which increase nitrogen-use efficiency[7].

The ZONO-conditioned ZSHH was then stored in sealed 1 kg polyethylene bags for six months at a temperature of 35 ± 5°C. The ZONO-conditioned ZSHH