Effects of Phorbol Esters in Carp (Cyprinus carpio L)

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ABSTRACT. Carp (Cyprinus carpio L) were fed diets containing phorbol esters at concentrations of 0, 3.75, 7.5, 15, 31, 62.5, 125, 250, 500 and 1,000 µg/g feed. Phorbol esters were from Jatropha curcas nuts. Jatropha curcas toxicity has been reported in humans, rodents and livestock, and phorbol esters have been identified as the main toxic agent. The adverse effects observed in carp at phorbol esters concentrations of 31 µg/g or higher were lower average metabolic growth rate, fecal mucus production and rejection of feed. Average metabolic growth rates (g/kg$^{0.8}$/d) in a 7-d experimental period during which diets containing phorbol esters were fed to carp (values with different letters being significantly different) were 15.4a, 14.4a, 12.5ab, 12.4ab, 10.9b, 3.4c, 0.2c, -3.8d, -4.9d and -5.6d, respectively, at the above mentioned concentrations. The values for the recovery phase of 9-d during which phorbol esters were not included in the diet were 16.0a, 15.6a, 14.9a, 15.6a, 5.3b, 1.6b, 4.6bc, 6.3bc, 7.8c and 8.2c, respectively. The adverse effects of phorbol esters were reversible since withdrawal of the esters from the diets led to gain in body mass. None of the fish died at any of the concentrations studied. Incorporation of vitamin C, an antioxidant, at levels of 0.4 and 2% in the feed did not prevent occurrence of the adverse effects of the phorbol esters. The threshold level at which phorbol esters appeared to cause adverse effects in carp was 15 µg/g feed or 15 ppm in the diet. Carp were highly sensitive to phorbol esters, thus making them a useful species for bioassay of these compounds. This bioassay together with other analytic procedures could be of immense use in the development of detoxification processes for agro-industrial products containing phorbol esters, such as jatropha meal or jatropha oil, and as a quality control method to monitor successive stages in industrial detoxification processes.

Jatropha curcas, also known as "physic nut, purging nut, pinoncillo, Habb-El-Meluk", is a member of the Euphobiaceae family. It is a shrub or small tree which can reach a height of up to 8 m. The plant grows quickly, survives in poor stony soil and is resistant to drought. It is thought originated in Central America, but presently grows in most of the tropics (1).

The seeds of J curcas weigh on average from 0.69 to 0.86 g depending on the variety. The kernel contains about 58% oil. The meal (oil-free kernels) contains about 58% crude protein (2). The seeds of J curcas are a good source of oil which is used as a diesel substitute and for medicines and cosmetics in various developing countries. However, although the meal is rich in protein, it is toxic to rats, mice and ruminants and therefore can not be used as an animal feed. Several cases of J curcas nut poisoning in humans after accidental consumption of the seeds have been recorded with symptoms of giddiness, vomition and diarrhea (3).

We are working to detoxify jatropha meal so that it can be used as a protein supplement for livestock feeding. The meal has high trypsin inhibitor and lectin activities which can be inactivated by heat treatment. Phorbol esters, present in high levels in the kernels, have been identified as the main toxic agent responsible for toxicity (3). It was not possible to destroy phorbol esters by heat treatment, but these were extracted using 92% aqueous methanol, and the meal after extraction was non-toxic to rats (3). Jatropha oil, which contains most of the phorbol esters, can also be used for human consumption after complete removal or destruction of the phorbol esters.

Fish are widely used in numerous fields of basic and applied research; they are currently the third largest laboratory animal group after mice and rats, and they will become increasingly important as there is a gradual shift to using lower vertebrates due to the European Community legislation which calls for the use of animals with a low degree of neurosensitivity (4). Common carp (Cyprinus carpio L) is a major species used mainly for routine toxicity tests. Our preliminary study suggested that carp are more sensitive to phorbol esters than rats, as the meal obtained after extraction with 92% aqueous methanol for removal of phorbol esters resulted in lower growth performance in carp (unpublished observations).

The objective of this present work was to systematically evaluate the effects of different levels of jatropha phorbol esters on carp. The results may lead to the use of carp as a valuable experimental model to study mechanisms of phorbol esters toxicity, help develop economically viable processes for destruction/extraction of phorbol esters in jatropha meal, and validate this process as a bioassay for monitoring the quality of detoxified jatropha meal or oil before it is used in animal rations or human diets. The effects of vitamin C incorporation in a diet containing phorbol esters were also studied.

MATERIALS AND METHODS

Feed

The ingredients and proximate analysis of the feed used are given in Table 1. Phorbol esters were extracted from J curcas oil using methanol. In brief, 100 ml of methanol was added to 50 ml of the oil in a separatory funnel. The contents were shaken thoroughly and the methanol phase collected. This procedure was repeated 4 times, and the pooled methanol phase (approximately 400 ml) was dried under vacuum at about 45 C using a rotary evaporator. The oily material (approximately 1.5 ml) left after removal of the methanol was dissolved in 3 ml tetrahydrofuran, and then 20 ml of water was added. This fraction, containing a total of 125 mg phorbol esters, was mixed uniformly to 50 g feed to give a phorbol ester level of 2.5 mg/g feed. This feed was pelleted with a small hand machine used for making spaghetti before being lyophilized. The phorbol ester content was determined using HPLC (5). The feed containing 2.5 mg phorbol esters/g was mixed with the standard feed in Table 1 in different proportions to obtain feed with phorbol ester levels of 3.75, 7.5, 15, 31, 62.5, 125, 250, 500 and 1,000 ug/g.

Fish Used and Experimental Phases

A recirculating system containing a set of aquaria, each with a capacity of approximately 25 L kept at 23 C, was used under a photoperiod of 12 h light:12 h dark. Rates of water flow were adjusted to maintain oxygen above 90% of the air saturation level. One aquaria containing 5 carp (C carpio L) was used/group. The fish were weighed individually on the first day of the experiment and each was marked by a cut at the top, lower, front-right or front-left fin for identification. The fin of 1 fish/group was not cut.

Table 1. Composition and proximate analysis of fish diet

Ingredients (%)	
Fishmeal	50
Wheat meal	42
Soyaoil	4
Standard vitamin mixture	2
Standard mineral mixture	2
Proximate composition (% in DM)	
Crude protein	40
Lipid	10
Neutral detergent fibre	4.5
Ash	10
Gross energy (MJ/kg DM)	20.1

The fish were fed diets containing phorbol esters at 5-times maintenance divided into 7 portions/d for 7 d. The chambers were cleaned every day to remove mucus, rejected feed particles and feces. A control feed (free of phorbol esters) was also fed. After 7 d, which was designated as the experimental phase, all fish were weighed and then fed the control feed for 9 d. During this recovery period the fish were fed 5-times maintenance (adjusted for weights recorded after the 7-d experimental phase) divided into 7 portions/d. This phase determined whether the changes induced by the phorbol esters in the experimental phase were reversible or not.

Experiment 1

Initially, a control and 3 phorbol ester groups (3.75, 7.5 and 15 ug/g feed) were studied for a total of 16 d, comprising the experimental and the recovery phases. After completion of this experiment, a control and 2 phorbol ester groups (31 and 62.5 ug/g feed) were studied. As there was no significant difference between body mass at the start and end of the experimental and recovery phases for the 2 control groups, the data (n = 10) were pooled and compared with those from the other groups (3.75, 7.5, 15, 31 and 62.5 ug phorbol esters/g feed). The initial weights of the control and phorbol ester groups were statistically similar (Table 2).

Experiment 2

Subsequently, a control and 2 phorbol ester groups (250 and 500 ug/g feed) were studied follwed by a control and 1 phorbol ester group (1,000 ug/g feed). Again, as there was no significant difference between weights at the start and end of the experimental and recovery phases for the 2 control groups, the data (n = 10) were pooled and compared with those from the other groups (250, 500 and

Table 2. Initial body mass and per cent gain in body mass during the experimental and recovery phases at different levels of phorbol esters in the diet

Phorbol esters (μg/g feed)	Initial body mass (g)	Body mass gain (%) in experimental phase	Body mass gain (%) in recovery phase
Experiment 1			
0	3.8 ± 0.25^{a}	36.8 ± 1.88^{a}	45.1 ± 3.49^{a}
3.75	3.9 ± 0.19^{a}	34.6 ± 1.71^{ab}	47.7 ± 2.55^{a}
7.5	4.0 ± 0.23^{a}	31.7 ± 1.66^{ab}	45.5 ± 2.02^{a}
15	4.3 ± 0.39^{a}	29.2 ± 3.76^{bc}	46.6 ± 3.04^{a}
31	4.2 ± 0.29^{a}	25.0 ± 3.76^{c}	14.6 ± 3.67^{b}
62.5	3.4 ± 0.11^{a}	7.7 ± 1.07^{d}	4.8 ± 3.67^{b}
125	3.5 ± 0.37^{a}	0.4 ± 1.24^{d}	13.8 ± 5.12^{b}
SEM	0.107	0.78	1.39
Experiment 2			
0	7.8 ± 0.79^{a}	33.4 ± 2.40^{a}	37.9 ± 3.27^{a}
250	6.4 ± 0.73^{a}	-5.9 ± 1.61^{b}	17.4 ± 8.19^{b}
500	6.3 ± 1.04^{a}	-6.8 ± 3.66^{b}	24.3 ± 5.1^{ab}
1000	8.8 ± 1.05^{a}	-9.2 ± 1.79^{b}	22.2 ± 6.95^{ab}
SEM	0.46	1.31	2.69

1,000 μg phorbol esters/g feed). The initial weights of the control and the phorbol ester groups were statistically similar. However, these weights were statistically higher than the initial weights of the groups in Experiment 1 (Table 2), and therefore the results from Experiments 1 and 2 are presented separately.

Effects of Vitamin C in Diet

Vitamin C, an antioxidant, was added to the standard fish feed at a level of 0.4 or 2% in the presence and absence of 125 μg phorbol esters/g feed. The body mass changes, mucus production and feed consumption were monitored for 7 d.

Statistical Analysis

The significance of differences between means was compared using Duncan's multiple range test after ANOVA for one-way classified data with the aid of the SAS/STAT program (6). Results were expressed as mean ± SE. A $P < 0.05$ was choosen as minimum for significance.

RESULTS

Percent Gain in Body Mass

Table 2 shows the initial mass of fish and percent body mass gain at the end of the experimental and recovery phases. Up to phorbol ester levels of 7.5 μg/g feed, the body mass gain in the experimental phase did not differ significantly from the control group. Phorbol esters at 15 μg/g feed or higher decreased the body mass gain significantly in the experimental phase. However, the body mass gain in the recovery phase did not differ between the control and phorbol ester groups up to 15 μg phorbol ester/g feed. Above this level, the body mass gain was significantly decreased.

Average Metabolic Growth Rate

As the initial body mass in Experiments 1 and 2 differed significantly, the average growth rate (g/kg $^{0.8}$/d) based on metabolic mass was calculated for the entire set of data and the results presented as Fig 1. The average metabolic growth rate for the control and phorbol ester groups up to 15 μg/g feed did not differ significantly in the experimental phase, but in the higher dose groups were significantly decreased. The average metabolic growth rates for the control and phorbol ester groups up to 15 μg/g feed were statistically similar in the recovery phase. The average metabolic growth rate in the recovery phase was statistically lower in phorbol ester groups at 31 μg phorbol esters/g feed or higher. The greater loss in body mass in the experimental phase and the higher gain in the recovery phase as the concentration of phorbol esters increased from 62.5 to 1,000 μg/g feed was notable. This was also evident from the percent body mass gain (Table 2).

Mucus Production and Feed Rejection

Production of fecal mucus or rejection of feed was not observed up to 15 μg phorbol

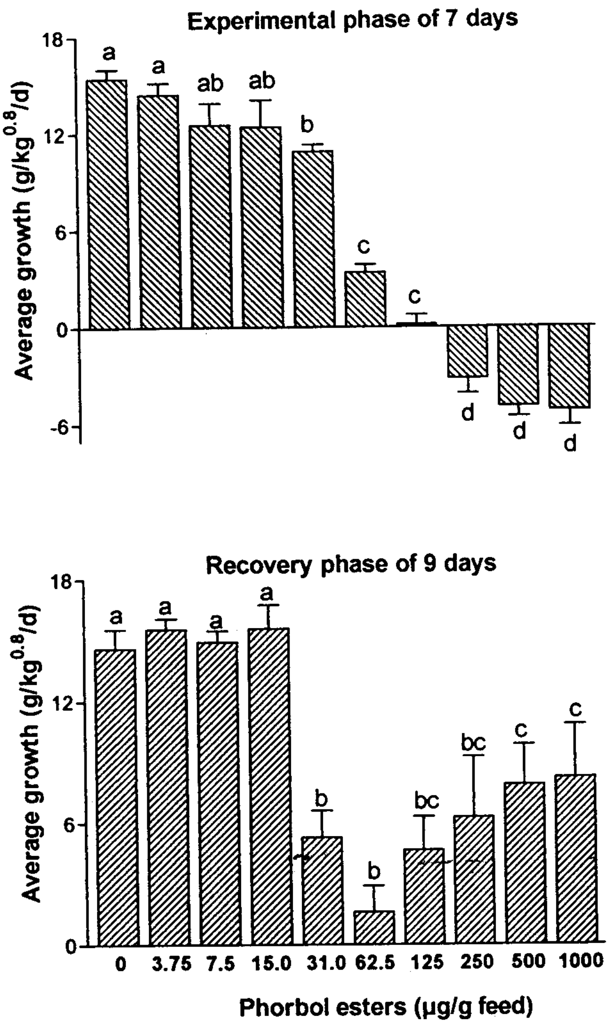

Figure 1. Effect of phorbol ester levels on average metabolic growth rate.

Table 3. Some observations on fecal mucus production and rejection of feed in fish fed a diet with and without different levels of phorbol esters

Phorbol esters (µg/g feed)	Observations	
	Mucus in feces	Rejection of feed
0	Nil	Nil
3.75	Nil	Nil
7.5	Nil	Nil
15	Nil	Nil
31	Mucus started on 4th day of experimental phase and continued but decreased till 4th day of the recovery phase; +	Nil
62.5	Mucus started from 3rd day onwards and continued but decreased till 8th day of the recovery phase; ++	Rejection (+) started from 6th day onwards; #
125	Mucus stated from 2nd day onwards and continued till the end of the experimental phase; +++	Rejection (++) started from 4th day onwards; #
250	Mucus started from 2nd day onwards of the experimental phase and continued till end of the experimental phase; +++	Rejection (+++) started from 3rd day onwards; #
500	Mucus started from 1st day onwards of the experimental phase and stopped after 3rd day; +++	Rejection (++++) started from 2nd day onwards; #
1000	Mucus started from 1st day onwards of the experimental phase and stopped after 3rd day; +++	Rejection (++++) started from 1st day onwards; #

+, ++, +++, ++++ increasing order of mucus production or rejection of feed
consumption of phorbol ester-free feed in the recovery phase started slowly

esters/g feed. Production of mucus in feces and rejection of feed started at 31 µg phorbol esters/g feed and higher. At the high levels of phorbol esters (250, 500 and 1,000 µg/g feed), mucus in the form of a fine tube, almost as long as the fish under investigation (as if the whole intestinal lining had become detached) was observed. At 1,000 µg phorbol esters/g feed, mucus appeared in the form of a tube approximately 2 h after the first portion of feed was consumed, suggesting strong irritating effects. At lower concentrations (62.5 and 125 µg/g), mucus also appeared in the same form, but these tube-like structures were smaller in length and more fragile in nature. As the concentration of phorbol esters in feed increased, the time to feed rejection and mucus production decreased (Table 3). No fish died during the experimental phase. However, the fish fed phorbol esters at concentrations of 31 µg/g feed or higher were sluggish and had a tendency to group together, often near the bottom of the aquarium.

Effect of Vitamin C

The results obtained using 2% vitamin C in the diet are presented in Table 4 (results with 0.4% ascorbic acid were similar and are not presented). The decrease in body mass induced by 125 µg/g phorbol esters was similar in the presence or absence of 0.4 or 2% vitamin C in the diet. At both these levels of vitamin C, mucus appeared from the 2nd d onwards and continued until the end of the 7-d experimental period. The mucus production decreased from the 5th d onwards, probably due to rejection of the feed. Feed rejection was also observed from the 4th d onwards in the presence or absence of vitamin C.

DISCUSSION

Carp are highly sensitive to phorbol esters. The threshold at which carp showed adverse effects was 15 µg/g feed (or 15 ppm). Phorbol esters at levels higher than 15 ppm in the diet can be detected by fish. The effects of phorbol esters on fish appear reversible in nature. The higher body mass gain in the recovery phase observed at 250 µg to 1,000 µg phorbol esters/g feed, as compared to 31 or 62.5 µg/g feed, appear due to lower phorbol ester intake by fish at higher dietary levels. As evident from Table 3, fish at high levels rejected feed after a brief exposure to the high phorbol ester levels, which presumably produced irritation of the intestine. The fish stopped eating the diets containing high phorbol esters and the damage produced by their brief exposure was repaired in a short time. On the other hand, diets containing lower levels of phorbol ester (31 or 62.5 µg/g feed) were consumed for longer durations, and fish on these diets probably ingested greater amounts of the phorbol esters. The adverse effects produced by low but continuous doses of phorbol esters seemed more severe and were reversible only after a long duration.

Phorbol esters, besides being co-carcinogens, bring about a wide range of biochemical and cellular effects, alter cell morphology, serve as lymphocyte mitogens and induce platellate aggregation (7). Phorbol esters elicite adverse effects by binding to a receptor site and activating protein kinase C, an enzyme that plays an important role in signal transduction and development processes of most cells and tissues. These are responsible for purgative and skin-irritant effects and tumor promotion (7,8). It would be interesting to investigate the histopathological changes in various organs of fish fed diets containing jatropha meal and different phorbol ester levels. The addition of vitamin C, an antioxidant, to the diet did not provide any protection to fish against the adverse effects of phorbol esters.

In our previous study (3), we showed that phorbol esters are the main toxic agent in jatropha meal, although the meal also has high activities of trypsin inhibitor and lectins (2). Symptoms such as decrease in body mass, mucus production and rejection of feed observed in fish feeding jatropha meal were reproduced by feeding a diet containing HPLC-purified phorbol esters (3) or the dichloromethane extract of oil containing phorbol esters (Tables 2, 3). These results suggest that carp (C carpio) are a suitable

Table 4. Effect of vitamin C addition (20 mg/g feed; 2.0 %) to the standard fish feed in presence and absence of phorbol esters, PBE (0.125 mg/g feed; 125 ppm) on body mass gain in carp

Feed	Initial weight (g)	Final weight (g)	Increase in body mass in 7 days (%)
Control	4.4 ± 0.3	5.9 ± 0.4	35.3 ± 0.9
Control + Vit C	4.3 ± 0.4	5.8 ± 0.6	36.4 ± 1.4
Control + PBE	4.0 ± 0.3	3.9 ± 0.3	-0.08 ± 2.0
Control + PBE + Vit C	3.8 ± 0.3	3.8 ± 0.3	-0.4 ± 0.4

model for studying the biological effects of phorbol esters or for studies on jatropha toxicity. Ingestion of some plants from the Euphorbiaceae and Thymelaeaceae families, which biosynthesize diterpene esters of the phorbol type, cause severe symptoms of toxicity in livestock (9). Fish can also be used for investigations on the toxicity of these plants. Besides satisfying the requirements of legislative bodies in controlling animals used for experimentation, the use of fish has several other advantages; eg they are easy to handle and require less feed, which is particularly important when the toxicity of a purified plant compound is under investigation. In most instances, these pure compounds are only available in minute quantities after tedious isolation and purification steps.

Phorbol esters in a diet at levels higher than 15 ppm can be detected by carp. Symptoms such as lower gain in body mass, mucus production and rejection of feed are easy to recognize, possibly rendering this bioassay attractive for applications other than studies of phorbol ester toxicity. The minimum detection limit in this bioassay was approximately 2-times higher than that of the HPLC analytical method presently available (3). In Nicaragua, jatropha oil is extracted on an industrial scale and is used for running diesel engines after transesterification. Due to shortage of fuel in developing countries, the cultivation of J curcas is becoming wide-spread in many tropical countries. Various approaches are being developed for detoxification of jatropha meal, a byproduct of the oil extraction (2,3). The bioassay employing carp appears to have potential as a process in the validation of extraction/destruction of the phorbol ester present in jatropha meal. This will help produce jatropha meal safe for feeding to livestock. In addition, this bioassay, together with other analytical techniques such as HPLC or TLC, could be used for monitoring batches of detoxified meal before their market release for animal consumption. Although jatropha oil is presently used as fuel, it may be suitable for human consumption after further detoxification, and the present bioassay would be useful for that application.

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