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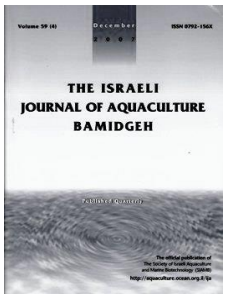

Dietary effect of *Quillaja saponaria* and/or *Yucca schidigera* extract on growth and survival of common carp *Cyprinus carpio*, their antioxidant capacity and metabolic response to hypoxic condition

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Key words: antioxidant capacity, *Cyprinus carpio*, metabolic response, saponins, yucca

Abstract

This study evaluated dietary effects of extracts of Quillay *Quillaja saponaria,* and/or Yucca *Yucca schidigera* on growth and survival of juvenile common carp *Cyprinus carpio,* and their antioxidant capacity and metabolic response to low dissolved oxygen (DO) stress. For 8 weeks, fish were fed one of 4 different diets. The diets were supplemented with either 150 mg/kg Quillay (QS), 150 mg/kg Yucca (YS), the combination of 75 mg/kg Quillay and 75 mg/kg Yucca (M), or control diet (C) without addition of Quillay or Yucca. Growth and survival were monitored periodically. After rearing, fish were subjected to low DO stress, and after a week, antioxidant capacity (superoxide dismutase, glutathione peroxidase and glutathione reductase) and metabolic response (glucose, triglycerides and lactate) were analyzed. QSfed fish had the highest weight gain among all the treatments. Treatments did not affect fish survival a week after low DO stress. Among antioxidant capacity and metabolic response, significant effects were found only on superoxide dismutase and glucose. QS fed fish had 39% lower plasma superoxide dismutase than the C and M groups. QS and M groups exhibited 29% and 26% lower plasma glucose than the C group, respectively. Overall, the QS diet improved growth and exhibited favorable antioxidant capacity and metabolic response of carp to low dissolved oxygen environment.

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Introduction

Natural plant products provide safe and acceptable alternatives to synthetic compounds when used in animal husbandry. Many synthetic antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene are available, however, their use is under strict regulation due to their potential health hazards (Bran, 1975). Therefore, the search for natural antioxidants as alternatives to synthetic products is of great interest, particularly in the aquaculture industry, since they affect growth, feed intake, and reproduction of fish (Francis et al., 2002b, Francis et al., 2001). Natural and renewable plant resources, such as Quillay *Quillaja saponaria* and Yucca *Yucca schidigera*, have been gaining attention because of their biological effects on fish and animals (Francis et al., 2002a).

Quillay is a large tree with thick bark, shiny, leathery leaves, and is native to China, Peru, and the arid zones of Chile (Leung, 1980). It is rich in saponins which are steroids or triterpene glycosides (Francis et al., 2002a). The bark of Quillay is one of the major sources of industrial triterpenoid saponins which play a role in promoting permeability of plasma membranes (Leung, 1997). The saponins possess a wide variety of immunological characteristics (Mimaki & Sashida, 1996) such as anti-inflammatory, anti-allergic, anti-viral, and molluscicidal activity (Lacaille-Dubois & Wagner, 1996; Hassan et al., 2010) .

Yucca is a flowering plant native to southwestern US and Mexico (Cheeke, 2000). It is a source of phenolic derivatives and is an antioxidant (Piacente et al., 2004). The main active compounds of Yucca are steroidal saponins and glycocomponents. Steroidal saponins are natural non-ionic surfactants, making them capable of enhancing nutrient absorption in ruminants (Oleszek et al., 1994). These also increase intestinal flora activity, and improve the digestive process. On the other hand, glycocomponents are molecular structures which are highly thermo stable with an ability to join ammonia, bind and neutralize it in the digestive tract. This neutralizes noxious effects and converts compounds into another type of non-toxic nitrogen compound, thus improving the metabolic process (Johnston et al., 1981). A Yucca-supplemented diet can improve not only the growth of Nile tilapia *Oreochromis niloticus* (El-Saidy and Gaber 2004) and white shrimp *Litopenaeus vannamei* but also the water quality (Yang et al., 2014).

Limited research has evaluated the antioxidant capacity, metabolic response, and their correlations to growth and survival of fish fed a diet with Quillay and/or Yucca extract. This study investigated the effect of dietary Quillay and/or Yucca on growth and survival of common carp *Cyprinus carpio* and antioxidant capacity and metabolic response under low oxygen environment.

Materials and Methods

Diet preparation. This study consisted of four treatments with three replicates of each. Control diet (C) was a commercial freshwater fish feed (B-Meg, San Miguel Foods, Incorporated, Philippines) containing 13 % moisture, 30 % crude protein, 8 % fat, 7 % fiber, and 16 % ash. The three other treatment diets were based on the control diet supplemented with: Diet QS - 150 mg/kg Quillay extract (product no. S-7900, Sigma Chemical Company, USA, from *Quillaja* bark, containing 11.0% sapogenin); Diet YS - 150 mg/kg Yucca extract (Desert King International, San Diego, California, USA) and Diet M; 75 mg/kg QS and 75 mg/kg YS. Diets QS, YS were diluted in 100 ml of distilled water. The solution was then sprayed onto 1 kg feed spread evenly with a garden sprayer on an aluminum pan. The control diet was sprayed with 100 ml distilled water only. The four experimental diets were dried overnight in an oven at 35 ^{o}C. Experimental diets were then stored dry in a plastic container at room temperature.

Fish rearing, feeding and sampling. Carp obtained from a commercial farm in San Mateo, Isabela, Philippines, were weighed and distributed into 12 (44 cm x 33 cm x 21.5 cm) glass aquaria (30 fish/ aquarium, initial fish weight 2.6 g ± 0.11 per treatment with no significant weight differences, $p > 0.05$). Fish were acclimated to aquarium conditions for one week prior to the 8-week rearing experiment during which they were fed a ration of 6% of their body weight twice daily at 0800 and 1500 h. Each aquarium was aerated. Feces and uneaten feed were siphoned out and one-third of the water was replaced daily.

Water quality parameters were monitored and kept within safe levels: dissolved oxygen (DO) 6 -7 mg/L, temperature 25-30 ^{o}C and pH 6.5-7.8.

Growth and survival. Fish were weighed every 2 weeks with a digital scale. The quantity of feed given was readjusted after each weighing and survival in each tank was monitored daily.

Weight gain (WG) and specific growth rate (SGR) were used as indices for the growth performance of fish.

$WG\ (\%) = 100 \times ((W_f - W_i)/W_i)$

$SGR\ (\%\ /day) = 100 \times ((\ln(W_f) - \ln(W_i))\ /T$

Where W_i is the initial body weight (g), W_f the final body weight (g), ln the natural logarithm and T the length of culture period (days).

% Survival= final count/ initial count *100

Low DO stress test. This experiment was conducted to test the premise that different experimental diets affect survival, antioxidant capacity and metabolic response of common carp under low DO stress conditions. At the completion of the previous experiment, 10 fish from each treatment were randomly selected and transferred to 12 10 L glass aquaria (four treatments with three replicates). Temperature was maintained at 26 C^{o} and DO at 5 mg/L. To induce respiration stress the aerator was removed and a cover placed on each aquarium causing the DO level to be reduced to 1.6-1.8 mg/L. Mortality was recorded daily for one week.

Antioxidant capacity and metabolic response. Blood samples were taken after one week of low DO stress. Sampled fish were quickly anesthetized with tricaine methanesulphonate (MS-222) at 100 mg/L. Approximately 200 µl heparinized blood was withdrawn from the caudal vessel of 3 fish per aquarium using 1ml sterile syringe with 25 gauge needles. Heparin was used to prevent blood coagulation. Blood was then centrifuged for 5 min at 1800 rpm and the plasma was withdrawn and immediately frozen (-4^{o}C) for later evaluation of antioxidant capacity and metabolic response.

The antioxidant capacity was analyzed with enzyme linked immunosorbent assay (ELISA) reader for superoxide dismutase (SOD) and SP-830 plus metertech spectrophotometer (Hitachi Ltd, Japan) for glutathione peroxidase (GPx) and glutathione reductase (GR). The volume of plasma used was 10, 10 and 20 µl for SOD, GPx and GR analysis, respectively.

SOD activity was measured by ability to inhibit superoxide radical dependent reactions. The reaction mixture (1.7 ml) contained xanthine (0.05 mM) and 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT, 0.025 mM) dissolved in 50 mM CAPS (pH 10.2) and 0.94 mM EDTA. In the presence of xanthine oxidase (80 U/L, 250 µl), superoxide and uric acid were produced from xanthine. The superoxide radical was then reacted with INT to produce a red formazan dye. The optical density was measured at 505 nm, 37 °C, and the rate of reaction was estimated from the absorbance readings at 30 s and 3 min after adding xanthine oxidase. A reference standard SOD was supplied with the Randox Kit (Crumlin, Co. Antrim, UK). One unit of SOD was defined as the amount required to inhibit the rate of xanthine reduction by 50% (Biagini, Sala & Zini 1995). One unit of activity was expressed in U/ml.

GPX activity was measured based on the method described by Paglia & Valentine (1967). GPx catalyses the oxidation of glutathione using cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized form of glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance at 340 nm is measured. 15 µl diluted plasma mixture was added to the reaction mixture containing 40 µl cumene hydroperoxide and 10 mM buffer. The optical density of NADPH was measured at 340 nm, 37 °C, and the rate of reaction was estimated from the absorbance readings in the first 3 min after adding cumene hydroperoxide. One unit of activity was expressed as U/ml.

GR catalyzes the reduction of glutathione in the presence of NADPH, which is oxidized to NADP+. The decrease in absorbance at 340 nm was measured. This assay was carried out using Randox laboratories kit according to manufacturer's instructions (Biagini, Sala & Zini 1995). One unit of activity was expressed in U/ml.

ELISA reader with individual Randox kits was used for determination of Gluc (Randox, GOD-PAP), Trig (Randox-GPO-PAP) and Lac (Randox, PAP). Methods were adapted to a 96-well plate using 3 µl samples and 300 enzyme reagents (Palacios et al., 2000). Gluc, Trig and Lac levels were expressed in mg/dl plasma.

Statistical Analysis. An arcsine transformation was used before processing percentage data (percentage survival). Kolmogorov–Smirnov and Levene's test for normality and homogeneity of variance were applied on the data of growth, survival, antioxidant capacity and metabolic response. The data conformed to normal distribution and homogenous variance. One-way analysis of variance (ANOVA) was then performed to determine the significance of the effects of Quillay and/or Yucca on growth, survival, antioxidant capacity and metabolic response. Duncan's Multiple Range Test was then carried out to compare differences among treatments. Correlation analysis was applied to growth, survival, antioxidant capacity and metabolic response. SAS v.9 was the software used in all the analysis. The significance level applied to all analysis was set at 5%.

Results

Feed intake and behavior. During weeks 1-4 of feeding experiment, fish consumed the entire daily ration of treated diets fed. However, in the 5th week, fish did not consume the entire daily ration and hence it was reduced to 3%. This feeding rate was adopted from week 5 until completion of the experiment.

Growth and survival. The effect of the treatments on growth (Table 1) was seen only after week 8, (for W_f, QS>M≧C≧YS and for WG, M=QS>C=YS). No difference was found when growth was expressed in terms of SGR and no significant difference was found on survival after eight weeks of the trial.

Table 1. Mean initial weight (W_i), final weight (W_f), weight gain (WG), specific growth rate (SGR) and survival of common carp *Cyprinus carpio* after fed experimental diet for eight weeks.

Treatment[1]	W_i	W_f	*WG*[2]	*SGR*[3]	*Survival*[4]
C	2.76^{a} (0.15)	5.20^{bc} (0.02)	77^{b} (4.6)	1.14^{a} (0.09)	83^{a} (1.93)
QS	2.62^{a} (0.06)	5.49^{a} (0.06)	93^{a} (2.2)	1.32^{a} (0.04)	86^{a} (1.11)
YS	2.76^{a} (0.16)	5.13^{c} (0.05)	75^{b} (3.6)	1.12^{a} (0.09)	82^{a} (1.11)
M	2.49^{a} (0.08)	5.33^{b} (0.06)	95^{a} (5.6)	1.36^{a} (0.05)	86^{a} (2.93)

Means (S.E) in the same column with a common superscript are not significantly different ($p \leq 0.05$).
[1] Fish were fed control diet (C), which was a commercial freshwater fish feed, diet supplemented with 150 mg/kg Quillay *Quillaja saponaria* (QS), 150 mg/kg Yucca *Yucca schidigera* extract (YS) or combination of 75 mg/kg Quillay and 75 mg/kg Yucca (M) for eight weeks.
[2] WG (%) =($W_f - W_i$)/ W_i) x 100
[3] SGR = ((ln mean final weight) − (ln mean initial weight)/no. of days) × 100.
[4] Survival (%)= final count/ initial count *100.

Antioxidant capacity. After exposure to low DO, SOD activity of the QS-group was lower than M and C groups but similar to YS group (Table 2).

Table 2. Mean activities of plasma antioxidant capacity of common carp *Cyprinus carpio* after fed experimental diet for eight weeks and then subjected to low dissolved oxygen for one week.

Treatment[1]	*Antioxidant capacity*[2]		
	SOD	*GPx*	*GR*
C	0.36^{b} (0.20)	1.969^{a} (0.16)	0.076^{a} (0.005)
QS	0.22^{a} (0.16)	2.167^{a} (0.36)	0.106^{a} (0.016)
YS	0.29^{ab} (0.04)	2.515^{a} (0.20)	0.074^{a} (0.0.18)
M	0.32^{b} (0.02)	2.797^{a} (0.05)	0.093^{a} (0.014)

Means (S.E.) in the same column with a common superscript are not significantly different ($p \leq 0.05$).
[1] Fish were fed control diet (C), which was a commercial freshwater fish feed, diet supplemented with 150 mg/kg Quillay *Quillaja saponaria* (QS), 150 mg/kg Yucca *Yucca schidigera* extract (YS) or combination of 75 mg/kg Quillay and 75 mg/kg Yucca (M) for eight weeks.
[2]Antioxidant parameters: SOD-Superoxide dismutase, GPx-Glutathione peroxidase and GR-Glutathione reductase.

Treatments had no effects on GPx and GR activity.

Metabolic response. After exposure to low DO, Gluc levels of QS and M fish were lower than those of C- and YS fish and there was no difference in Gluc between the former or the latter two levels (Table 3).

Table 3. Mean activities of plasma metabolic response of common carp *Cyprinus carpio* after fed experimental diets for eight weeks and then subjected to low dissolved oxygen for one week.

Treatment[1]	*Metabolic response*[2]		
	Gluc	*Trig*	*Lac*
C	94.20[a] (4.35)	120.65[a] (4.70)	30.31[a] (2.11)
QS	67.10[b] (3.8)	131.09[a] (14.15)	33.37[a] (3.57)
YS	91.81[a] (9.37)	110.65[a] (10.80)	36.38[a] (3.18)
M	70.78[b] (5.94)	126.96[a] (9.97)	35.71[a] (4.00)

Means (S.E.) in the same column with a common superscript are not significantly different ($p \leq 0.05$).
[1] Fish were fed control diet (C), which was a commercial freshwater fish feed, diet supplemented with 150 mg/kg Quillay *Quillaja saponaria* (QS), 150 mg/kg Yucca *Yucca schidigera* extract (YS) or combination of 75 mg/kg Quillay and 75 mg/kg Yucca (M) for eight weeks.
[2]Metabolic Parameters: Gluc-Glucose, Trig-Triglycerides and Lac-Lactate.

Treatments had no effects on Trig and Lac.

Survival under low DO stress. There was no significant difference in survival of fish fed experimental diets for eight weeks and a week after exposure to DO stress (Fig. 1).

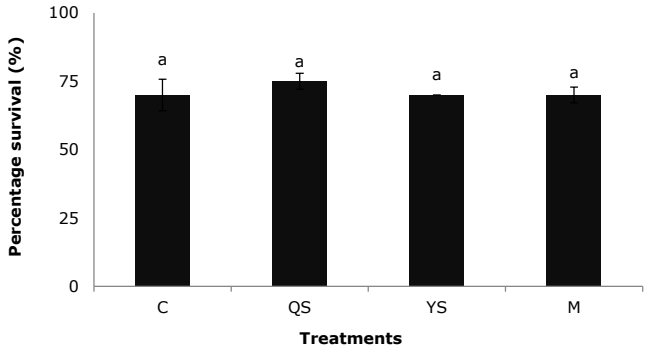

Fig. 1. Mean (S.E.) percentage survival of common carp *Cyprinus carpio* fed diet with control (C), 150 mg/kg Quillay *Quillaja saponaria* (QS), 150 mg/kg Yucca *Yucca schidigera* extract (YS) or combination of 75 mg/kg Quillay and 75 mg/kg Yucca (M) for eight weeks and then subjected to low dissolved oxygen for one week.

Correlation analysis. Correlation analysis was conducted among W_f, WG, SGR, survival, antioxidant capacity and metabolic response of fish fed experimental diet for eight weeks and a week after exposure to DO stress (Table 4).

Table 4. Correlation matrix among Final weight (W_f), weight gain (WG), survival, antioxidant capacity and metabolic response of common carp *Cyprinus carpio* fed diet with Quillay *Quillaja saponaria* and /or Yucca *Yucca schidigera* extract for eight weeks and then subjected to low dissolved oxygen for one week.

Parameters[1]	*W_f*	*WG*	*SGR*	*Sur*	*SOD*	*GPx*	*GR*	*Gluc*	*Trig*	*Lac*
	W_f	0.71*	0.53*				0.57*	-		
		WG	0.94*							
			SGR							
				Sur			0.60*			
					SOD			0.65*		
						GPx				
							GR	-		
								Gluc		
									Trig	
										Lac

The correlation between two parameters is shown by correlation coefficient (r) value; * significant ($p \leq 0.05$); Blank- not significant.
[1] W_f- Final Weight; WG- Weight gain; SGR- Specific growth rate; Sur- Percentage survival after DO stress; SOD- Superoxide dismutase; GPx- Glutathione peroxidase; GR- Glutathione reductase; Gluc- Glucose; Trig- Triglycerides; Lac- Lactate

W_f was positively correlated to WG, SGR and GR and negatively correlated to Gluc. On the other hand, Gluc was positively correlated to SOD and negatively correlated to GR. Finally, GR was positively correlated to survival.

Discussion

Optimum level of saponins helps to enhance the growth performance of fish. In this study, the WG of fish fed QS and M diets increased by 20% ((93-77/77)*100) and 23% ((95-77/77)*100) compared to fish fed the C diet, respectively. Positive effect of QS on growth of carp was also observed with supplementation with Quillay at 150 mg/kg which

caused an 18% increase in the average weight of carp over the control (Francis et al., 2002b). The increased growth performance can be attributed to the effect of dietary saponins on increased permeability of intestinal wall and nutrient absorption (Francis et al., 2001; Francis et al., 2002b).

Antioxidant enzymes counteract oxidative stress to some extent. In this study, the SOD activity of QS-fish was 39% lower compared to C fish. The lowest SOD activity observed in QS fed fish suggested that QS fed fish exhibited strong antioxidant defense capacity through feeding of dietary saponins. Lower SOD may indicate higher cell protection (Hartog et al., 2003). With some antioxidants, like astaxanthin (AX), lowest level of GPx was observed in AX-fish which indicates that AX could be effective in removing H_2O_2 induced by hypoxia (Pan et al., 2010). SOD and GPx activity were increased under hypoxia stress showing a synergistic relationship between SOD and GPx (Lushchak et al., 2001). It was reported also that dietary carotenoid reduced SOD activity in characins *Hyphessobrycon callistus* but had no effect on GPx when fish were under hypoxia stress (Pan et al., 2010). On the other hand, the decrease in the activity of GR may lead to formation of O_2 and H_2O_2 (Yu, 1994) and hydroxyl radical (OH) which harms the cell membrane. Other research showed that inhibition of GR activity could be somehow indication of failure in antioxidant defense due to oxidative damage (Hermes-Lima & Storey, 1993).

Elevation of plasma Gluc, Trig, and Lac levels are used as indicators of the secondary response of animals to stress (Barton & Iwama, 1991). These responses to stressors are considered adaptive and important for fish to regain homeostasis (Mommsen et al., 1999). In this study, Gluc was the only metabolic parameter that responded to the different treatments during low DO stress. The Gluc of QS- and M- fish was 29% and 26% lower compared to the C-fish, respectively. The elevated plasma Gluc level observed in C-fish probably due to cortisol stimulation of glyconeogenesis, especially from amino acids mobilized from peripheral stores (Mommsen et al., 1999). This demonstrates that QS contributes to stabilizing the metabolic response of fish to attain homeostasis under DO stress.

Antioxidant capacity and metabolic response that make up the antioxidant defense system and metabolic processes are expected to increase under stress in order to detoxify ROS and stabilize the overall metabolism, respectively. The antioxidant enzymes are intrinsically linked and dependent upon the activity of one another as well as in metabolic response. One would therefore expect to see correlative changes among the tested parameters. Results of the correlation analysis showed that the higher GR may account for better growth and survival of the fish. This study also found that lower Gluc levels correspond to lower SOD activity and better growth performance.

The scope of the study is limited to the effect of Quillay and Yucca on carp held in hypoxic conditions. Nevertheless, these findings can be used as reference for future studies regarding not only the potential of Quillay and Yucca but also of other natural antioxidants in enhancing performance characteristics of other commercially important aquaculture species and stressors. Furthermore, while dietary antioxidants are being used more widely in animal feeds, safety and consumer perception restrict their application. Natural antioxidants such as Quillay which is a generally recognized as safe (GRAS) product may be added to the feed of farm animals to enhance growth performance, antioxidant capacity, and metabolic response. However, the economic feasibility remains to be evaluated on a commercial scale.

In conclusion, QS supplementation improved growth performance of carp. Furthermore, while carp was exposed to DO stress for a week, the prior dietary supplementation of QS at 150 mg/kg for eight weeks enhanced fish antioxidant capacity (SOD) and stabilized metabolic response (Gluc). Natural, biologically active and renewable plant products, like QS could be used to replace hazardous synthetic growth stimulants to increase carp production. Supplementation of QS, which possesses antioxidant properties that minimizes stress, is an avenue for carp aquaculture development. Further investigations on the effects of QS and YS on metabolic response and antioxidant capacity of fish under more stressful conditions are recommended. Exposing fish to more stressful conditions will enable further characterization of the biochemical indicators which will produce a clearer metabolic and antioxidant defense response in fish.

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