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Cynodon dactylon methanol extract potentiates stress mitigation in response to acidic stress *in Labeo calbasu* fingerlings

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ABSTRACT

A 30-day feeding trial was conducted to study the antistress ability of dietary *Cynodon dactylon* methanol extract against acid stress in *Labeo calbasu* fingerlings. Fish fingerlings (average weight 5.57±0.04 g) were randomly distributed into 4 groups; each with 3 replicates were fed with either of 4 diets containing different levels of *C. dactylon* methanol extract (0, 0.05, 0.5 or 5%). The possible antistress ability of *C. dactylon* methanol extract was assessed in terms of serum cortisol, serum glucose, serum cholesterol, serum alanine amino transferase (ALT), serum aspartate amino transferase (AST), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), ATPase, superoxide dismutase (SOD), catalase (CAT) and Glutathione S transferase by exposing to acidic water with pH 5.5 for 24 h. The serum cortisol level was significantly higher in the acid stress group than that in without acid stress counterpart. However, gradual supplementation of *C. dactylon* methanol extract in diet significantly reduced the cortisol level before as well as after acid stress. Similar trend was also found in serum glucose, serum cholesterol, ALT and AST after acid stress. However, serum cholesterol showed no significant effect before acid stress. Acid stress increased the glycolytic, gluconeogenic, protein metabolic and antioxidative enzymes to cope up with acid stress. Our results indicated that methanol extract of *C. dactylon* at different level possesses good anti stress activity. Hence, supplementation of *C. dactylon* methanol extract in fish diet at a minimum level of ≥0.5% concomitantly reduced the stress in *L. calbasu* exposed to acid stress.

Key words: Acid stress, Biochemical parameters, *Cynodon dactylon*, *Labeo calbasu*, Methanol extract

Fish, like other vertebrates, respond to a stressor by eliciting a generalized physiological stress response, which is characterized by an increase in stress hormone secretion and consequent changes that help maintain the animal's normal or homeostatic state (Iwama *et al.* 1999, Barton 2002). This generalized response is considered to be adaptive and represents the natural capacity of the fish to respond to stress. This response includes increase in plasma cortisol, catecholamines and glucose levels (Barton and Iwama 1991). Measurement of metabolic enzyme activity can also serve as a valuable stress indicator in marine invertebrate and fishes, where the accurate determination of field's metabolic rate is difficult (Dahlhoff 2004).

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Water pH causes disturbances in acid–base and ion regulation and ammonia excretion (Wood *et al*. 1989, Wilkie and Wood 1991, Jensen and Brahm 1995), which causes stress in fish affecting its body physiology and growth (Pickering 1981). Information on the effects of water pH on fish physiology is scarce (Scott *et al.* 2003) and little is known about the physiological responses of fish to water pH changes (acidic and alkaline). The carp culture industry is currently the most important sub-sector of fisheries in India and its input cost is higher, however, a change in water pH could cause stress in fish affecting its body physiology and growth (Das *et al.* 2006).

Natural plant products promote antistress, growth promotion, appetite stimulation, tonic and immuno-stimulation, and to have aphrodisiac and antimicrobial properties in finfish and shrimp larviculture due to alkaloids, flavanoids, pigments, phenolics, terpenoids, steroids and essential oils (Citarasu *et al*. 1999, 2001, 2002; Sivaram *et al.* 2004). *Cynodon dactylon* (*Dhub* grass, Bermuda, Bahama grass, Hariali, Durva, Haritali) grows throughout India and in almost all parts of the world. It forms an important part of Ayurvedic medicine. The juice is used in hysteria, epilepsy and insanity (Rajvaidya 1935), and has antioxidant properties (Auddy *et al.* 2003). The plant

possess antimicrobial and antiviral activity (Dhar *et al.* 1968) and is also used to treat urinary tract infection, calculi and prostatitis. The aqueous plant extract is used as anti-inflammatory, diuretic, anti-emetic and purifying agent (Ahmed *et al*. 1994). It also has significant application in treating dysentery, dropsy and secondary syphilis (Chopra and Handa 1982). *C. dactylon* is used as an antidiabetic agent in traditional system of medicine in India (Kirtikar and Basu 1980). Methanol extract of *C. dactylon* is also used as immunostimulants against WSSV virus infected shrimp (Citarasu *et al.* 2006). Therefore, the present work was intended to investigate the antistress activity of methanol extract of *C. dactylon* in *L. calbasu* fingerlings exposed to acid stress by analysing the serum hormones and selected enzyme activities of different biochemical pathways.

MATERIALS AND METHODS

Collection of plant and preparation of plant extracts: The plant *Cynodon dactylon* was collected from Versova, Mumbai, India. Fresh plants of *C. dactylon* were cleaned and shade dried. The dried plants were pulverized by an electrical blender and passed through 20 µm mesh sieve. The individual sieved powder was soaked in methanol (1:1 ratio) for 48 h. The slurry was filtered, washed to remove non-soluble fractions and filtrate was centrifuged (20,000 × g for 30 min) for clarification. The clarified extracts were condensed at 35°C until the solvent residue had evaporated (Eloff 1998, Citarasu *et al*. 2006). The yield of the extract was 14.6% in terms of dried starting material.

Isocaloric artifitial diets (4) were formulated containing 0, 0.05, 0.5 and 5% *Cynodon dactylon* methanol extracts. Ingredient composition of the experimental diets are presented in Table 1. All the ingredients except vitamin mineral mixture and *C. dactylon* methanol extract were mixed in a big plastic bowl to get homogeneous ingredient mixture. The dough was allowed for 1 h at room temperature for proper conditioning followed by steaming for 20 min in a pressure cooker at 15 psi. The vitamin mineral mixture and *C. dactylon methanol* extract was added after cooling and thoroughly mixed with other ingredients. Pellets were prepared by hand pelletizer having 2 mm diameter. Finally, the pellets were air dried for 24 h and kept in hot air oven at 50°C till the desired moisture level (10%) was reached. After drying, the pellets were packed in airtight polythene bags, labelled and stored properly.

The proximate composition of the experimental diets (Table 1) was determined following AOAC (1995). The moisture content was determined by drying at 105°C to a constant weight. Nitrogen content was estimated by Kjeldahl method (Kjeltec Auto distillation; Foss Tecator, Hoganas, Sweden) method and crude protein was calculated by multiplying nitrogen percentage by 6.25. Crude lipid (CL) was measured by solvent extraction method (1045 Soxtec extraction unit, Tecator, Sweden) using diethyl ether (boiling point, 40–60°C) as a solvent, and ash content was determined by incinerating the samples in a muffle furnace at 600°C for 6 h. Total carbohydrate was calculated by difference, i.e. total carbohydrate % = 100 – (CP% + EE% + Ash%). The digestible energy value of experimental diets was calculated by Halver (1976).

Table 1. Feed formulation of the different experimental diets (per kg feed)

Ingredient	Experimental diet (g)			
	Control (0%)	T_1 (0.05%)	T_2 (0.5%)	T_3 (5%)
Groundnut oil cake[a]	400.0	400.0	400.0	400.0
Fish meal[a]	250.0	250.0	250.0	250.0
Rice bran[a]	200.0	199.5	195.0	150.0
Soybean meal[a]	120.0	120.0	120.0	120.0
Vitamin and mineral mix[b]	20.0	20.0	20.0	20.0
Starch	10.0	10.0	10.0	10.0
C dactylon methanol extract	0.0	0.5	5.0	50.0
Proximate composition of diet (% DM basis)				
Crude protein (CP)	39.26± 0.12	38.73± 1.45	38.18± 1.48	38.24± 1.43
Ether extract (EE)	5.66± 0.35	5.89± 0.56	5.14± 0.76	5.07± 0.32
Total carbohydrate	44.14± 0.76	46.37± 0.94	44.52± 1.53	46.54± 2.02
Total ash	10.80± 0.82	10.01± 0.01	10.22± 0.46	10.15± 1.05
Organic matter	89.20± 1.23	89.99± 1.45	89.78± 2.38	89.85± 3.45

[a]Procured from Local market, Mumbai, India. [b]Vitamin–mineral mix (EmixTM plus) (quantity/2.5 kg): vitamin A, 55,00,000 IU; vitamin D3, 11,00,000 IU; vitamin B2, 2,000 mg; vitamin E, 750 mg; vitamin K, 1,000 mg; vitamin B6, 1,000 mg; vitamin B12, 6 mg; calcium panthothenate, 2,500 mg; niacin amide, 10 g; choline chloride, 150 g; Mn, 27,000 mg; iodine, 1,000 mg; Fe, 7,500 mg; Cu, 2,000; Zn, 5,000 mg; Co, 450 mg; Ca, 500 g; P, 300 g; Se, 50 ppm; L, Lysine-10 g; DL, methionine-10 g.

Fingerlings *of Labeo calbasu* were brought from Lembucherra fish farm of Tripura (India) to the laboratory of the institute, and were acclimatized to the experimental rearing conditions for 15 days. Acid stress model was used for evaluating antistress activity. *L. calbasu* fingerlings (225; average weight 5.57±0.04 g) were randomly distributed into 4 groups in triplicates each with 15 fishes (15 fishes/group) in a plastic tank of 150 L capacity following a completely randomized design (CRD). The experiment lasted for 30 days. Round-the-clock aeration was provided to all the tanks from a compressed air pump, and water was exchanged every other day. All the fish were fed manually to satiation twice a day (08:00 and 18:00 h) either one of the diets containing 0, 0.05, 0.5 or 5% level of methanol extract of *C. dactylon* @ 3% body weight/day (Table 1). The water temperature of all the treatments were within the range of 26–27°C throughout the experimental period. At the end of 30 days, 6 fish from each replicate (i.e. 18 fish/treatment) were used for the estimation of

cortisol, glucose, cholesterol, and enzyme activities, and rest nine fish from each replicate (i.e. 27 fish/treatment) were given acid stress by exposing to acidic water for 24 h and again, the same parameters were analysed. Water pH in tanks was decreased gradually from pH 7.5 to 5.5 pH with hydrochloric acid. Water quality parameters were checked every week (APHA 1998) and were within the recommended range for rearing of carps.

The fish were anaesthetized with CIFECALM (50 µL/L) (Verma *et al.* 2007), and blood was withdrawn from the caudal vein within 2 min after capture by using a 2 mL syringe with 26-G needle and immediately transferred to dried Eppendorf tubes. The tubes were allowed to stand in a tilted position for 1 h to collect the serum, which was used subsequently for serum cortisol, glucose cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST). Liver, muscle and gill samples were collected and 5% homogenate was prepared in chilled sucrose solution (0.25 M) in a mechanical tissue homogenizer. The homogenate was centrifuged at 5,000×g at 4°C for 10 min, supernatant collected and stored at –20°C for enzymatic studies. The fresh tissue homogenate were used immediately for catalase, glutathion S transferase (GST) and superoxide dismutase (SOD) enzyme analysis.

Cortisol in fish serum was estimated by using a validated cortisol ELISA (LDN) MS E-5000 kit method. Serum cortisol was expressed as µg/dL. Serum glucose was estimated by using glucose kit (glucose oxidase/peroxidase method) purchased from Qualigens Fine Chemicals, Mumbai (India). Serum cholesterol of fish was estimated by using cholesterol kit (CHOD-POD. Liquid) purchased from Merck Chemical Mumbai (India). Serum cholesterol was expressed as mg/dL. Alanine aminotransferase (ALT) or glutamate pyruvate transaminase (GPT) in fish serum was estimated using GPT/ALT (LDH-NADH. Kinetic UV) kit purchased from Merck Pvt. Ltd. Mumbai (India). The procedure follows the principle where ALT/GPT catalyses the reversible transfer of an amino group from alanine to alpha-ketoglutarate forming glutamate and pyruvate. The pyruvate produced is reduced to lactate by lactate dehydrogenase (LDH) and NADH. The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of ALT present in the sample. Serum ALT was expressed as U/L. Aspartate aminotransferase (AST) formerly called glutamate oxaloacetate (GOT) was assayed by same procedure as for ALT except the substrate L-aspartic acid instead of alanine.

Enzyme assay of tissue samples: Lactate dehydrogenase (LDH) (E.C.1.1.1.27) was assayed in 100 mM phosphate buffer (pH 7.5) and 0.1 mM NADH. The reaction was initiated by adding 0.2 M Na pyruvate and monitored at 340 nm (Wroblewski and La Due 1951). A similar reaction mixture was used for the estimation of malate dehydrogenase (MDH) (E.C.1.1.1.37) except for the substrate, 0.1 mM oxaloacetate (Ochoa 1955).

Alkaline phosphatase (ALP) (EC 3.1.3.1) was assayed in mixture comprising 0.2 mL bicarbonate buffer (0.2 M), 0.1 ml of 0.1 M $MgCl_2$, 0.1 mL tissue homogenate, 0.5 mL of DW and 0.1 mL of freshly prepared 0.1 M PNPP. Incubate the reaction mixture in water bath at 37°C for 15 min and then stop the reaction by adding 1 mL of 0.1 N NaOH. OD value was recorded at 410 nm (King and Macpherson 1966). Acid phosphatase (ACP) was assayed by the same method except for the buffer, acetate buffer instead of bicarbonate buffer.

Total adenosine triphosphatase (ATPase) (E.C.3.6.1.3) was assayed in a reaction mixture of 0.1 M Tris–HCl buffer (pH 7.8), 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$ and 5 mM ATP. The mixture was incubated for 15 min, and the reaction was terminated by means of 10% trichloroacetic acid (Post and Sen 1967). Phosphate liberated was estimated at OD of 660 nm (Fiske and Subbarow 1925).

The activity of superoxide dismutase (SOD) was assayed with the oxidation of epinephrine–adrenochrome transition and estimated at 480 nm (Misra and Fridovich 1972). Catalase (CAT) activity was estimated as per Takahara *et al.* (1960), using phosphate buffer (50 mM, pH 7.0). Glutathione S-transferase activity towards CDNB was determined spectrophotometrically at 340 nm (Habig *et al.* 1974). The assay was performed at 25°C using 100 mM potassium phosphate buffer, pH 6.5, with GSH and CDNB (dissolved in ethanol) at a final concentration of 1 mM each. Activity was calculated from the changes in absorbance at 340 nm (ε 340 nm=9.6 mM/ cm). One unit of GST activity was defined as the amount of enzyme catalyzing the conjugation of 1 µmoL of CDNB with GSH/min at 25°C.

Total protein content was analysed from the supernatant (Lowry *et al.* 1951) for calculating enzyme activities. All the colorimetric assays were carried out using UV–VIS spectrophotometer (E-Merck, Germany).

Statistical analyses: All the parameters were analysed for one-way ANOVA with respect to dietary *C. dactylon* methanol extract. Post hoc test was carried out using Duncan's multiple comparison procedures, if they were significantly different. Student's t test was performed to assess the significance between the before and after acid stress. All the statistical analyses were performed by using SPSS 16.0 for Windows.

RESULTS AND DISCUSSION

Stress response in fish includes a cascade of reactions such as primary, secondary and tertiary responses. It is initiated after the perception of stress in hypothalamus, which in turn activates the pituitary inter-renal axis, resulting in the release of the hormone cortisol and catecholamine. The secondary stress response includes metabolic, haematological and immunological changes due to the action of cortisol and catecholamine. The tertiary response is the final stage, which leads to disease or exhaustion, growth retardation and finally death (Chatterjee *et al.* 2006).

Stress hormones: The serum cortisol level was significantly higher in acid stress group than that in without acid stress counterpart (Fig. 1). The highest cortisol level

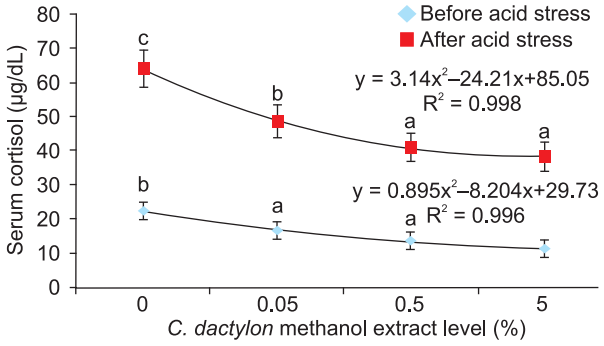

Fig. 1. Impact of *Cynodon dactylon* methanol extract level (%) in feed on serum cortisol in *Labeo calbasu* before and after acid stress. Mean values bearing different superscripts (a, b, c, d) differ significantly (P<0.05).

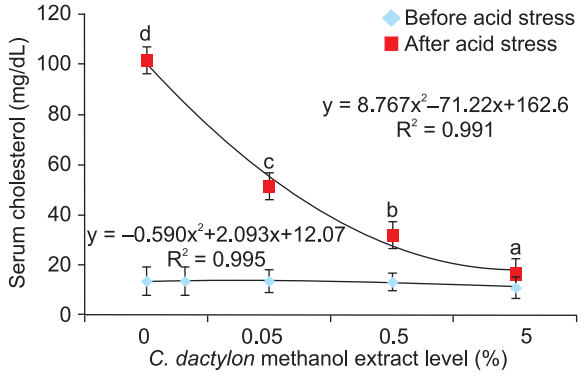

Fig. 2. Impact of *Cynodon dactylon* methanol extract level (%) in feed on serum glucose in *Labeo calbasu* before and after acid stress. Mean values bearing different superscripts (a, b, c, d) differ significantly (P<0.05).

was in control fed without *C. dactylon* methanol extract indicating secretion of cortisol due to the stress caused by acid stress. Previous studies showed that acid stress enhanced plasma cortisol level in rainbow trout (Brown *et al*. 1990) and greenback flounder (Barnett and Pankhurst 1998). The increased cortisol level after stress is to elicit several compensatory physiological responses that help the fish to deal with the stress (Mommsen *et al*. 1999). However, in this study, gradual supplementation of *C. dactylon* methanol extract in diet significantly reduced the cortisol level both in acid stress and without stress group indicating that *C. dactylon* methanol extract possess antistress ability in *L. calbasu* fingerlings.

Similar trend was also observed for serum glucose (Fig. 2) level and serum cholesterol level after acid stress. However, cholesterol level showed no significant effect (P>0.05) before acid stress (Fig. 3). These alterations are due to the stimulation of hypothalamo-pituitary axis (HPA) and sympathetic nervous system resulting in liberation of catecholamines and glucocorticoids (Prabhakaran *et al.* 2003). The preventive effects of *C. dactylon* methanol extract on the acid stress that induced biochemical changes indicate its anti-stress activity. The effects of *C. dactylon* methanol extract may be due to its effect on the central nervous system or endocrines and antioxidant effect that prevent stress induced damage caused by generation of free radicals (Cotran *et al.* 2000).

The induction of acid stress significantly increased ALT (Fig. 4) and AST (Fig. 5) levels after acid stress compared to before acid stress. All the 3 treatments produced a significant reduction in serum ALT and AST activity as compared to control before as well as after acid stress. A decreased trend in serum ALT activity was recorded with increasing level of *C. dactylon* methanol extract in all the treatment groups both before and after acid stress. ALT and AST are markers of hepatic functional status as they indicate

Table 2. Activity of lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and adenosine triphosphatase (ATPase) in different tissues of *Labeo calbasu* before and after acid stress with different *Cynodon dactylon* methanol extract levels

Parameter	Tissue	Acid stress	Treatment			
			Control (0%)	T_1 (0.05%)	T_2 (0.5%)	T_3 (5%)
LDH[1]	Liver	Before stress	1.88^{Xa}±0.02	1.81^{Xab}±0.05	1.08^{Xa}±0.02	1.85^{Xa}±0.02
		After stress	5.76^{Yc}±0.61	3.49^{Yb}±0.14	1.77^{Ya}±0.12	2.45^{Yb}±0.14
	Muscle	Before stress	2.67^{Xc}±0.37	2.39^{Xbc}±0.25	0.52^{Xa}±0.03	1.55^{Xb}±0.01
		After stress	$6.62Y^{d}$±0.14	3.02^{Yc}±0.14	1.71^{Ya}±0.03	2.79^{Yb}±.35
MDH[1]	Liver	Before stress	0.29^{Xb}±0.03	0.25^{Xb}±0.03	0.21^{Xb}±0.02	0.14^{Xa}±0.01
		After stress	0.48^{Yc}±0.01	0.28^{Yab}±0.02	0.25^{Ya}±0.01	0.24^{Ya}±0.01
	Muscle	Before stress	0.26^{X}±0.02	0.25^{X}±0.01	0.18^{X}±0.01	0.10^{X}±0.05
		After stress	0.57^{Yb}±0.10	0.38^{Yb}±0.01	0.27^{Ya}±0.02	0.19^{Ya}±0.01
ATPase[2]	Liver	Before stress	69.17^{X}±0.495	92.45^{X}±6.015	80.84^{X}±5.61	88.52^{X}±4.96
		After stress	20.09^{Ya}±1.60	56.04^{Yb}±0.020	62.898^{Yb}±0.145	80.28^{Yc}±3.27
	Gill	Before stress	267.44^{Xb}±8.82	181.75^{Xa}±16.63	299.91^{Xb}±1.45	324.14^{Xb}±21.51
		After stress	91.05^{Ya}±4.65	116.30^{Ya}±1.74	224.17^{Yb}±2.96	268.54^{Yb}±2.04

Values represent mean±SE. Mean values in a row under each parameter bearing different superscripts (a, b, c, d) differ significantly (P<0.05). Mean values in a column (before and after heat shock) under each parameter bearing different superscripts (X and Y) differ significantly (P<0.05). [1]Activities expressed as micro moles min/mg/protein; [2]Activities expressed as nanogram phosphorus released per mg protein per min.

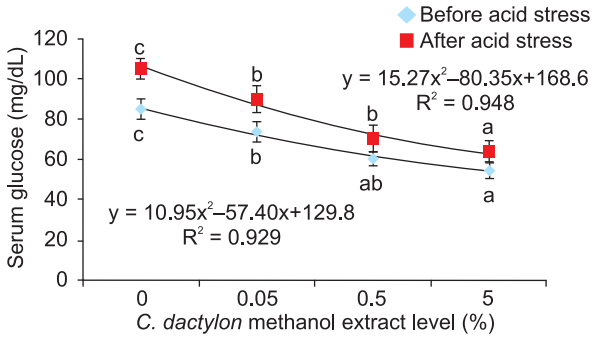

Fig. 3. Impact of *Cynodon dactylon* methanol extract level (%) in feed on serum cholesterol in *Labeo calbasu* before and after acid stress. Mean values bearing different superscripts (a, b, c, d) differ significantly (P<0.05).

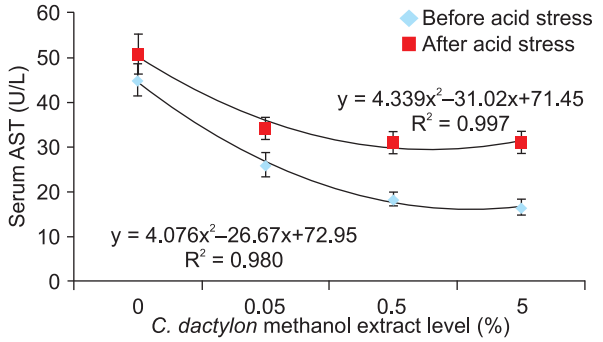

Fig. 4. Impact of *Cynodon dactylon* methanol extract level (%) in feed on serum ALT in *Labeo calbasu* before and after acid stress. Mean values bearing different superscripts (a, b, c, d) differ significantly (P<0.05).

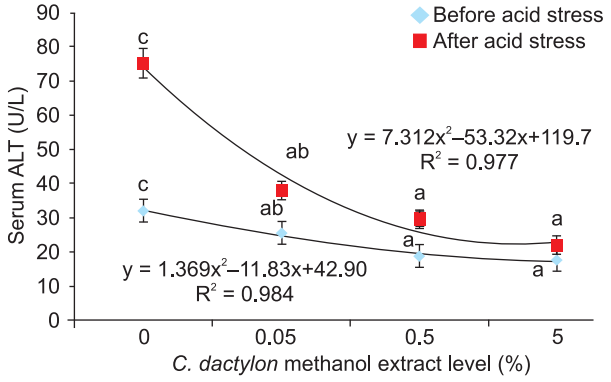

Fig. 5. Impact of *Cynodon dactylon* methanol extract level (%) in feed on serum AST in *Labeo calbasu* before and after acid stress. Mean values bearing different superscripts (a, b, c, d) differ significantly (P<0.05).

parenchymal liver damage. The study of different enzyme activities such as ALT, AST have great value in the assessment of clinical and experimental liver damage (Vaishwanar and Kowale 1976). ALT and AST activities increased in acid stress group possibly because of the alteration in the membrane permeability, which might occur in the cell during stress. These changes also represent a functional alteration in the cell membrane due to steroidal storms, which occur during stress (Nayanatara *et al*. 2009).

Enzymes in tissue samples: A significant difference (P<0.05) in LDH activity was observed in the liver and muscle before and after acid stress (Table 2). LDH activity of control both before and after acid stress was higher, which decreased with increasing level of dietary *C. dactylon* extract upto 0.5% level and further increased in 5% level. Similarly, MDH activity in liver and muscle significantly increased (P<0.05) after acid stress compared to before acid stress. In liver, MDH activity significantly decreased (P<0.05) as treatment levels increased both before and after acid stress. MDH activity was unaffected in muscle before acid stress whereas after acid stress MDH activity decreased significantly with increase in treatments level. Metabolism is a physiological process reflecting the energy expenditure of living organisms. The increased activity of LDH due to acid stress may be due to the higher production of lactate, which is the preferred source for gluconeogenesis in fish during anaerobic metabolism (Moon and Foster 1995). Lower activity in the treatment groups compared to control after acid stress suggested that *C. dactylon* methanol extract helps in reducing stress in *L. calbasu*. MDH is an enzyme of TCA cycle that was increased after the acid stress in order to use the product (oxaloacetate) due to the higher activity of AST for the production of more energy to cope up with the stress. In the present study, there was a significant enhancement in the MDH activity in the liver and muscle of post stressed fish, which strengthens the above hypothesis. Lower activity in the treatment groups support our findings that the supplementation of *C. dactylon* methanol extract reduced the energy demands in the *L. calbasu* fingerlings as stress level was reduced.

The ATPase activity decreased significantly (P<0.05) in both liver and gill after acid stress compared to before acid stress (Table 2). Increased in dietary *C. dactylon* methanol extract levels significantly increased (P<0.05) ATPase activity in liver and gill before as well as after acid stress. ATPase hydrolyses the high-energy phosphate (ATP) and utilizes that energy to maintain ionic gradient across the plasma membrane (Das 2002). ATPase activity decreased in both liver and gill after acid stress which may be because of the utilization of available ATP, which is needed for the enzyme activity during stressed condition to maintain energy balance. Present study also showed that increase in ATPase activity in both liver and gill in fish fed with *C. dactylon* extract incorporated diet might be due to the higher substrate availability in treatment groups. On the contrary, the ATPase activity in gill did not reflect any variation with respect to *C. dactylon* extract before acid stress. Similar pattern of increase in ATPase activity was reported when *Channa punctatus* was exposed to sublethal doses of endosulphan (Sarma *et al.* 2009).

The ALP activity after acid stress significantly increased (P<0.05) in liver, muscle, and gill compared to before acid stress (Table 3). This increase is due to response to increased lysosomal activity in the liver and muscle. Lysosomes play an important role in the function of eukaryotic cells, many

Table 3. Activity of alkaline phosphatase (ALP), acid phosphatase (ACP), adenosine triphosphatase (ATPase) in different tissues of *L. calbasu* before and after acid stress with different *C. dactylon* methanol extract levels

Parameter	Tissue	Acid stress	Treatment			
			Control (0%)	T_1 (0.05%)	T_2 (0.5%)	T_3 (5%)
ALP[1]	Liver	Before stress	190.92^{Xa}±5.16	220.25^{Xab}±9.25	319.69^{Xb}±9.62	308.87^{Xb}±11.67
		After stress	435.28^{Yb}±28.53	331.77^{Ya}±23.13	366.18^{Ya}±13.67	392.40^{Ya}±6.02
	Muscle	Before stress	251.41^{Xa}±21.95	248.13^{Xc}±14.95	189.00^{Xb}±3.95	128.82^{Xa}±7.09
		After stress	413.69^{Yd}±3.96	376.52^{Yc}±5.11	300.06^{Yb}±1.52	244.52^{Ya}±9.74
	Gill	Before stress	174.60^{Xa}±4.97	189.58^{Xa}±6.10	235.68^{Xb}±5.98	164.53^{Xa}±3.20
		After stress	331.89^{Yb}±9.34	316.10^{Yb}±8.53	329.49^{Yb}±18.19	277.15^{Yab}±12.41
ACP[1]	Liver	Before stress	283.41^{X}±7.88	233.44^{X}±3.66	290.93^{X}±4.26	328.60^{X}±14.08
		After stress	575.64^{Yc}±5.33	279.98^{Ya}±4.45	337.10^{Ya}±9.67	370.30^{Yb}±4.55
	Muscle	Before stress	355.09^{Xa}±6.75	501.99^{Xb}±9.47	440.22^{Xab}±13.87	467.45^{Xab}±7.67
		After stress	689.63^{Yb}±27.39	567.77^{Yb}±30.99	491.84^{Yab}±5.52	517.90^{Yab}±5.85
	Gill	Before stress	494.84^{Xc}±4.15	431.13^{Xab}±21.83	320.43^{Xa}±5.48	291.22^{Xa}±2.41
		After stress	515.98^{Yc}±3.68	474.96^{Yb}±17.49	394.17^{Yab}±7.26	366.02^{Ya}±.23.54

Values represent mean±SE. Mean values in a row under each parameter bearing different superscripts (a, b, c, d) differ significantly (P<0.05). Mean values in a column (before and after heat shock) under each parameter bearing different superscripts (X and Y) differ significantly (P<0.05). [1]Activities expressed as micro mole of PNP released/mg protein/min at 37°C.

Table 4. Activity of superoxide dismutase (SOD), catalase (CAT), glutathione S transferase (GST) in different tissues of *L. calbasu* before and after acid stress with different *C. dactylon* methanol extract level

Parameter	Tissue	Acid stress	Treatment			
			Control (0%)	T_1 (0.05%)	T_2 (0.5%)	T_3 (5%)
CAT[1]	Liver	Before stress	0.31^{Xa}±0.025	0.55^{Xb}±0.005	0.76^{Xc}±0.085	0.80^{Xc}±0.06
		After stress	0.62^{Ya}±0.03	0.82^{Yab}±0.01	1.08^{Yb}±0.02	1.19^{Yb}±0.02
	Muscle	Before stress	0.31^{Xa}±0.01	1.38^{Xb}±0.06	0.80^{Xa}±0.04	0.69^{Xa}±0.03
		After stress	0.67^{Ya}±0.01	1.59^{Yd}±0.09	1.10^{Yc}±0.03	0.92^{Yc}±0.07
	Gill	Before stress	0.27^{Xa}±0.01	0.73^{Xc}±0.05	0.46^{Xab}±0.02	0.51^{Xbc}±0.01
		After stress	0.60^{Ya}±0.09	1.14^{Yb}±0.01	0.80^{Ya}±0.04	0.80^{Ya}±0.01
SOD[2]	Liver	Before stress	9.21^{X}±0.77	11.60^{X}±0.23	15.65^{X}±5.57	15.17^{X}±0.34
		After stress	13.20^{Ya}±0.15	19.17^{Yc}±0.21	24.02^{Yd}±0.34	16.80^{Yb}±0.68
	Muscle	Before stress	20.49^{X}±0.22	21.36^{X}±0.54	22.94^{X}±2.72	19.51^{X}±0.89
		After stress	29.52^{Y}±0.52	23.20^{Y}±0.03	19.08^{Y}±2.82	20.22^{Y}±1.59
	Gill	Before stress	35.63^{X}±0.91	31.57^{X}±0.72	37.94^{X}±0.52	35.35^{X}±0.93
		After stress	24.06^{Ya}±1.20	25.42^{Ya}±1.40	55.62^{Yc}±3.60	39.09^{Yb}±0.71
GST[3]	Liver	Before stress	0.40^{Xa}±0.02	0.58^{Xa}±0.06	0.61^{Xa}±0.10	1.45^{Xb}±0.06
		After stress	0.51^{Ya}±0.02	0.65^{Yab}±0.02	0.77^{Yb}±0.03	1.81^{Yc}±0.02
	Muscle	Before stress	0.955^{Xa}±0.05	1.36^{Xb}±0.10	1.50^{Xc}±0.36	1.10^{Xb}±0.16
		After stress	1.249^{Ya}±0.01	1.90^{Ybc}±0.07	2.23^{Yc}±0.05	1.62^{Yb}±0.06
	Gill	Before stress	1.18^{Xa}±0.07	2.34^{Xb}±0.22	2.91^{Xb}±0.01	2.87^{Xb}±0.11
		After stress	2.44^{Ya}±0.01	4.72^{Yb}±0.05	7.34^{Yc}±0.06	6.36^{Ybc}±0.07

Values represent mean±SE. Mean values in a row under each parameter bearing different superscripts (a, b, c, d) differ significantly (P<0.05). Mean values in a column (before and after heat shock) under each parameter bearing different superscripts (X and Y) differ significantly (P<0.05). [1]Activities expressed as micro moles H_2O_2 decomposed/min/mg protein. [2]Activities expressed as U/min/mg protein. [3]Micro moles of CDNB-GSH conjugate formed/min/mg protein.

environmental contaminates including heavy metals and organic xenobiotics are known to be sequestered in lysosomes and their stability reduced in vertebrates with increasing contamination (Alireza *et al.* 2010). Increase in the dietary *C. dactylon* methanol extract level significantly decreased (P<0.05) the ALP activity in liver and muscle for both before and after acid stress. But in gill, higher ALP activity was observed at 0.5% level compared to 0.05% and 5% levels. The ACP activity increased significantly (P<0.05) in liver, muscle and gill after acid stress compared to before acid stress (Table 3). Ghorpade *et al*. (2002) reported that ACP levels were significantly increased in a plasticizer (diethyl phthalate) treated fish. ACP activity decreased significantly (P<0.05) up to 0.5% level and again increased in 5% level. ALP and ACP catalyse the hydrolysis of various phosphate-containing compounds and act as

transphosphorylases at alkaline and acid pH, respectively. Acid phosphatases act as marker enzymes for the detection of lysosomes in cell fractions and can be altered by the presence of xenobiotics (Cajaraville *et al.* 2000), whilst alkaline phosphatases are intrinsic plasma membrane enzymes found on the membranes of almost all animal cells. Both enzymatic activities have been studied in several organisms and the influence of heavy metals stress has been reported (Blasco *et al.* 1993). These enzymatic activities are involved in a variety of metabolic processes, such as molecule permeability, growth, cell differentiation and steroidogenesis (Ram and Sathayanesan 1985). For fish, in laboratory conditions, liver alkaline phosphatase activity changes in response to waterborne metal making it useful as indicator of heavy metal exposure (Ram and Sathayanesan 1985).

SOD catalyses the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide. Subsequently, catalase (CAT) activity decomposes this hydrogen peroxide into oxygen and water, and this constitutes a crucial part of the cellular antioxidant defence mechanism. In general, stress causes oxidative damage to cells, which overcomes that effect through the increased activity of antioxidative enzymes (SOD and CAT). The increases in SOD activity with exposure to acid stress in the present study possibly indicate a role for scavenging O_2^- (Parihar *et al*. 1997). However, the activity was higher in high dietary C. *dactylon* extract–fed groups, indicating the defence response of *C. dactylon* to acid stress through the increased activity of SOD and CAT. In the present study, 0.5% and 5% show higher SOD and CAT activity in stressed fish. Similar pattern of increased SOD and CAT was reported when *L. rohita* was exposed to higher temperature (Shivendra *et al.* 2011). This also suggests that different levels of protein interact in characterizing oxidative status of the fish. But the area needs more research.

The activity of GST also got decreased significantly in the tissues, viz. liver, muscle and gill of *L. calbasu* both before and after acid stress. However, upon treatment with *C. dactylon* methanol extract, GST activity increased significantly compared to control. The decreased activity of GST observed in the fish exposed to acid stress in liver, muscle and gill may be because of the inactivation caused by ROS (Andalu and Vadacharlu 2003). The significant recoveries in the activities of these enzymes (CAT, SOD and GST) because of treatment of stressed fish with the *C. dactylon* methanol extract reflect the antioxidant potential of this herbal preparation. There may be a possibility that methanol extract from *C. dactylon* might have contributed in preventing glycation and inactivation of these enzymes. Phytochemical investigation of *C. dactylon* reveals the presences of flavonoids and sterols (Aishah *et al.* 1997, Patil *et al.* 2005). Total phenolic content in *C. dactylon* is 20 mg/g which also had positive correlation between the total phenolic content and antioxidant activity (Djeridane *et al*. 2006).

From this study, we can conclusively state that *C. dactylon* methanol extract showed remarkable effects on serum cholesterol, serum glucose, serum triglycerides and serum cortisol level, and reduction in different enzymes level besides marked improvement of oxidative stress enzymes. The extract seems to be non toxic as no death was reported up to 10 times of effective dose. Thus it can be concluded that the supplementation of *C. dactylon* methanol extract in feed can reduce stress and can act as antioxidant in aquaculture. Further pharmacological and biochemical investigations are underway to elucidate the mechanism of the antistress effect in methanol extract of *C. dactylon.* However, the application of *C. dactylon* extract in large-scale field condition requires the detailed study of the route of administration and effective doses for different age group of fish. Further work needs to be carried out to isolate and characterize the active principles of *C. dactylon* plant extract as well as delineate its specific properties.

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