VIDYASAGAR UNIVERSITY

INDIAN JOURNAL OF BIOLOGICAL SCIENCES

ISSN 0972-8503

OLUME - 11 2005

INDIAN JOURNAL OF BIOLOGICAL SCIENCES

Vol. 11 2005

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EFFECT OF NIFEDIPINE ON THE TRANSMISSION OF NERVE IMPULSE AT THE SKELETAL NEUROMUSCULAR JUNCTION IN CATS

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ABSTRACT

The present study was undertaken to investigate in cats the effects of nifedipine on the transmission of nerve impulse at the skeletal neuromuscular junction. In sodium pentobarbitone anesthetized cats, intraarterially administered nifedipine (50-200 μg / kg, i.a.) significantly reduced the isometric twitch amplitude of gastrocnemius muscle stimulated indirectly and directly from control values. This result suggests that nifedipine may exert its blocking action both on the neuromuscular junction and the muscle. Further, indirect twitch amplitude was reduced more than direct twitch suggesting that nifedipine may act directly on the nerve impulse transmission at the neuromuscular junction. In the present study nifedipine (50-200 μg / kg, i.a.) also blocked indirectly elicited posttetanic potentiation and tetanic contraction of gastrocnemius presynaptic site of neuromuscular junction. In anesthetized cats, nifedipine (50-100 μg / kg, i.a.) blocked the acetylcholine induced contraction of gastrocnemius muscle. The result indicates that nifedipine blocked acetylcholoine induced contraction by inhibiting post-junctional acetylcholine receptors of the neuromuscular junction. In the present study nifedipine (50-200μg / kg, i.a.) produced an initial facilitation followed by a significant depression of gastrocnemius monosynaptic reflex. Significant recovery of nifedipine depressed gastrocnemius monosynaptic reflex was observed by 60 seconds with a dose of calcium chloride (25 mg /kg, i.a.) or caffeine (10 mg /kg, i.a.). From the result it is suggested that nifedipine may reduce the release of acetylcholine from motor nerve terminal of neuromuscular junction presumably by decreasing calcium and cyclic AMP levels in the motor nerve terminal. In conclusion, it appears that nifedipine inhibits nerve impulse transmission at the skeletal neuromuscular junction in intact cats by inhibiting the release of acetylcholine from presynaptic motor nerve terminals and the sensitivity of endplate receptors to acetylcholine.

Key words : nifedipine, nerve impulse, neuromuscular junction, monosynaptic reflex, acetylcholine.

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INTRODUCTION

Nifedipine, a dihydropyridine organic calcium channel blocker, inhibits the ionic current carried through the slow channel by calcium ions in the vascular and cardiac excitable membranes [1,2]. This pharmacologic property has made it useful in the treatment of several cardiovascular diseases in man [1,3,4]. Despite the wide spectrum of biological functions dependent on calcium ions, the pharmacologic effects of nifedipine and other organic calcium channel blockers appears restricted to the cardiovascular system [1,5]. Significant blocking effect of nifedipine on noncardiovascular tissues has not been reported to date. So, the knowledge of its effects on noncardiovascular tissues may be of clinical importance. Moreover, the studies about the effects of nifedipine on noncardiovascular tissues may not only throw light on the function of Ca^{++} in the cells, but also illucidate the property of nifedipine which is not fully understood, though these have little clinical significance. The present study was undertaken to investigate in anesthetized cats the effects of intra-arterially administered nifedipine on the transmission of the nerve impulse at the skeletal neuromuscular junction.

It has been reported that organic calcium channel blockers even in a larger dose producing no or only a slight depression on skeletal muscle contraction in toad [6] and frog [7]. Sato and Ono [8] studied the effect of nifedipine on the neuromuscular junction in the in situ anterior tibial muscle preparation of the dog and concluded that nifedipine facilitates neuromuscular transmission by acting directly on the motor nerve terminals of neuromuscular junctions. It has also been reported that nifedipine facilitates neurotransmitter release independently of calcium channels in central synapse of cats [9]. We report here that nifedipine inhibited nerve impulse transmission at the skeletal neuromuscular junction in intact cats for doses below the therapeutic dose range recommended for man.

METHODS

Surgical preparations

The experiments were performed on 39 adult cats (2.5-3.5kg body weight) of either sex. Anesthesia was induced with an intraperitoneal injection of sodium pentobarbitone (35-40mg/kg). Subsequently anesthesia was maintained with an intravenous dose of sodium pentobarbitone (10mg/kg) throughout the experiment. The trachea was exposed and a 'T' shaped polyethylene cannula was inserted into the trachea after a low tracheotomy and connected to the respiratory pump for artificial respiration when required. A catheter was placed in right femoral artery for intraarterial respiration when required. A catheter was placed in right femoral artery for intraarterial pressure monitoring. The blood pressure was continuously monitored with a Bell and Howell (Type 4-327-0129) pressure transducer. A second catheter was placed in the left femoral artery so that its tip lay at or above the bifurcation of the abdominal aorta, for intraarterial drug injection. The position of the catheter

was checked postmortem. The electrocardiogram (lead II) continuously recorded cardiac rate and rhythm. Each animal received approximately 10ml. $kg^{-1}.h^{-1}$ of 0.9 percent saline with 5 percent glucose.

Recording of isometric muscle twitch twitch amplitude stimulated indirectly and directly

After initial surgical preparations, cat was suspended in a cat frame. The left hind limb was fixed to a rigid framework with a nail driven into the distal end of the femur. The tendon of the gastrocnemius muscle was cut distally and connected with a silk thread to a Beckman isometric force displacement transducer (Type-4151) to record isometric twitch amplitude of the muscle under a preload. The sciatic nerve of gastrocnemius muscle was transected centrally and the motor filament of the nerve was isolated from the peripheral cut end. The exposed gastrocnemius muscle and desheathed motor filaments of the sciatic nerve were immersed in a liquid paraffin pool maintained at 37.5°C. Branches of the femoral artery not serving the gastrocnemius muscle were also occluded. In this experiment the gastrocnemius muscle was stimulated alternately by indirect stimulation to the peripheral cut end of the motor filament of sciatic nerve and a direct one to the muscle. The peripheral cut end of the motor filament of sciatic nerve was stimulated with monophasic square wave pulses of 0.5 millisecond duration and supra-maximal voltage at a frequency of 0.2 Hz through a bipolar silver-silver chloride electrode by means of an electric stimulator (Grass, SD9). The direct stimulation to the muscle was achieved with square wave pulses of 0.5 millisecond duration applied through a bipolar silver-silver chloride electrode being set at the midportion of the muscle by means of a second stimulator (Grass, S48). The pulse output of the first stimulator (SD9) was connected with the trigger input point of second stimulator (S48) and in this arrangement the delay was adjusted to 2 seconds [8,10]. Isometric muscle twitch amplitude to direct and indirect stimulation was alternately recorded on a Beckman RM Dynograph recorder. Viability of the preparation was tested in another group of cats, which received the saline injection. The twitch heights were recorded continuously up to 30 minutes after drug injections; then every 15 minutes up to 3 hours. The experiment was terminated at 3 hours.

Recording of tetanic contraction and post-tetanic potentiation of muscle elicited by indirect tetanic stimulation

After surgical preparations, isometric muscle twitch amplitudes to indirect stimulation were recorded on Beckman RM Dynograph recorder. As the tetanic stimulation, a frequency of 80Hz was applied for 5 seconds. During repetitive stimulations of the nerve at 80Hz the gastrocnemius muscle effectively maintained the tetanic contraction and after the frequency of stimulation was switched to 0.2Hz, the twitch amplitudes were potentiated as compared with pretetanic level over a few minutes [8]. Tetanic contraction and posttetanic potentiations were recorded every 2 minutes up to 30 minutes after drug injections and then every 15 minutes up to 3 hours. The experiment was terminated after 3 hours.

Recording of acetylcholine induced contraction of muscle

After initial surgical preparations for recording indirectly elicited twitch amplitude of gastrocnemius muscle, a dose of acetylcholine (1-2mg/kg) was injected close intraarterially to the muscle through a catheter placed in the left femoral artery. As a result, a reproducible acetylcholine induced isometric contraction of gastrocnemius muscle was recorded on Beckman recorder. When peripheral portion of motor filament of sciatic nerve was stimulated, the indirectly elicited isometric twitch amplitudes of muscle were potentiated as compared with pre-acetylcholine twitch amplitudes and gradually returned to the pre-acetylcholine level over a few minutes. Acetylcholine induced contractions and indirectly elicited isometric muscle twitch amplitudes were recorded every 2 minutes up to 30 minutes after drug injections and then every 15 minutes up to 3 hours. The experiment was terminated after 3 hours.

Recording of monosynaptic reflex

After initial surgical preparations, cat was suspended in cat frame. The left hind limb was fixed to a rigid framework with a nail driven into the distal end of the femur. The gastrocnemius muscle of this limb was surgically exposed and immersed in a liquid paraffin pool maintained at 37.5°C. Spinal cord was exposed from L3 to cauda equina by laminectomy. The dura was opened to expose the nerve trunk of the spinal cord under the stereoscopic dissecting microscope (Vicker's, UK). The dorsal and ventral roots of L6, L7 and S1 innervating the gastrocnemius muscle were sectioned as far from their entry to the spinal cord as possible to record the monosynaptic reflex from the ventral root by stimulating appropriate dorsal root. The central cut end of the appropriate dorsal and ventral root were desheathed and split into fine filaments with watchmaker's tweezers (made in Switzerland) under dissecting microscope. The dorsal and ventral roots innervating the gastrocnemius muscle were identified by observing the response of dorsal root (peripheral cut end) to stretch of the gastrocnemius muscle and the response of gastrocnemius muscle to ventral root (peripheral cut end) stimulation. A bipolar silver-silver chloride electrode was placed on central cut end of appropriate dorsal root filaments for electrical stimulation, and a second bipolar silver-silver chloride electrode was placed on central cut end of appropriate ventral root filaments for recording monosynaptic reflex. The exposed nerve trunk, dorsal and ventral root filaments were immersed in a liquid paraffin pool maintained at 37.5°C. The central cut end of the dorsal root filament was stimulated with square wave pulses of 5 milliseconds duration and one and a half to twice threshold strength at intervals of 2 seconds through bipolar electrode by means of an electric stimulator (Grass SD9). The isolation unit within the stimulator helps to reduce the electrical noise and other interferences. The resulting monosynaptic reflexes picked up with the bipolar electrode placed on central cut end of appropriate ventral root filament were initially amplified with a preamplifier (Tektronix AM502) and displayed on dual beam oscilloscope (Tektronix 5112 or 5113) for photograph and further analysis [11]. Several control records of the monosynaptic reflex were observed. The monosynaptic reflexes were recorded every 5

seconds up to 30 minutes after drug injections; then every 5 minutes up to 3 hours. The experiment was terminated at 3 hours.

We ensured that the anesthesia was adequate during surgery and recording by checking at regular intervals that neither heart rate nor blood pressure were altered in response to noxious stimuli and that the pupils were not dilated. At the end of the experiment, a lethal dose of barbiturate was given intravenously.

Drugs and chemicals used

Nifedipine, acetylcholine chloride and caffeine were from RBI (Natick, MA, USA). Calcium chloride was from E. Merck (India). Nifedipine was dissolved in ethanol and the stock solution (10mg/ml) was diluted to a desired concentration with 0.9% saline. The other chemicals were dissolved in normal saline before use. The doses given are refereed to the bases. Nifedipine, calcium chloride, acetylcholine chloride, and caffeine were injected close intraarterially to the muscle in a volume of 0.1-1 ml through a catheter placed in the left femoral artery.

Statistics

The heights of the isometric muscle twitches and monosynaptic reflexes were measured and expressed as a percentage of the peak height of the average control twitch and reflexes. Results were expressed as the mean ± standard error of mean (SEM). Statistical evaluation of the result was done with Student's 't' test.

RESULTS

Effect of nifedipine on indirect and direct muscle twitch amplitude

The effect of intraarterially administered nifedipine (50-200μg/kg) on isometric twitch amplitude of gastrocnemius muscle, elicited by indirect and direct electrical stimulation, was observed in vivo of the cat. At all doses tested, nifedipine significantly reduced isometric twitch amplitude of gastrocnemius muscle stimulated indirectly and directly from control values. A representative illustration of this phenomenon is shown in Figure 1 (Tracing A, B, C and D). It will be seen that 20 minutes after the administration of nifedipine (100μg/kg; i.a), the indirectly and directly elicited isometric twitch amplitude of gastrocnemius muscle were reduced to 88% and 84% of the control amplitude (Figure 2). The magnitude of effect and duration of response were dose dependent (Figure 3). The maximal twitch reduction was observed between 15 and 20 minutes of nifedipine administration and persisted at the same level for the duration of the experiment (180 minutes) for all doses of nifedipine. Indirect isometric twitch was reduced more than direct isometric twitch. No significant changes in blood pressure, cardiac rate or rhythm were observed. In the saline treated control group of cats, no change in twitch amplitude or cardiac parameters (rate, rhythm, blood pressure)

were observed over the time course of the experiment.

In an attempt to find out whether a recovery was involved or not in the nifedipine induced reduction of twitch amplitude by calcium supplementation, a standard dose of calcium chloride

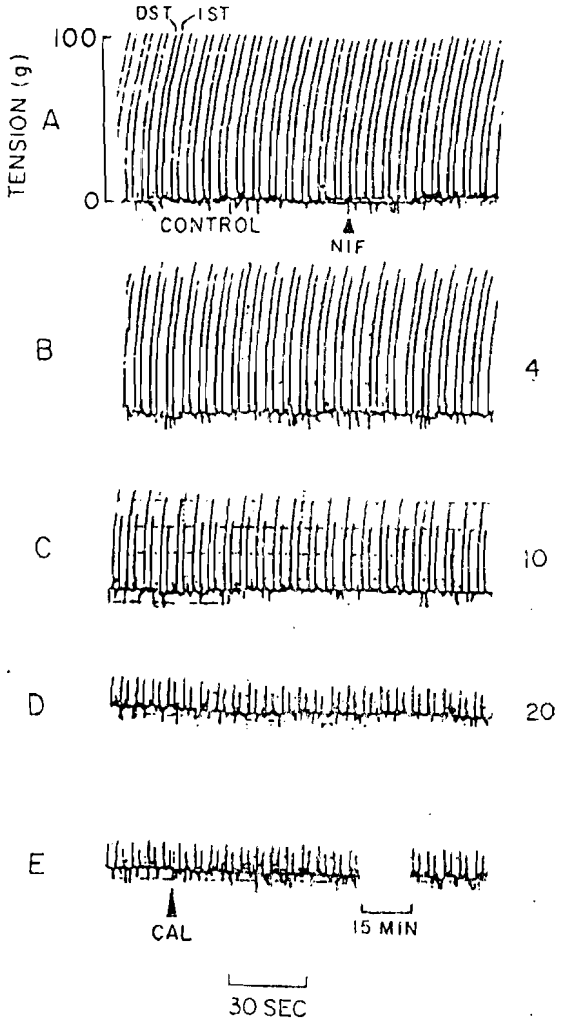

Figure 1. Typical record showing the effect of nifedipine (100μg/kg, i.a.) on isometric twitch amplitude of gastrocnemius muscle elicited by indirect and direct electrical stimulation. Nifedipine was administered at the arrow (σ). The numbers on the right represent the interval (min) after the nifedipine administration. NIF is nifedipine. Tracing **A** shows pre-drug control twitches in which first record of a pair is directly stimulated twitch (DST) and second record is indirectly stimulated twitch (IST) and the records immediately after the nifedipine administration. Tracing **B, C,** and **D** show the successive stages of the effect of nifedipine on isometric twitch amplitude of gastrocnemius muscle. Tracing **E** shows the effect of intraarterially administered calcium chloride (25mg/kg) on the recovery of nifedipine depressed isometric twitch amplitude of gastrocnemius muscle. Calcium chloride (CAL) was administered at the arrow (σ).

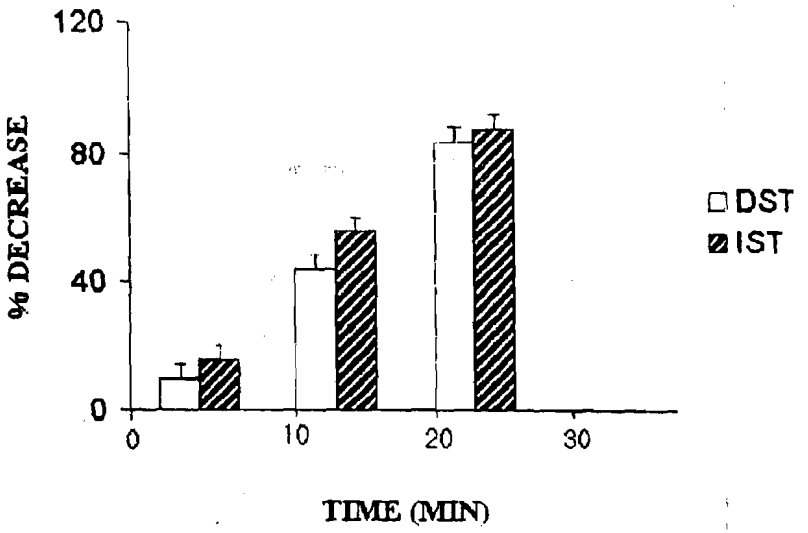

Figure 2. Shows percent decrease in the isometric twitch amplitude elicited by indirect and direct electrical stimulation after the administration of nifedipine (100µg/kg, i.a.) DST is directly stimulated twitch and IST is indirectly stimulated twitch. The values represented the Mean ± SEM, n=7.

(25mg/kg) was administered intraarterially to the muscle under alternate stimulations of indirect and direct. No recovery of the nifedipine depressed indirect and direct isometric twitch were obseved after calcium chloride administration for all doses of nifedipine for the duration of the experiment (180 minutes). An illustration of this phenomenon is shown in Figure 1 (Tracing E). In the saline treated control group of cats, no change in nifedipine depressed indirect and direct twitch were observed over the time course of the experiment.

Effect of nifedipine on post-tetanic potentiation and tetanic contraction of gastrocnemius

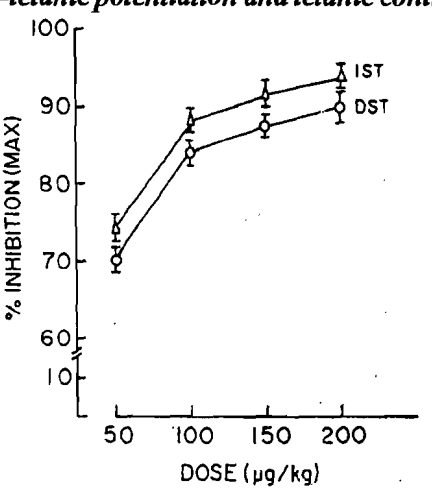

Figure 3. Shows dose response relationship of nifedipine on the percent inhibition of indirectly and directly stimulated isometric twitch amplitude. **DST** is directly stimulated twitch and **IST** is indirectly stimulated twitch. The values represented the Mean ± SEM, n=7.

muscle

The effect of intraaterially administered nifedipine (50-200μg/kg) on post-tetanic potentiation and tetanic contraction of gastrocnemius muscle, elicited by indirect tetanic stimulation, were observed. The post-tetanic potentiation and tetanic contraction were completely inhibited between 4 and 6 minutes of nifedipine administration, and persisted at the same level for the duration of the experiment (180 minutes) for all doses. An illustration of this phenomenon is shown in Figure 4. The magnitude of effect and duration of response were dose dependent. In saline treated control group of cats, no changes in posttetanic potentiation and tetanic contraction were observed over the time course of the experiment.

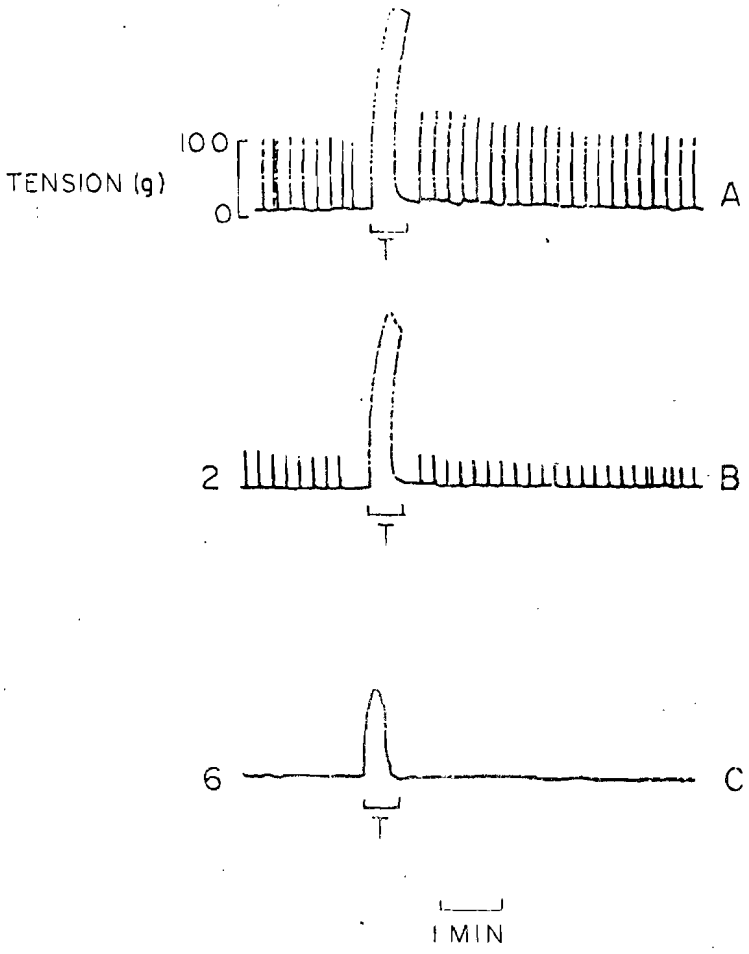

Figure 4. Typical record showing the effect of nifedipine (100μg/kg, i.a) on posttetanic potentiation and tetanic contraction of gastrocnemius muscle. **T** is tetanic stimulation. Tracing **A** shows pre-drug control posttetanic potentiation and tetanic contraction. Tracing **B** and **C** show the stages of the effect of nifedipine on the same. The numbers on the left represent the interval (min) after nifedipine administration.

Effect of nifedipine on acetylcholine induced contraction of gastrocnemius muscle

In anesthetized cats, the effect of intraarterially administered nifedipine (50-100μg/kg) on acetylcholine induced contraction of gastrocnemius muscle, was observed. A single close arterial injection of acetylcholine (1-2mg/kg) elicited a reproducible contraction of the gastrocnemius muscle. Nifedipine, at each dose tested, abolished the acetylcholine induced contraction and depressed the indirectly elicited isometric twitch amplitude of gastrocnemius muscle between 6 and 8 minutes of intraarterial administration. Both the changes of the acetylcholine induced contraction and the indirectly elicited isometric twitch amplitude persisted at the same level for the duration of the experiment (180 minutes) for all doses of nifedipine. A representative illustration of this phenomenon is shown in Figure 5. The magnitude of effect and duration of response were dose dependent. No significant changes in blood pressure, cardiac rate or rhythm were observed. In saline treated control group of cats, no change in acetylcholine induced contraction and indirectly elicited isometric twitch amplitude were observed over the time course of the experiment.

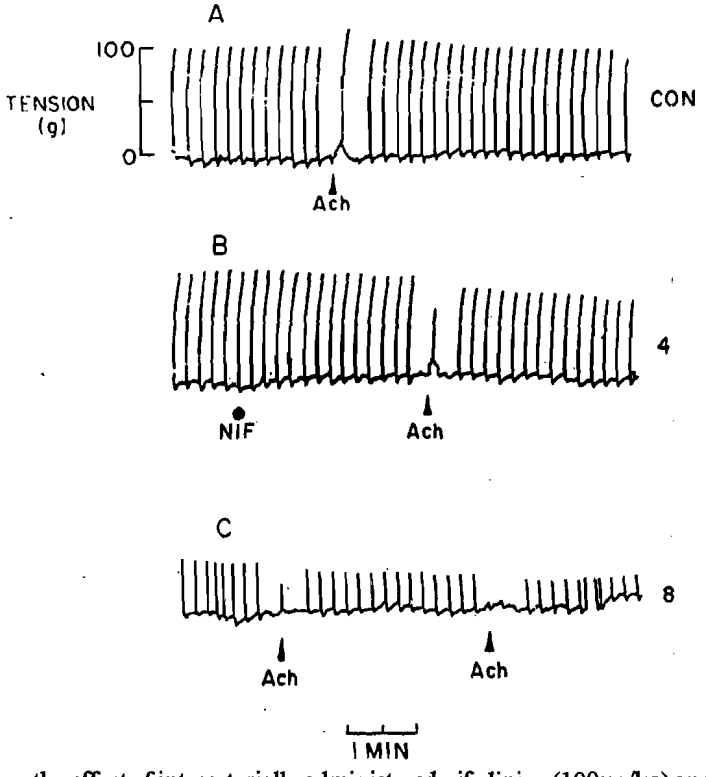

Figure 5. Showing the effect of intraarterially administered nifedipine (100μg/kg) on the acetylcholine (1mg/kg) induced contraction and indirectly elicited twitch amplitude of gastrocnemius muscle. Nifedipine (NIF) was administered at the circle (λ) and Acetylcholine (ACh) was administered at arrow (σ). **CON** is control. Tracing **A** shows the pre-drug control acetylcholine induced contraction and indirectly elicited isometric twitch. Tracing **B** and **C** show the stages of the effect of nifedipine on the same. The numbers on the right represent the interval (min) after the nifedipine administration.

Effect of nifedipine on gastrocnemius monosynaptic reflex.

In anesthetized cats, the effect of intraarterially administered nifedipine (50-200μg/kg) on gastrocnemius monosynaptic reflex, was observed. A dose of nifedipine sufficient to significantly decrease the indirectly stimulated isometric twitch amplitude of gastrocnemius muscle (100μg/kg) produced an initial facilitation followed by a significant depression of gastrocnemius monosynaptic reflex for the duration of experiments (180 minutes). Figure 6 illustrates a typical result. It will be seen that 10 seconds after the administration of nifedipine the gastrocnemius monosynaptic reflex was potentiated to 25% of the control height, and 40 seconds after the administration of nifedipine the reflex was depressed to 67% of the control height (Figure 7). The peak of the depression was between 35 and 45 seconds of nifedipine

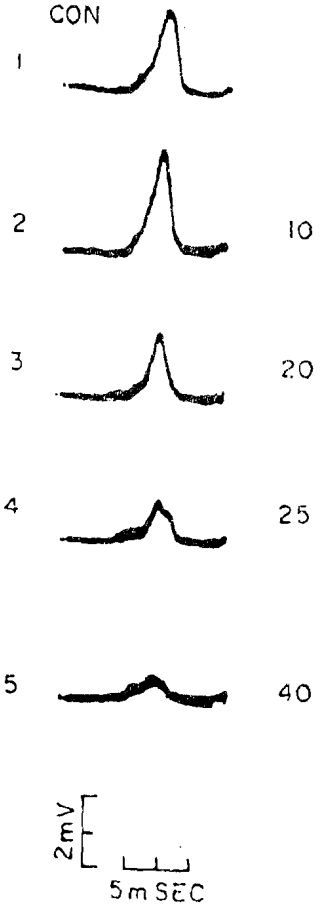

Figure 6. Typical record showing the effect of nifedipine (100μg/kg, i.a.) on gastrocnemius monosynaptic reflex recorded from L7 ventral root. The numbers on the right represent the interval (sec) after nifedipine administration. Tracing **1** shows the control (**CON**) gastrocnemius monosynaptic reflex. Tracing **2,3,4** and **5** show the effect of intraarterially administered nifedipine on the same monosynaptic reflex.

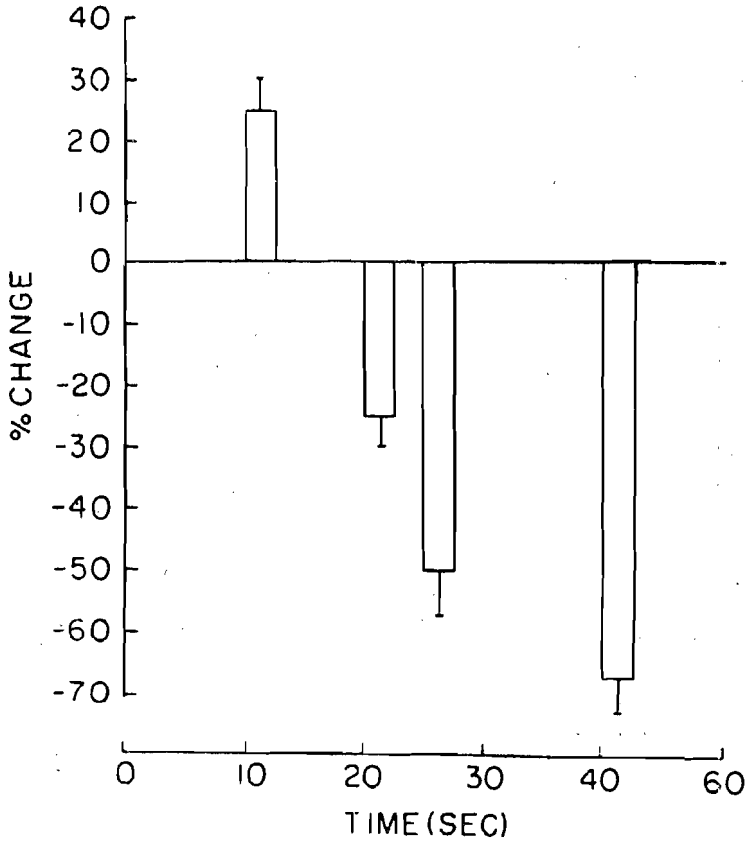

Figure 7. Shows percent changes in the amplitude (Peak) of gastrocnemius monosynaptic reflex after the administration of nifedipine (100μg/kg, i.a). The values represented the Mean ± SEM, n=7.

administration. Increasing doses of nifedipine up to 200μg/kg increased the magnitude of effect and decreased the duration of response. In saline administered control group of cats, no change in gastrocnemius monosynaptic reflex was observed over the time course of the experiment. Significant recovery of the nifedipine depressed gastrocnemius monosynaptic reflex to control was observed with a dose of calcium chloride (25mg/kg, i.a.) or caffeine (10mg/kg, i.a.). An illustration of this phenomenon is shown in Figure 8. It will be noticed that 50 seconds after the injection of calcium chloride the nifedipine depressed gastrocnemius monosynaptic reflex was recovered to 93% of the control height, and 50 seconds after the injection of caffeine the nifedipine depressed monosynaptic reflex was recovered to 71% of the control height (Figure 9). In saline injected control group of cats, no recovery in the nifedipine depressed gastrocnemius monosynaptic reflex was observed over the time course of the experiment.

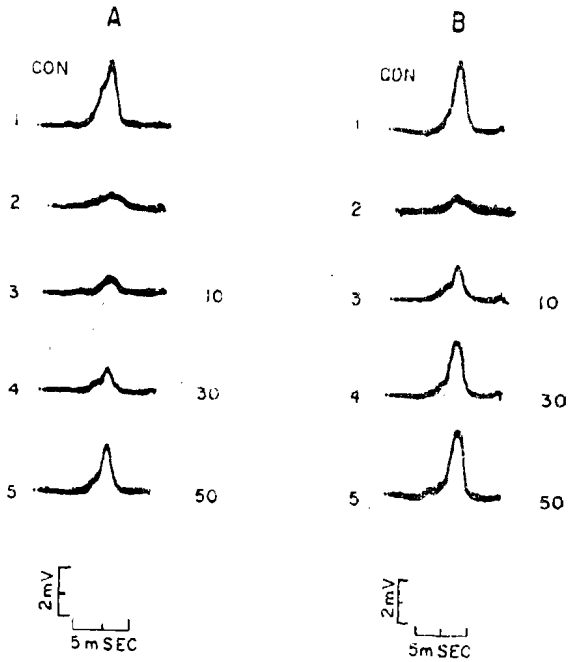

Figure 8. Showing the effect of intraarterially administered calcium chloride (25mg/kg) and caffeine (10mg/kg) on the recovery of nifedipine depressed gastrocnemius monosynaptic reflex. **CON** is control. The numbers on the right represent the interval (sec) after the nifedipine administration. Tracing **1** of row **A** and **B** show the control monosynaptic reflex. Tracing **2** of **A** and **B** show the maximal depressed monosynaptic reflex after nifedipine administration (100μg/kg, i.a). Tracing **3, 4,** and **5** of row **A** show the effect of caffeine and of row **B** show the effect of calcium chloride on the recovery of nifedipine depressed monosynaptic reflex.

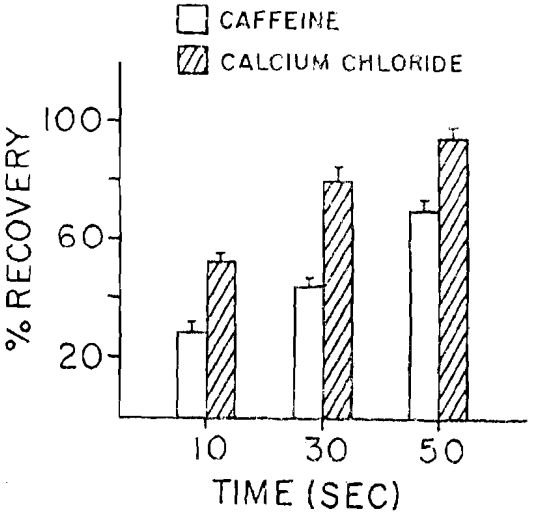

Figure 9. Shows percent recovery of the nifedipine depressed gastrocnemius monosynaptic reflex after intraarterial administration of calcium chloride (25mg/kg) and caffeine (10mg/kg). The values represented the Mean ± SEM, n=7.

DISCUSSION

In the present study we investigated the effect of nifedipine, a representative dihydropyridine organic calcium channel blocker (L-type), on the transmission of the nerve impulse at the skeletal neuromuscular junction in anesthetized cats. A method of close arterial injection was employed in this study. This route of administration was expected to deliver a drug selectively for the muscle in adequate concentration to exert the action.

The organic calcium channel blocker nifedipine, employed in man for the medical management of cardiovascular diseases, dose dependently reduced the isometric twitch amplitude of gastrocnemius muscle elicited by indirect and direct electrical stimulation for the duration of the experiment. In order to determine the effect of nifedipine on the neuromuscular junction and the muscle, nifedipine was administered to the muscle under alternate stimulations of indirect and direct. The result suggests that nifedipine may exert its blocking action both on the neuromuscular junction and the muscle. These findings are similar to those described earlier [10,12,13] but contrary to those which report that calcium channel blockers increased the force of contraction of soleus and gastrocnemius muscle of the cat [14] and twitch tension of the anterior tibial muscle of the dog [8]. Differences between our findings and those of other investigators might reflect the species difference, though it is almost unbelievable that skeletal muscles of the cat and dog make such a radical difference, dose of nifedipine, mode of electrical stimulation, and type of skeletal muscle used for recording isometric contraction. Since the reduction with the nifedipine of the isometric twitch amplitude of gastrocnemius muscle of the cat in the present study was much greater when the muscle was stimulated indirectly via the motor nerve than stimulated directly, the principal site of the blocking action of the nifedipine is thought to be neuromuscular junction. In order to investigate whether a recovery was involved or not in the nifedipine induced reduction of twitch amplitude by calcium supplementation, calcium chloride was administered to the muscle under alternate stimulations of indirect and direct. In the present study no recovery to control of the nifedipine depressed isometric twitch was observed by calcium supplementation. This result suggests that calcium supplementation fails to counteract the blocking action of nifedipine on the neuromuscular junction and the muscle.

Nifedipine may exert its effect directly at the synaptic level of the neuromuscular junction. Publicover and Duncan [15] observed *in vitro* that organic calcium channel blocker verapamil abolished indirectly elicited muscle twitch in frog nerve-muscle preparations. Similarly Kraynack *et al.* [16] observed that verapamil and pancuronium equally reduced indirect twitch amplitude in isolated bullfrog sciatic nerve-sartorious muscle preparations. The neuromuscular blocking action in the present study could result from (1) a presynaptic action of nifedipine. Nifedipine may reduce calcium conductance of the presynaptic membrane, alter intracellular presynaptic calcium pools, alter cyclic AMP levels or inhibit the membrane calcium pump. These actions may interfere with the mobilization of acetylcholine or its actual

release. Alternatively, (2) a post-junctional site of action could also be responsible. The blocking action of nifedipine on muscle may be brought about by one or more of the following reasons (1) action on muscle spindle activity, (2) action on contractile mechanism, or (3) action on muscle membranes.

In order to ascertain the effects of nifedipine on the presynaptic motor nerve terminals at the neuromuscular junction, the effect of nifedipine on posttetanic potentiation and tetanic contraction were observed since these phenomena have been shown to be presynaptic events [17,18]. In the present study with the use of nifedipine the posttetanic potentiation and tetanic contraction were not sustained. Posttetanic potentiation and tetanic contraction were completely blocked through a gradual depression after intraarterial administration of nifedipine. It seems that the motor nerve becomes unable to maintain the transmitter release for posttetanic twitch amplitude potentiation to a high frequency stimulation under the influence of nifedipine. Because tetanizing stimulation causes calcium to accumulate in the motor nerve terminals to such a degree that the intracellular binding sites that keep cytoplasmic calcium low are overwhelmed. Increased amount of calcium promotes to release excess amount of acetylcholine from presynaptic motor nerve terminals which in turn potentiates twitch amplitude in absence of nifedipine. Results also suggest that complete inhibition of tetanic contraction may be due to inhibition of transmitter release from presynaptic motor nerve terminals as a result of the exhaustion of calcium level under the influence of nifedipine. In this regard it is assumed that nifedipine may exert its blocking action on the accumulation of calcium in the presynaptic motor nerve terminals at the neuromuscular junction.

Though the contractile response to exogenous acetylcholine has been considered chiefly due to its depolarizing action on the motor nerve terminals by Riker and his colleagues [19,20], reexamination using alpha and beta-bungarotoxin by Lee and Tsai [21] had presented evidence indicating that it is a post-junctional event. Therefore, in order to find out the effect of nifedipine on post-junctional plasma membrane of muscle at neuromuscular junction, the effect of nifedipine on acetylcholine induced contraction of gastrocnemius muscle was investigated. In the present study with the use of nifedipine the acetylcholine induced contraction of gastrocnemius muscle was blocked. This result suggests that nifedipine injected close arterially to the muscle depressed the sensitivity of post-junctional acetylcholine receptors on the external surface of the muscle plasma membrane of the motor endplate which normally responds to the acetylcholine released from the motor nerve endings. In this regard chiarandini and Bentley [6] reported that organic calcium channel blocker verapamil blocked the acetylcholine induced contraction in toad skeletal muscle, though this effect could not be antagonized by increasing calcium concentration in bathing solution. Sato and Ono [8] also reported that nifedipine either abolished or depressed the acetylcholine induced contraction in dog skeletal muscle, although they reported that nifedipine facilitated the neuromuscular transmission in dog skeletal muscle. In this study significant depression of indirectly elicited isometric twitch

amplitude was also observed with nifedipine. From this result it is suggested that nifedipine depressed the sensitivity of extrajunctional acetylcholoine receptors of the muscle plasma membrane in addition to its blocking action of post-junctional acetylcholoine receptors.

In order to elicit a possible mechanism for the observed effect of nifedipine on motor nerve terminals at the neuromuscular junction, the effect of intraarterially administered nifedipine on gastrocnemius monosynaptic reflex was observed. In the present study nifedipine produced an initial facilitation followed by a significant depression of gastrocnemius monosynaptic reflex for the duration of the experiment. This result suggests that nifedipine induced depression of gastrocnemius monosynaptic reflex could result from a depression of spinal monosynaptic transmission. It is suggested that the depression of monosynaptic transmission is due to reduction of transmitter release from the presynaptic site (sensory axon terminal) of spinal synapse. In the present study intraarterially administered calcium or caffeine significantly recovered the nifedipine depressed gastrocnemius monosynaptic reflex. Significant recovery of nifedipine induced depression of gastrocnemius monosynaptic reflex by calcium supplementation suggests that calcium may increase transmitter release by elevating local calcium level in the presynaptic site. Because transmitter release from the nerve terminal is known to be increased by elevating local calcium concentration which will increase the influx of calcium into the terminal during the excitation [22]. It is suggested that nifedipine depresses monosynaptic transmission by blocking calcium entry into the presynaptic site, because calcium is necessary to release transmitter substance by means of exocytosis. In this study caffeine also significantly counteracts nifedipine induced depression of gastrocnemius monosynaptic reflex. This result suggest that caffeine may enhance transmitter release by increasing cyclic adenosine monophosphate (cyclic **AMP**) concentration in the presynaptic site. Thus it appears that nifedipine depresses gastrocnemius monosynaptic reflex by blocking calcium entry into the presynaptic site and reducing cyclic **AMP** level in the presynaptic site of spinal synapse. These actions may interfere with the mobilization of transmitter or its actual release. Initial potentiation of gastrocnemius monosynaptic reflex might be due to facilitation of synaptic transmission by local elevation of calcium concentration. In this regard, nifedipine can either promotes the release of calcium from intracellular storage site in the sensory axon terminal or inhibits the membrane calcium pump to put out the intracellular calcium, both resulting in increase in intracellular calcium level. Such an effect of nifedipine is expected to increase transmitter release on each nerve impulse at the spinal synapse in spite of a possible inhibitory effect of calcium influx [15]. From the result it is suggested that nifedipine reduces indirectly elicited isometric muscle twitch amplitude of gastrocnemius muscle by depressing neuromuscular transmission. The primary site of the nifedipine induced depression of neuromuscular transmission is the motor nerve terminal i.e., the presynaptic site of neuromuscular junction. Nifedipine depresses neuromuscular transmission presumably by reducing the release of acetylcholine, the transmitter substance, from motor nerve terminal. Nifedipine may reduce the release of acetylcholine by decreasing

calcium and cyclic AMP levels in the motor nerve terminal.

In conclusion, it appears that nifedipine inhibits nerve impulse transmission at the skeletal neuromuscular junction in intact cats for doses below the therapeutic dose range recommended for man by inhibiting the release of acetylcholine from motor nerve terminals and the sensitivity of end plate receptors to acetylcholine at the neuromuscular junction. In clinical situations where potent inhalation agents, antibiotics, neuromuscular blocking agents and other drugs that possess neuromuscular blocking action may be used, therapeutic doses of nifedipine may interact to promote muscle weakness. Until appropriate clinical studies or deny these possibilities anticipation and caution may be suggested in such situations.

We thank the University Grants commission, New Delhi, India for their financial assistance.

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PALMAR INTERDIGITAL RIDGE AMONG THE BRAHMIN OF MADHYA PRADESH

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Abstract

The present study deals with palmar interdigital ridge counts of the Brahmin of Madhya Pradesh. The study reveals no bilateral and bisexual difference except the bilateral difference in b-c ridge count among males. For the sake of comparison four different population groups are taken in the present study.

Introduction

Anthropometric traits are often used for taxonomic classification of human population. Of late, the emphasis is being given to genetic traits for such purposes. All human physical traits, as for example, skin colour, nasal profile, height or dermatoglyphic traits (ridge count and pattern on fingers, palms and soles), are determined by many loci, which are subject to selection procedure (Narain, 1993). Ridge count scores between various interdigital triradii of palmar and plantar surfaces are an interesting dermatoglyphic marker in the population variation studies due to their non- adaptivness and variability.

The qualitative and quantitative variabilities in palmar dermatoglyphics have been studied widely in different ways. However, few studies have so far been reported on palmar interdigital ridge counts in India. Distribution of various palmar dermatoglyphic traits among the Relli and Manne Dora has been reported by Pratheesam et al (1993). Pateria (1967) studied a-b, b-c, c-d, palmar interdigital ridge counts among the Bhangi of Sagar, Madhya Pradesh Side by side, Mandal and Sharma (2000) have reported the same type of study among the Lodha of Midnapore, West Bengal. The present study is an endeavour to deal with interdigital ridge counts of palm among the Brahmin of Madhya Pradesh.

Material and methods

The sample of the present study consists of bilateral palmar prints of 90 males and 73 females belonging to the Brahmin caste of Cheechli Block of Narsingpur district, Madhya Pradesh. For the present study field work was conducted by A.M.M (first author of this paper). Care was taken to exclule related individuals from the present sample, though no

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statistical sampling was done. Prints were taken by ink and roller method as suggested by Cummins and Midlo (1961). To obtain morphological distance, data on Bhangi (Pateria, 1976), Relli and Manne Dora (Parvatheesam et al., 1993), and Lodha (Mandal and Sharma, 2000) have been taken into consideration, according to the methods given by Penrose (1947, 1954) and Rao (1952). While to find out bilateral ridge count asymmetry for each inter-digital areas two measures namely mean directional asymmetty (MDA) and mean absolute asymmetry (MAA) have been used.

Results and discussion

Mean ridge counts of a-b, and c-d interdigital areas of the Brahmin are shown sex-wise in Table 1. It reveals that the mean values are slightly higher among the males that of the females, which is true for all the three ridge counts. Considering all these three counts separately, no significant difference is observed.

Table 1 : Distribution of mean a-b, b-c and c-d palmar ridge counts among the Brahmin

Inter	Male			Female			t-test
digital area	Mean	C.V.	S.D.	Mean	C.V.	S.D.	value
a-b	65.60	13.71	9.00	64.55	892	5.76	0.90
b-c	44.02	17.35	7.64	42.22	21.95	9.27	1.33
c-d	59.59	14.71	8.77	57.00	15.15	8.64	1.89

In Table 2 mean interdigital ridge counts are shown by sex and side-wise of the study population. It is interesting to note that mean values in left palms are slightly higher than that of the right palms in both the sexes, which is true again in all the ridge counts. However, in both the sexes no significant bilateral difference is noticed, excepting b-c ridge counts among the males.

Table 2 : Bilateral differences in mean a-b, b-c and c-d palmar ridge counts among the Brahmin

Palm	Male			Female		
	a-b	b-c	c-d	a-b	b-c	c-d
Right	32.74	20.75	29.74	32.04	21.03	28.07
S.E.	±0.77	±0.46	±0.61	±0.40	±0.56	±0.69
Left	32.83	23.27	29.89	32.51	21.19	29.20
S.E.	±0.66	±0.61	±0.54	±0.39	±0.60	±0.58
t-test	0.09	3.29*	0.18	0.84	0.19	.25

* Significant at 0.05 level of probability

Ridge count Asymmetry

Bilateral ridge count asymmetry for each interdigital areas was ascertained by two measure, namely mean directional asymmetry (MDA) and mean absolute asymmetry (MAA). MDA is obtained by calculating by calculating the mean of the differences in ridge counts of the right palm from the left palm. The formula is :

$$MDA = \sum_{i=1}^{n}\left[\frac{(R_i - L_i)}{N}\right]$$

Where Ri = ridge count of concerned area of right palm of the individual "i",

Li = ridge count of homologous area of left palm of the (same) individual "i" and

N = number of palm.

MAA is also the mean of the differences and calculated using the same formula but the signs of individual values are ignored in summation during calculating the mean.

Values of MDA and MAA of three palmar interdigital areas are presented sex wise in Table 3. The highest mean MDA is noticed in a-b in males and b-c in females Whereas, the maximum value of MAA is noticed in b-c in males and a-b in females. The bisexual difference in MDA is significant in a-b and b-c interdigital area, while in MAA significant difference is noticed in b-c interdigital area.

Table 3 : Distribution of mean directional asymmetry (MDA) and mean absolute asymmetry (MAA) among the Brahmin

Interdigital area	MDA (Mean + S.E.) Male	MDA (Mean + S.E.) Female	t-test value	MAA (Mean + S.E.) Male	MAA (Mean + S.E.) Female	t-test value
a-b	0.61±0.31	-0.20±0.14	2.41*	0.64±0.61	0.69±0.28	0.08
b-c	-1.23±0.22	0.11±0.15	5.06*	2.21±0.45	0.15±0.30	3.81*
c-d	0.04±0.20	0.53±0.24	1.83	-0.05±0.40	-1.02±0.48	1.71

* Significant at 0.05 level of probability

Comparison

The mean palmar interdigital ridge counts of the Brahmin have been presented in Table 4. Among the males a-b mean value is considerably higher in the Relli than those exist among other four populations. In the Brahmin however, the mean of this interdigital area is lowest than other four population in this sex. Among the females the highest mean of a-b is again

Table 4 : Mean a-b, b-c, and c-d ridge counts of palmar interdigital areas among the populations considered for the purpose of comparison.

Inter digital areas		Bhangi			Relli			Manne Dora			Lodha			Brahmin (Present study)		
		Male (131)	Female (131)	t-test value	Male (50)	Female (50)	t-test value	Male (50)	Female (50)	t-test value	Male (168)	Female (155)	t-test value	Male (90)	Female (73)	t-test value
a-b	Mean	74.49	73.62	0.60	88.60	75.80	0.46	74.6	62.10	1.18	75.58	74.10	0.77	65.6	64.55	0.90
	C.V.	15.10	14.40		16.09	10.45		15.36	28.81		23.73	22.99		13.71	8.92	
	S.D.	11.20	10.6		13.93	7.92		11.46	17.89		8.97	8.52		9.00	5.76	
a-b	Mean	47.36	48.27	0.58	56.60	48.60	1.12	52.80	62.50	2.12*	44.96	46.54	0.48	44.02	42.22	1.33
	C.V.	24.80	23.80		23.37	20.95		15.8	9.96		78.31	48.47		17.35	21.95	
	S.D.	11.70	11.50		13.22	10.18		8.34	6.22		17.59	11.28		7.64	9.27]	
a-b	Mean	66.17	69.15	0.87	72.20	74.40	0.87	77.00	64.10	1.18	55.94	64.64	0.89	59.59	57.00	1.89
	C.V.	16.90	16.40		20.76	16.92		16.92	29.23		79.69	45.53		14.71	15.15	
	S.D.	11.20	16.40		14.99	12.58		12.80	18.74		22.29	14.07		8.77	8.64	

* Significant at 0.05 level of probability

ISSN 0972-8503

recorded in the Relli but its lowest mean is recorded in the Manne Dora. Lowest mean of b-c interdigital ridge counts is recorded in the Brahmin, which is true for both the sexes, whereas its highest value is recorded in the Relli among the males and Manne Dora among the females and the Lodha show the lowest for the males. Side by side, Manne Dora show the highest mean in this ridge count for the males and the Relli show the highest for the females. The study population Brahmin, however, show a lower level of mean values in all the three counts in general than other four populations.

Morphological Distance

Morphological distances have been estimated sex-wise considering all the five populations. For this purpose all possible pairs in terms of three palmar interdigital ridge counts have been taken into consideration. In Tables 5 and 6 the values of size (C^2_Q) and shape (C^2_Z) distances are presented. These values are obtained to trace out the extent of divergence among the population groups. The computed mean values for size distance between ten possible pairs are 1.08 and 0.81 for males and females respectively. While for shape distance the mean values are 0.85 and 1.40 in the same sequence. It appears that the value of size distance is larger than the shape distance among the males and the reverse is true among the females. Thus these five populations show a tendency to differ more in size distance than in shape distance in the males, whereas the situation is just reverse in the females. However, in males the size distance and in females the shape distance play some important role for overall morphological differences among these populations.

Table 5 : Values of size distance (C^2_Q) between populations

Population	Bhangi	Relli	Manne Dora	Lodha	Brahmin
Bhangi	–	1.65	0.29	0.18	0.75
Relli	0.12	–	0.42	2.88	0.99
Manne Dora	0.00	0.18	–	1.08	2.28
Lodha	0.06	0.33	0.03	–	0.21
Brahmin	1.77	2.82	1.59	1.20	-

Upper triangular matrix : Male, Lower triangular matrix : Female

Table 6 : Value of shape distance (C^2_Z) between populations

Population	Bhangi	Relli	Manne Dora	Lodha	Brahmin
Bhangi	–	0.33	0.24	0.27	0.21
Relli	0.06	–	1.08	0.03	4.56
Manne Dora	3.24	3.90	–	0.99	0.09
Lodha	0.06	0.18	3.66	–	0.75
Brahmin	0.06	0.21	2.46	0.15	–

Upper triangular matrix : Male, Lower triangular matrix : Female

Matrix of C^2_H (morphological distance) values for both the sexes are shown in Table 7. In Table 8 the values of C^2_H between all the ten pairs have been shown in increasing order of magnitude. It reveals that in the males the Bhangi maintain a very close distance with the Lodha (0.45), whereas the Brahmin are comparatively distantly related with the Bhangi (0.96), Manne Dora (2.37) and Relli (5.55). Likewise, in case of females also the Bhangi maintain a very close distance with the Lodha (0.12) and the Brahmin maintain a distant relation with the Lodha (1.35) Bhangi (1.83), Relli (3.03) and Manne Dora (4.05).

Table 7 : Value of morphological distance (C^2_H) between populations

Population	Bhangi	Relli	Manne Dora	Lodha	Brahmin
Bhangi	-	1.95	0.63	0.45	.96
Relli	0.18	-	1.50	2.91	5.55
Manne Dora	3.24	4.08	-	2.027	2.37
Lodha	0.12	0.51	3.69	-	0.96
Brahmin	1.83	3.03	4.05	1.35	-

Upper triangular matrix : Male
Lower triangular matrix : Female

Table 8 : Values of (C^2_H) between population pairs by sex in increasing order of magnitude.

Bhangi		Relli		Manne Dora		Lodha		Brahmin	
M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
0.45	0.12	1.50	0.18	0.63	3.24	0.45	0.12	0.96	1.35
Lodha	Lodha	M. Dora	Bhangi	Bhangi	Bhangi	Bhangi	Bhangi	Bhangi	Lodha
0.63	0.18	1.95	0.51	1.50	3.69	0.96	0.51	0.96	1.83
M. Dora	Relli	Bhangi	Lodha	Relli	Lodha	Brahmin	Relli	Lodha	Bhangi
0.96	1.83	2.91	3.03	2.07	4.05	2.07	1.35	2.37	3.03
Brahmin	Brahmin	Lodha	Brahmin	M. Dora	Brahmin	M. Dora	Brahmin	M. Dora	Relli
1.95	3.24	5.55	4.08	2.37	4.08	2.91	3.69	5.55	4.05
Relli	M. Dora	Brahmin	M. Dora	Brahmin	Relli	Relli	M. Dora	Relli	M. Dora

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ANTHROPOMETRIC AND PHYSIOMETRIC ASSESSMENT OF ADULT DHIMALS OF NAXALBARI, WEST BENGAL

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Key Words : Dhimals; Anthropometry; Physiometric; Health; Nutritional Status

Abstract

This paper presents new anthropometric and physiometric data of Dhimals, an ethnic group resident in Naxalbari, West Bengal. It also discusses the health and nutritional implications of these data.

Introduction :

Dhimal, Mech, Koch, Toto, Garo, Chakma, Bhutia, Lepcha, Rabha and Limbu are some of the Mongoloid tribes of North Bengal. Dhimals are Tibeto-Burman language speaking Indo- Mongoloid tribe of North Bengal like the Meches (Basu 1922). Dhimal is a less known small community in North Bengal. Origin and ethnicity of Dhimals raised controversy in literatures over centuries. Dhimals, Meches, Totos, Limbus and Bodos have many similarities in their language and other behavioral patterns in their socio-economic and political life, religious beliefs, customs and practices and other folk traditions and other cultural ways of life. History of migration indicate that Bodos and Kacharis of Assam and Meches, Rai, Limbu and Koch of West Bengal in India and Nepal belong to the same race and they resemble closely in features and complexion (Hodgson 1880). Dhimals and Totos have very close affinity to this common stock. "*Dhimal, Dhemal* or *Maulik*, a non-Aryan tribe of the Darjeeling and Nepal Terai, classed by Fr. Muller as Lohitic. They belong to the same main stock as the Kochh and are rapidly losing their tribal identity" (Risley 1891). Because of their facial features, language, and religious practices, Dhimals are also called the Limbus of the Nepalese plains (Sanyal 1973). Linguistic analyses through glutochornology method (Maitra 2001) predict that the bifurcation of Dhimal lineage from the main Mongoloid stock took place in and around 500 B.C. Dhimals and Totos got separated from each other in between 800 - 1200 A.D.

Dhimal is a small community in North Bengal. Evidence of bio-anthropological research on this community is not available. Dhimals live beside the river Mechi in the bordering areas of India and Nepal. The total population of the Dhimals in India is below one thousand. Unofficial records count 908 (done by the Dhimal people themselves in 2005). However, from official records (Table 1), it is evident that the strength of Dhimal population in North

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Bengal is around 700. In 1931 Census, Dhimal population was 621. Since the Census of India 1941, separate enumeration for Dhimals had been excluded. It was 1060 as recorded by Maitra (2001). Dhimals have their own unique language, dress preferences and culture. Inspite of having indigenous cultural practices and folk traditions over the centuries, Dhimals have not yet been enlisted in the Scheduled Tribe (ST) category. They are enlisted in Other Backward Class or OBC category in West Bengal.

It has long been well established that the use of anthropometry is an efficient indicator of nutritional and health status of adults (WHO, 1995). The body mass index (BMI) is indicative of overall adiposity (Bose 1996). Low BMI and high levels of undernutrition (based on BMI) is a major public health problem especially among rural underprivileged adults of developing countries (WHO, 1995). Although adult nutritional status can be evaluated in many ways, the BMI is most widely used because its use is inexpensive, non-invasive and suitable for large-scale surveys (Lohman, 1998; Ferro-Luzzi, 1992; James et. al. 1994). Therefore, BMI is the most established anthropometric indicator used for assessment of adult nutrition status (Lee, 2003). BMI is generally considered a good indicator of not only the nutritional status but also the socio-economic condition of a population, especially adult populations of developing countries (Ferro-Luzzi, 1992; Shetty, 1994; Khongsdier, 2002). A BMI < 18.5 kg/m^2 is widely used as a practical measure of chronic energy deficiency (CED), i.e., a 'steady' underweight in which an individual is in energy balance irrespective of a loss in body weight or body energy stores (Khongsdier, 2005). Such a 'steady' underweight is likely to be associated with morbidity or other physiological and functional impairments (WHO, 1995; Shetty, 1994; James, 1988)

In general, data are scarce on the anthropometric and nutritional status of various tribal populations of India (Bose, 2005; Bose, 2006a,b,c). It has been recently suggested (Bose, 2005; Bose, 2006a,b,c) that there is urgent need to evaluate the nutritional status of various tribes of India. In view of this, the objective of the present study was to report nutritional status, based on BMI, of adult Dhimals. To the best of our knowledge, this is the first report on the nutritional profile of adult Dhimals.

Materials and Methods :

Dhimal villages ('Busti' or 'Dera') are located in Mallabari under Naxalbari police station of Darjeeling district in West Bengal. Dhimals are concentrated in ten hamlets under three villages, viz. Maniram, Hatighisa and Buraganj. Dhimals are not found elsewhere in India. These villages are located around 15 kms. from Siliguri town which is approximately 580 kms. from Kolkata, the provincial capital of West Bengal. Dhimals of India however, is essentially a part of a larger gene pool of the Dhimals of Jhapa district of Nepal (called *Purbi* Dhimals) with whom they have regular matrimonial exchanges. Hence the Dhimals in North Bengal and Jhapa district of Nepal are genetically constituting a single population

or gene pool. Dhimals are also found in Morang district (*Paschimi* Dhimals) of Nepal but marriage beteween the Dhimals of Morang districts and the Dhimals Jhapa districs of Nepal and West Bengal are not socially sanctioned. These two demes (Purbi Dhimal including the Dhimals of North Bengal and Paschimi Dhimals) also vary in their dialects and cultural practices. Marriage between Dhimals and other neighbouring communities like the Rajbangshis, Meches or Limbus is also not custmarily approved by the traditional Dhimal society.

In our present study, we have considered the Dhimal population of the age-group 18 years and above. In the entire region, the exhaustive survey records 309 individuals (159 male and 150 female) in these age-groups. However, in this present investigation, the spouses, belonging to other community (ies) and married to a Dhimal and his or her offspring have been excluded in order to maintain the appropriate genetic structure of the population under study. Most of the Dhimals are land less and marginal labourers. However, farming is their specialization. Previously they were involved in slash and burn ('Jhum") cultivation. They are exceptionally poor in socio-economic and literacy status.

Anthropometric measurements were taken among adult ($\geqslant$ 18 years) residents of all houses in the three villages were contacted and a total of 313 subjects were studied. Out of these, 4 individuals were excluded because of missing data. Therefore the final sample size of the study was 309 (159 men and 150 women). The vast majority of the subjects were illiterate. They were predominantly settled cultivators or very low-wage earning manual labourers. Thus, they belonged to the low socio-economic class.

Ethical approval and prior permission was obtained from Vidyasagar University Ethics Committee and local community leaders, respectively, before commencement of the study. Informed consent was also obtained from each participant. Information on ethnicity, age, occupation and educational status were obtained from all subjects with the help of a questionnaire.

Anthropometric measurements

All anthropometric measurements of lightly clothed subjects were taken by the investigators using standard anthropometric techniques followed by Lohman et. al., (1988). Height and weight were taken to the nearest 0.1 cm and 0.5 kg, using standard Martin's anthropometer and weighing scale (Doctor Beliram and Sons, New Delhi, India), respectively. Technical errors of measurements (TEM) were computed and they were found to be within acceptable limits (Ulijaszek and Kerr 1999). BMI was computed using the following standard equation :

$$BMI = \text{Weight (kg)} / \text{height (m}^2\text{)}$$

Chronic energy deficiency (CED) was evaluated using internationally accepted BMI guidelines (WHO 1995). The following cut-off points were used :

CED Grade III :	BMI < 16.0
CED Grade II :	BMI = 16.0 – 16.9

CED Grade I : BMI = 17.0 – 18.4

Normal : BMI = 18.5 – 24.9

We followed the World Health Organization's classification (WHO, 1995) of the public health problem of low BMI, based on adult populations worldwide. This classification categorises prevalence according to percentage of a population with BMI < 18.5.

1) Low (5–9%): warning sign, monitoring required.
2) Medium (10–19%): poor situation.
3) High (20–39%): serious situation.
4) Very high (≥ 40%): critical situation.

The distributions of the anthropometric variables were not significantly skewed in both sexes. Student's t-tests were performed to test for sex differences in mean anthropometric characteristics. Sex differences in CED/non CED were determined by chi-square test (including odds ratio, OR). Correlation coefficient was calculated by standard statistical method. All statistical analyses were undertaken using the SPSS Statistical Package. Statistical significance was set at $p < 0.05$.

Measurement of Blood Pressure

Blood pressure measurements were taken on left arm with a sphygmomanometer and stethoscope placed at the heart level of the subject who as been rested in relaxed and supine position on a bed. Systolic (SBP) and diastolic (DBP) blood pressures were recorded to the nearest mmHg as the appearance (Phase I) and disappearance (Phase V) of Korotkoff sounds, respectively.

Mean arterial blood pressure (MABP) have been calculated out of values of the systolic and diastolic blood pressures. The calculation of MBP is 1/3 of the systolic blood pressure (SBP) + 2/3 of the diastolic blood pressure (DBP) (Lam et. al. 2001). However, the calculated values of both the mean arterial blood pressure MABP = (1/3 SBP + 2/3 DBP) as well as mean arterial blood pressure MABP = [DBP +1/3 (SBP-DBP)] are same (Bhadra et.al.2002).

Estimation of haemoglobin level

Sahli's method of haemoglobin level (gm/dl) estimation by haemoglobinometer (made in Germany) was followed in this investigation. This method is based on conversion of haemoglobin to acid haematin of brown colour with the help of mixing of N/10 HCl with 20µl of blood sucked in haemoglobin pipette. Normal haemoglobin values are as follows which reported by (Ganong 1981; Guyton 1981) :

adult male: 15.5 ± 2.5 gm/dl (14 - 18 gm %)

adult female: 14.0 ± 2.5 gm/dl (11.5 - 16.5 gm %)

Therefore, sex ratio was calculated using the following standard formula:

Sex ratio= (Male / Female) × 1000

Results and Discussion

The Dhimal community of Naxalbari region of Northern part of West Bengal, is essentially a small population and may be considered as a genetic isolate in the midst of other

ethnic groups in that region. Age-sex distributions (table –1) of the adult Dhimals exhibit majority of male (65.13%) and female (72.37%) sections of the population belong to the age-group, 18-39 years. From demographic point of view, the high proportion of the Dhimal population of both the sexes of this age-group, indicate significant labour force by the young-adult section along with a sizeable strength of population in reproductive age. Hence, this kind of structure of the Dhimal population has important socio-economic correlates and also indicates high status of reproductive health. This situation may help in solving the questions of survival of the small population when the entire Dhimal community is suffering from severe undernutrition (figure 1) and high degree of anaemia due to frequent infestations of malarial parasites in the population.

Table1: Sex-Ratio Patterns of the adult Dhimals in West Bengal

AGE-GROUP	MALE (%) (N=159)	FEMALE (%) (N=150)	SEX-RATIO
18-39	65.13	72.37	1111
40-59	28.30	20.39	689
60 +	9.43	5.92	600
TOTAL	100.00	100.00	943

Among the Dhimals, the young adults exhibiting high sex-ratio (female to male ratio or FMR) in the age-group 18-39 years, has also put a reasonable account from the demographic point of view of women's participation and their significant involvement and roles in socio-economic pursuits of the community. However, remarkable decline of FMR from 1111 to 689 (in the age-group 40-59 years) and 600 in the age group of 60 years and above, indicate higher rate of female mortality in the post-reproductive ages. This information may be correlated with the health-related issues like onset of menopause and lowering of immunity along with the other factors like severe malnutrition (figure 1) and higher degree of anaemia among the womenfolk, compared to the Men.

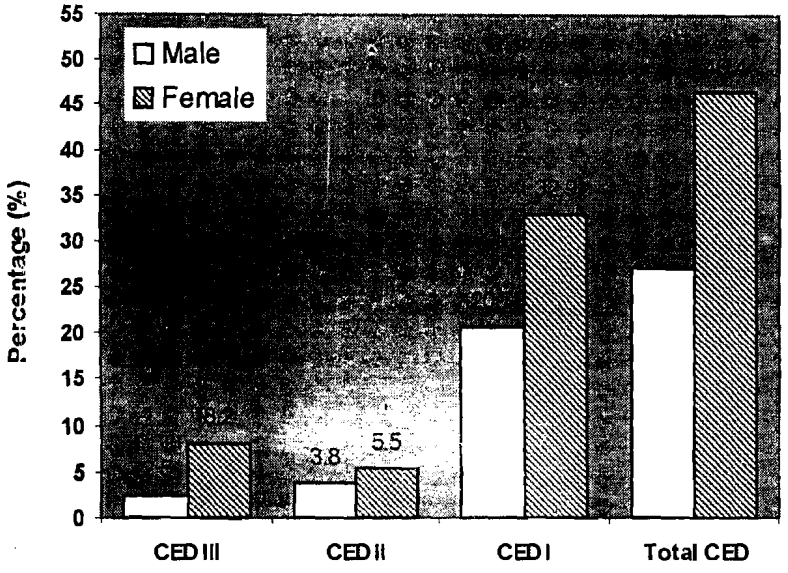
Table 2: Anthropometric and physiometric characteristics of adult Dhimals of Naxalbari, West Bengal, India.

VARIABLES	MALE (N= 159)			FEMALE (N = 150)			t
	Mean	± se	CV	Mean	± se	CV	
Age (years)	35.93	±1.14	40.13	32.59	±1.06	40.03	2.14*
Height (cm)	163.3	±0.50	3.85	152.40	±0.51	6.60	21.37***
Weight (kg)	52.2	±0.57	13.80	44.6	±0.57	15.50	9.43***
BMI (kg/m^2)	19.54	±0.16	10.33	19.20	±0.21	13.58	1.30
Hb level (gm/dl)	9.69	±0.13	16.55	8.82	±0.12	16.30	4.83***
SBP (mm/Hg)	128.84	±1.46	14.27	122.51	±1.14	11.36	3.42***
DBP (mm/Hg)	79.45	±0.88	14.03	78.99	±0.73	11.35	0.40
MABP (mm/Hg)	95.75	±0.98	12.92	92.56	±0.73	9.70	2.61**

Sex difference: level of significance: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

The mean (± se) values along with coefficient of variation (CV) of age (in years), height (in cm), weight (in kg.), body mass index (BMI) haemoglobin (Hb) level (gm/dl) and blood pressures (systolic=SBP; diastolic=DBP and mean=MABP) of both men and women are presented in table 2. Mean age of both sexes represent standard adult population. The mean age of men (35.93 ±1.14) and women (32.59 ± 1.06) were not similar. Results show that the males are taller and heavier than females, and also have higher systolic (SBP), please delete and mean (MABP) blood pressures. Highly significant sex differences were found in height, weight, haemoglobin level, systolic and mean blood pressures. Whereas, sex difference in BMI and in diastolic blood pressure was found to be non-significant.

Figure 1: Chronic energy deficiency (%) among adult Dhimals at Naxalbari in West Bengal.



Overall sex combined CED = 36.4 %,
Sex differences of CED: chi-square = 12.54, $p < 0.001$.
OR = 2.35; 95% CI = 1.42 – 3.90.

Figure 1 presents the frequency of CED among the subjects. The overall (sex combined) frequency of CED was 36.4 %. The prevalence of CED was much higher among women (46.4 %) as compared to men (27.0 %). This sex difference was statistically highly significant ($\chi 2 = 12.54$, $p < 0.001$). The odds ratio (OR) was 2.35 (95% CI = 1.42 –3.90). This implied that among adult Dhimals, women had 2.35 greater chance of being chronic energy deficient then men.

Recent studies from India (Bose and Chakraborty, 2005, Bose et al., 2006a,b,c) Yadav et al., 1999; Gogoi and Sengupta, 2002; Khongsdier, 2002; 2005; Sahani, 2003; Dash Sharma, 2004) have utilized BMI to study nutritional status of tribal populations. Therefore, the use of BMI and WHO (1995) BMI-based cut-off points for the evaluation of CED are valid for use among tribal populations of India.

Figure 1 also compares the rate of CED among the Dhimals. The population has a very high rate of undernutrition. The frequency of undernutrition among men is found among

Dhimals to be 27.0 %. In women, the rate is observed among Dhimals is 46.6. Hence, women have higher rate compared to men. Within-group sex difference in undernutrition rate among the Dhimals is 19.4 %. In fact, in case of Dhimals, women have 2.35 fold chances of being chronic energy deficient compared with males. Using the WHO (1995) classification of public health problem of low BMI, based on adult populations worldwide, it is observed that females of this populations have very high (≥ 40%) rates of undernutrition and the situation is critical. Dhimal males have high (20-39%) rates of undernutrition with the situation being serious.

These results clearly indicated that

i) this population is under critical or severe nutritional stress,
ii) this stress is more in women
iii) sex difference in CED is very high among Dhimals.

Table 3 : age-related changes of blood pressure among the adult Dhimals at Naxalbari in West Bengal.

AGE GROUPS	BLOOD PRESSURE	MALE (N = 159)		FEMALE (N = 150)		t
		Mean	± se	Mean	± se	
18 - 39	SBP	124.43	± 2.45	122.05	± 1.58	0.82
	DBP	78.0	± 1.27	78.65	± 1.04	0.54
	MABP	93.17	± 1.43	92.97	± 1.07	0.12
40 - 59	SBP	133.37	± 3.62	122.00	± 2.20	2.70*
	DBP	82.37	± 1.74	80.96	± 1.49	0.27
	MABP	99.20	± 2.28	94.50	± 1.25	1.82
60 +	SBP	140.60	± 9.06	136.44	± 6.79	0.37
	DBP	81.20	± 3.68	82.67	± 4.48	0.04
	MABP	100.80	± 5.08	100.41	± 4.86	0.06

SBP = Systolic blood pressure; DBP = Diastolic blood pressure; MABP = Mean arterial blood presure. Sex difference: level of significance: $^{*}p < 0.05$.

Consistent rise of blood pressures (both SBP and DBP) are observed with advancement of age in both the sexes, except a minor decline of DBP among the males of 60 years and above (table 3). The results of mean blood pressure (MABP) also indicate that the Dhimal individuals of both the sexes exhibit physiologically normal blood pressure and a tendency of its rise in accordance with age (Ganong 1981; Guyton 1981). Sex difference in blood pressure (both SBP and DBP) in different age-groups was found to be non-significant. However, in case of systolic blood pressure (SBP), significant sex difference ($t = 2.70$; $p<0.001$) was observed in the age-group 40-59 years.

Table 4 : Distribution of haemoglobin level (gm/dl) in different age-groups of the adult Dhimals at Naxalbari in West Bengal.

AGE GROUPS (YEARS)	MALE (N = 159) Hb LEVEL (gm/dl)		FEMALE (N = 150) Hb LEVEL (gm/dl)		t
	Mean	± se	Mean	± se	
18 - 39	10.07	± 0.19	8.86	± 0.18	4.48*
40 - 59	9.89	± 0.27	9.29	± 0.30	1.50
60 +	8.22	± 0.48	8.07	± 0.34	0.25
TOTAL	9.69	± 0.13	8.82	± 0.12	4.83*

Hb = Haemoglobin. Sex difference: level of significance *p < 0.001;

Results show that the haemoglobin levels in both the sexes (male: 9.69gm/dl Vs female: 8.82gm/dl, t=4.83, p<0.001) among the adult Dhimals were very low (tables 2 and 4). This is also true in all age groups. Females are found to be more anaemic than males in all ages. Significant sex differences (p<0.001) in this context were recorded in 18 – 39 years of age. In both sexes, it has been noted that there is a trend of decline of haemoglobin level with the advancement of senility.

Table 5: Distribution of haemoglobin level (gm/dl) among the adult Dhimals at Naxalbari in West Bengal.

HAEMOGLOBIN LEVEL (RANGE)	MALE			FEMALE			t
	% of persons	Mean	± se	% of persons	Mean	± se	
Below 5.0	n.f.	n.f.	n.f.	1.16	4.8	±0.0	N.A.
5.0 – 8.9	31.12	7.03	±0.13	50.88	7.09	±0.08	0.37
9.0 – 12.9	67.56	10.75	±0.08	47.96	10.55	±0.08	2.22*
13.0 - 14.9	1.32	13.75	±0.53	n.f.	n.f.	n.f.	N.A.

n.f. = None found. N.A = Not applicable. Sex difference: level of significance: *p < 0.02.

Distribution (table 5) shows that highest proportion of females (50.88%) is having haemoglobin level 7.09gm/dl (±0.08) in the range of 5.0-8.9gm./dl., which is very low. Severe undernutritions along with malarial parasite infections are the major reasons behind this situation. On the other hand, 67.35% of males with mean haemoglobin level 10.75gm/dl. (±0.08) and 47.96% of females with haemoglobin level 10.55 gm./dl (±0.08) are found in the range of 9.0-12.9gm/dl. No females and only 1.32% of males are observed to have haemoglobin level 13.75gm/dl. (±0.53) in the range of 13.0 - 14.9 gm/dl. The entire adult Dhimal population is suffering from severe undernutrition as well as acute anaemia due to

malarial parasite infection and females are also found to be more affected than the males. This may reflect negative bias for women in Dhimal society who have to play major roles in nursing and feeding of men folk during malarial parasite infection in epidemic form and are also underprivileged in the context of distribution of food for both the sexes. These social and cultural factors behind the health and nutritional status of both the sexes in Dhimal society need further investigation.

Table 6: Pearson correlation analysis of age, BMI, SBP, DBP, MABP and haemoglobin level (HB) among the adult male Dhimals (N=159) at Naxalbari in West Bengal.

		AGE	BMI	DBP	HB	MABP	SBP
Pearson Correlation	AGE	-	-.152	.253**	.306**	.285**	.268**
	BMI	-.152	-	.244**	-.184*	.220**	.148
	DBP	.253**	.244**	-	.093	.931**	.667**
	HB	.306**	-.184*	.093	-	.123	.137
	MABP	.285**	.220**	.931**	.123	-	.893**
	SBP	.268**	.148	.667**	.137	.893**	-

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Pearson correlation analysis (table 6 for males and table 7 for females) between age, BMI, and blood pressures (SBP, DBP and MABP) has been done. Results show that, for both the sexes, age has negative correlation with BMI ($r = -0.152$ with no significance in males and $r = -0.405$ in females with level of significance $p<0.01$). Correlation between age and haemoglobin level is significant ($p<0.01$) for the males ($r = 0.306$) but negative correlation among the females ($r = -0.003$) has no significance. Among adult Dhimal males, blood pressures (SBP, DBP and MABP) show significant ($p<0.01$) correlation with age (table 7). But in case of the females, SBP and MABP has significant correlation with age at 5% level ($r = 0.188$ for SBP and $r = 0.199$ for MABP).

Table 7: Pearson correlation analysis of age, BMI, SBP, DBP, MABP and haemoglobin level (HB) among the adult female Dhimals (N=150) at Naxalbari in West Bengal.

		AGE	BMI	DBP	HB	MABP	SBP
Pearson Correlation	AGE	-	-.405**	.157	-.003	.199*	.188*
	BMI	-.405**	-	-.026	.019	-.048	-.060
	DBP	.157	-.026	-	.011	.890**	.452**
	HB	-.003	.019	.011	-	.046	.077
	MABP	.199*	-.048	.890**	.046	-	.809**
	SBP	.188*	-.060	.452**	.077	.809**	-

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Further, correlation between BMI and haemoglobin was found to be negative ($r = -0.184$) with level of significance, $p < 0.01$ among males. No significant correlations are noted between haemoglobin level and both BMI and blood pressures (SBP, DBP and MBP) among the female section of adult Dhimal population (table 7). In case of males, DBP ($r = 0.244$) and MBP ($r = 0.220$) are found to have significant correlations ($p<0.001$) with BMI (table 6). But among females, blood pressures (SBP, DBP and MBP) have no significant correlations with BMI (table 7)

From the public health perspective, most importantly, immediate nutritional intervention programs need to be implemented among the Dhimals. Although priority must be given to tribal groups having the highest rates of undernutrition, all groups must be incorporated in these food supplementation programs. The recommendations should include not only adequate dietary intake but also various ways in which they can enhance their socio-economic status through improved education and employment opportunities. It is expected that better educational attainment will lead to more scope for employment and healthier dietary practices. It is here that the government should play a proactive role in reducing the rates of undernutrition among tribals. It has already been emphasized (Topal and Samal, 2001) that there exists variation in social and economic conditions among tribes of India. This variation must be taken into account before tribal-specific intervention programmes are initiated.

The economic and health burden of high frequencies of adult undernutrition have been well documented (Ferro-Luzzi et al., 1992; Campbell and Ulijaszek, 1994; James et al., 1994; Naidu and Rao, 1994; Khongsdier, 2005). Serious endeavours should be made to study the consequences of the functional impairments commonly associated with low BMI in these ethnic groups. It is also imperative to ascertain the relationship of the high rate of undernutrition with morbidity and mortality. Similar studies should also be undertaken with the other tribal populations in India, since they constitute a sizeable portion of India's entire population. In the case of Dhimals, similar studies should be undertaken in Nepal since the majority of the Dhimals reside in that country. Moreover, since undernutrition has several underlying causes (WHO, 1995; Lee and Nieman, 2003), future investigations should aim at identifying the likely cause(s) of high rates of undernutrition among the Indian tribal populations.

Lastly, since nutritional status is intricately linked with dietary habits as well as the ecology of the population, further research should be undertaken to investigate, in details, these factors. Each tribal population has its unique food habits (Mandal et al., 2002). Moreover, there are distinct inter-tribal differences in the environment in which they reside, i.e. ecology of the population (Mandal et al., 2002). The present report did not deal with these factors as they were beyond the scope of study. However, it is imperative that future studies on tribal populations include these parameters when investigating their nutritional status.

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Acknowledgement

The authors would like to thank Garjan Mullick and Prasanjit Mullick for all their help and cooperation. All subjects who participated in the study are also acknowledged.

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MAKING AQUATIC WEEDS USEFUL V: NUTRITIONAL EVALUATION OF *Nymphoides cristatum* (Roxb.) O. Kuntz LEAF MEAL AS A FEED INGREDIENT FOR *Cirrhinus mrigala* (Hamilton)

*Shampa Patra, Joydev Maity, Jogendra Mohan Debnath and Bidhan C. Patra**

ABSTRACT

An eight weeks laboratory feeding trial was conducted at the ambient water temperature of 28.0 - 30.0°C, pH 7.1 - 7.5, DO_2 8.9 - 9.5 mg lit^{-1} and alkalinity 105.7 - 110.0 mg lit^{-1} on fingerlings of *Cirrhinus mrigala* of mean length 95.0 ± 5.0 mm and weight 5.3 ± 0.6g maintained on isocaloric diets (Crude Protein Level ≅ 35.0%) with increasing substitution levels of 20.0% to 60.0% of aquatic weed, *Nymphoides cristatum*. Daily food consumption (g per 100g fish day^{-1}) was variable but the mean consumption did not vary significantly between plant-protein substituted diets (EN_{20}, EN_{30}, EN_{45}, En_{60}) and control (EN_{00}).

The best growth in terms of SGR%, weight gain% and % ADG, food conversion ratio (FCR), protein efficiency ratio (PER) and net protein utilization (NPU%) was observed with the control diet and up to the level of 30.0% leaf meal substitution. However, with an increase in *Nymphoides* leaf meal incorporation the growth performance and feed utilization efficiency were reduced. Carcass composition also indicated that protein deposition was higher with lower incorporation leaf meal.

Nutrient agar culture of intestinal mucosa revealed the activity of cellulolytic symbiotic micro flora, the count per cm. being higher in the intestinal bulb, followed by middle and posterior intestine and was positively correlated with the amount of plant fibre present in the diet.

Key words : *Nymphoides*, Nutritional evaluation, *Cirrhinus*

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INTRODUCTION

The menace of aquatic weeds is reaching alarming proportions in many parts of the World and is particularly severe in tropical countries where warm water and increasing number of dams foster aquatic plant growth. The problem is worsened by increasing enrichment of natural waters by fertilizer run off and by nutrients from human and agricultural wastes. Unfortunately, there is no simple way to reduce the infestations.

The increasing cost of fish feed and unavailability of fish meal prompted to initiate research on cheaper source of protein. Algal meals or single-cell proteins (Matty and Smith, 1978; Beck *et al.*, 1979; Appler, 1985), soy protein concentrate (Escaffre *et al.*, 1997), leaf protein concentrate (LPC) and aquatic weeds (NAS, 1976; Hepher *et al.*, 1978; Ogino *et al.*, 1978; Almazon *et al.*, 1986; Wee and Wang, 1987; De Silva and Gunasekera, 1989) have been tried as substitutes for fish meal. Observations revealed that the Indian major carps can utilize some of the aquatic weeds to a limited extent (Alikunhi, 1957; Mishra *et al.*, 1988; Patra and Ray, 1988; Ray and Das, 1992; Singh and Kumar, 1996; Pandey *et al.*, 1997; Medda *et al.*, 1993; Keshavanath and Matty, 1994; Das *et al.*, 1994; Patra *et al.*, 2000; Patra *et al.*, 2001; Patra *et al.*, 2001; Patra *et al.*, 2002.; Maity, and Patra, 2003).

The present study is one in a series aimed at developing a low-cost feed using readily available plant ingredients for *Cirrhinus mrigala* (Ham.). The influence of the aquatic weed, *Nymphoides cristatum* on growth performance, conversion efficiency and biochemical composition of fish flesh was evaluated.

MATERIAL AND METHODS

Diets

Ground fish meal and *Nymphoides* leaf meal were used as the major protein sources in diets supplemented with animal fat, mustard oil cake and rice bran. Five experimental diets designated as EN_{00}, EN_{20}, EN_{30}, EN_{45} and EN_{60} (subscripts represent the percentage of leaf meal incorporated in the diet) were prepared containing varying proportions of plant and animal proteins [Table 1]. The diets were isonitrogenous and isocaloric, except diet EN_{60} (24.70% crude protein) [Table 1B].

Experimental design

The feeding trial was conducted in specially designed fibre glass aquaria (Ray and Patra, 1987) of 100 l capacity in which a water flow of 1 l min^{-1} was maintained. Each experiment was duplicated. Water temperature, dissolved oxygen, alkalinity, total ammonia and pH were monitored at one week interval and varied from 28.0 - 30.0°C, 8.9 -9.5 mg. l^{-1}, 105.7 - 110.0 mg l^{-1}, and 7.1 - 7.5, respectively (APHA, 1976).

Cirrhinus mrigala fingerlings (average length 95 mm and weight 5.3 g) were acclimatized for 15 days in the laboratory feeding with the control diet (30.0% crude protein).

The fingerlings were randomly distributed between the aquaria at a stocking density of 20 fish per aquarium. Fish were fed twice daily to satiation (6.0% bw) between 9.00 & 10.00 hr. and 16.00 and 17.00 hr. The quantity of feed was adjusted every week on the basis of the average weight of fish. The experimental fish were weighed individually at the beginning and end of the feeding trial. However batch weighing was done at weekly intervals for growth rate calculation. The study was conducted for 60 days, after which the experimental fish were sacrificed, and the carcass taken for gross bio-chemical analyses. In the last week of the experiment, faeces were collected from the experimental aquaria. The faeces collected from replicate treatments were pooled, dried in an air-oven (at 60°C), and stored for subsequent proximate analyses.

Analytical Methods

Feed ingredients, experimental diets, and fish carcasses were analyzed for their proximate composition by the methods of AOAC (1984), in triplicate: moisture, determined by oven-drying at 85.0°C to constant weight; crude protein, determined indirectly from the analysis of total Kjeldahl nitrogen (crude protein = N × 6.25) by the micro-Kjeldahl method; crude lipid, determined by extraction with petroleum ether (60.0 - 62.0°C bp) for 6 hr. in a Soxhlet apparatus; ash, determined from weighed samples in a porcelain - silica crucible placed in a muffle furnace 500.0° ± 50°C for 4 hr. (minimum); fibre content determined using acid-based digestion. The apparent nutrient digestibility measurements, chromic oxide (Cr_2O_3) were estimated in the diets and faeces using the rapid method of Furukawa and Tsukahara (1966).

Culture of intestinal microflora

Nutrient agar culture medium (Beef extract – 1.5 g, peptone – 2.5 g, sodium chloride – 2.5 g, distilled water – 500 ml., agar-agar – 2 %, pH – 7.2) was used for isolation and culture of cellulolytic symbiotic microflora. The medium was sterilized at 15 lb pressure and 121°C temperature for 15 minutes. The inoculate was spread over the solidified nutrient agar medium kept on the sterilized petridish (plate culture). Incubation period was 24 hrs – 72 hrs and then the separate colony with different morphology was transferred into nutrient agar slant.

Statistical analyses

Statistical analyses of the results of the feeding trials were made by using ANOVA and Duncan's Multiple Range Test (Duncan, 1955) to evaluate the mean differences among individual diets at 0.05 significance level.

RESULTS

Proximate Composition of *Nymphoides cristatum*

N. cristatum is a small, water-lily like aquatic herb with floating leaves. Rhizome short, erect, with petiole like branches. Leaves 2.5 - 10 x 2 - 10 cm, nearly orbicular, deeply cordate, green and shining above, purplish beneath. Flowers white, sweet scented, in clusters. Calyx deeply divided. Corolla lobes subrotate with a tuft of white hairs around the yellow throat. Stamens 5. Style very short, Capsule subglobose. Common in tanks, ponds and other swampy areas. The composition of diets EN_{00}-EN_{60} and their proximate composition on dry matter basis are presented in Table 1.

Feed intake, weight gain and condition factor

The performance of fish fed on diets EN_{00}-EN_{60} are given in Table 2 and 3. The mean daily food consumption was variable in all groups of fish as seen in the consumption patterns in the three *Nymphoides* meal based diets and EN_{00}. The growth performance in terms of weight gain (%), ADG, SGR, length and weight are presented in Tables 2, 3, 5 and were significantly ($P<0.05$) higher with diet EN_{00} as compared to EN_{20}, EN_{30}, EN_{45} and EN_{60}. There was a trend of reduced growth performance with increase in the level of *Nechamandra* leaf meal on the basis of daily weight gain, percentage weight gain and specific growth rate. At the end of the 60 day experimental period, a high value of c.f.(W/L^3 X 100) was observed as compared to initial one, and the value increased with the decreased rate of growth of fish. (Table 3).

Digestibility of nutrients

The apparent digestibility values of crude protein, crude lipid and gross energy were significantly higher ($P<0.05$) for the diet EN_{00} as compared to EN_{20}, EN_{30}, EN_{45} and EN_{60} respectively (Table 2). An increase in the level of plant protein, regardless of treatment, resulted in a significant reduction in apparent nutrient digestibility.

Nitrogen and energy balance

The intake and absorption of feed N_2 (mg per 100 g fish d^{-1}) was significantly higher ($P<0.05$) in EN_{00}, EN_{20}, EN_{30} and EN_{45} respectively as compared to EN_{60} (60.0% *Nymphoides* leaf meal diet). Whereas, the absorbed feed energies (cal per 100 g fish d^{-1}) was significantly higher ($P<0.01$) with the diet EN_{20}, EN_{30} and EN_{00} in comparison with EN_{45} and EN_{60} (60.0% plant protein) (Table 2).

Percentage weight gain, FCR, PER and % NPU

The mean weight and percentage weight gain ($F_w - I_w / I_w \times 100$), at 15 days interval, of *Cirrhinus* fry maintained on different diets (EN_{00} - EN_{60}) is shown in Table 5 and Figure 1. The FCR (g dry weight of food consumed/g increase in biomass), the PER (g increase in

biomass/g dry weight of protein consumed) and the % NPU (net increase in carcass protein x 100)/protein consumed) ranged from 1.62 (EN_{00}) to 2.19 (EN_{45}) to 2.32 (EN_{60}) and 20.16 (EN_{45}) to 27.30 (EN_{60}), respectively (Table 4). The trends of change in PER and % NPU in relation to the substitution level are shown in Table 4, i.e. within each treatment the PER and NPU values were significantly reduced with an increase in the level of leaf meal incorporation for all treatments (except EN_{60}).

Carcass composition

The carcass composition of experimental fish at the beginning and end of the experiment is shown in Table 6. An increase in the *Nymphoides* leaf meal inclusion resulted in a decrease in carcass protein, dry matter, and energy contents and an increase (not significant) in carcass moisture, fat, and ash content in all treatments.

Micro flora population in the gut

Culture of intestinal mucosa revealed the presence of cellulolytic bacteria, the count per cm being higher in the intestinal bulb ($3.44 \times 10^4 - 4.76 \times 10^4$) followed by middle intestine ($2.18 \times 10^4 - 2.37 \times 10^4$) and posterior intestine $1.36 \times 10^3 - 1.81 \times 10^3$). Microscopical observation after Gram staining revealed the presence of gram positive and gram negative microflora like *Cellulobacta* sp., *Cellulomonas* sp., *Cytopaga* sp., *Cellvibrio* sp., *Pseudomonas* sp., *Cellfalcicula* sp., etc.

DISCUSSION

Proximate Composition of *Nymphoides cristatum*

The protein (15.17%) and caloric content (3.85 Kcal g^{-1}) of *N. cristatum* is comparable to other aquatic weeds (*Nechamandra* : 19.17, 3.87; *Hydrilla* :14.67, 3.96; *Spirodela* : 13.60, 3.17; *Ceratophylum* : 14.37, 3.71; *Cynodon* : 14.80, 3.94; etc.) as per the results found by Edwards *et al.*(1985), Hajra (1987), Hajra and Tripathi (1985), Patra and Ray (1988a), Venkatesh and Shetty (1978) and Wee and Wang (1987).

Feed intake, weight gain and condition factor

The average daily dry matter intake per 100 g of fish was significantly higher with EN_{00} (fish meal based diet) as compared with the diet EN_{20}, EN_{30}, EN_{45} and EN_{60} ($P<0.05 - 0.01$). Generally, a day or two of high consumption was followed by a low consumption. Consumption in g per g fish day^{-1} decreased with increasing body weight and as g/fish day^{-1} increased with increasing weight. Similar observations were recorded by De Silva and Gunasekera (1989) in *Oreochromis* fry and (Patra, 1993) in *Anabas* fry.

The growth of *Cirrhinus* fed *Nymphoides* leaf meal (60.0%) is comparable with *Labeo* fed pelleted *Hydrilla* (Patra and Ray, 1988). In general, there were no significant differences in growth responses for fish fed 30.0% or 45.0% plant protein, but there were

significantly better than those fed 60.0% plant protein diet, EN_{60} (*Nymphoides*). Since, the diets were isonitrogenous (except EN_{60}) on dry matter basis, the differences in growth performance were related to protein quality.

A high value of c.f.($W/L^3 \times 100$) was observed as compared to initial one, and the value increased with the decreased rate of growth of fish. Dey and Sharma, (1982), however, did not find any change in the c.f. values of *Tilapia* fed with rice bran-oil cake mixture and water hyacinth petiole.

Digestibility of nutrients

The increased crude protein digestibility is related to reduced fibre content and slightly higher protein content in the diets of the present study as has been reported by Wannigama *et al.*, (1985) in *Sarotherodon*, for diets devoid of rice bran. High values of lipid digestibility with all the diets may also be a result of high temperature (28 - 30°C) and quality of fat as has been reported by Andrews *et al.*, (1978) and differ significantly with the level of incorporation of *Nymphoides* leaf meal. An increase in the level of plant protein, resulted in a significant reduction in apparent nutrient digestibility.

Nitrogen and energy balance

The intake and absorption of feed nitrogen was significantly higher ($P<0.05$) except EN_{60} whereas, the absorbed feed energies was significantly higher ($P<0.01$) with the diet EN_{20}, EN_{30} and EN_{00} in comparison with EN_{45} and EN_{60} (60.0% plant protein). The net dietary nitrogen and energy intake increased with increasing body weight. However, these were dependent on the dietary protein level.

Percentage weight gain, FCR, PER and % NPU

The fry maintained on the formulated diet EN_{00} grew significantly ($P<0.05$) better. Among the four levels of plant meal incorporation the best performance was observed in fry maintained on diets with substitution up to 20.0% (EN_{20}) followed by 30.0% (EN_{30}), 45.0% (EN_{45}) and 60.0% (EN_{60}).

The best FCR of 1.62 was observed in fry maintained on the diet EN_{00}. The FCR of diets with up to 30.0% *Nymphoides* leaf meal substitution was not significantly different from the formulated diet (EN_{00}). However, at higher plant protein levels , the FCR was substantially poor. The PER and NPU values were significantly reduced with an increase in the level of leaf meal incorporation for all treatments (except EN_{60}). The result of the present study are in agreement with the observations of Beamis and Medland (1986) in respect of dietary protein level and FCR, and negative correlation of dietary protein levels and PER (Patra and Ray, 1988).

Carcass composition

At the low level of incorporation of 20.0% to 30.0% plant protein, the carcass composition did not differ significantly from diet EN_{00}. A positive correlation was observed between the dietary protein level and that of the carcass composition (not significant) as reported by Patra and Ray (1988) in *Anabas*. The present investigation did not show a reciprocal relationship between moisture and lipid content in the carcass, although reported by Alexis *et al.* (1986) and Patra (1989). Ash and lipid contents, showed a direct relationship with those of diets, which agreed with the observations of Viola and Zohar (1984). Wee and Wang (1987) reported that fish meal could be replaced up to 50.0% by *Leucaena* leaf meal (soaked) in practical diets for Nile Tilapia.

Microflora population in the gut of *Cirrhinus*

The presence of *Cellulobacta, Cellulomonas, Cytophaga, Cellvibrio, Pseudomonas* in the digestive tract of *Cirrhinus* proves their ability to digest cellulose, at least to a limited extent. The highest count per cm in the intestinal bulb followed by the middle and posterior intestine revealed that the rate of digestion of cellulose is much higher in the intestinal bulb as compared to middle and posterior regions of the intestine and absorption is mostly associated with the distal portion of the intestine of *C. mrigala*.

CONCLUSION

The present study indicates that *Nymphoides* leaf meal could be incorporated up to 30.0% into practical diets of *C. mrigala*, as a non-conventional source of protein thus reducing the cost of feed.

ACKNOWLEDGEMENTS

We are grateful to the University Grants Commission (UGC), New Delhi, India for financial support; Calcutta University, India for laboratory and field facilities and the Department of Botany, Visva-Bharati, India for the culture of intestinal micro flora and identification of the same.

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Table 1 : Composition (g 100g^{-1}) and proximate analysis (%, dry weight basis) of experimental diets (EN_{00}, EN_{20}, EN_{30}, EN_{45} and EN_{60}).

A. Composition of Experimental Diets :

Diet	EN_{00}	EN_{20}	EN_{30}	EN_{45}	EN_{60}
Nymphoides Meal	0.0	20.0	30.0	45.0	60.0
Fish Meal	50.0	30.0	30.0	30.0	30.0
Mustard Oil Cake	20.0	15.0	10.0	5.0	0.0
Rice Bran	23.0	28.0	23.0	13.0	3.0
Cod Liver Oil	1.0	1.2	2.0	2.5	2.0
Corn Oil	2.0	1.8	1.0	0.5	1.0
Binder[1]	1.5	1.5	1.5	1.5	1.5
Premix[2]	2.0	2.0	2.0	2.0	2.0
Cromic Oxide	0.5	0.5	0.5	0.5	0.5

[1] Wheat Flour.

[2] Vitamin and mineral mixture (Vitaminate Forte; Roche India Ltd.), Each 0.8g contains : Vit. A I.P. 2500 I.U.; Vit. B_1 I.P. 2.0 mg.; Vit. B_2 I.P. 3.0 mg.; Nicotinamide I.P. 25.0 mg.; Vit. B_6 I.P. 1.5 mg., Calcium Pantothenate U.S.P. 5.0 mg.; Vit. B_{12} I.P. 1.0 mcg.; Vit. C I.P. 50.0 mg.; Vit. D_3 U.S.P. 200 I.U.; Vit. E.N.F. 100 mg.; Vit. H 0.05 mg.; Calcium Phosphate I.P. 208.0 mg.; Dried Ferrous sulphate I.P. 10.6 mg.; Magnesium Phosphate 48.0 mg.; Manganese hypophosphite 0.6 mg.; total phosphorus in the preparation 44.6 mg.

B. Proximate Analysis of experimental diets :

Components	EN_{00}	EN_{20}	EN_{30}	EN_{45}	EN_{60}
Moisture	03.36	03.66	04.00	04.97	04.92
Ash	11.08	13.47	14.68	16.62	21.01
Crude protein	35.55	34.37	34.07	33.21	24.70
Crude lipid	11.23	10.95	10.33	10.29	09.98
Crude fibre	08.31	07.50	08.36	10.12	14.47
Nitrogen-free extract	36.51	33.32	30.74	27.05	26.59
Gross energy (cal)	375.6	400.2	416.2	399.5	375.3

Each value is a mean of 10 separate determinations

Table - 2 : Performance of the fish fed experimental diets for 60 days.

	Parameters	EN_{00}	EN_{20}	EN_{30}	EN_{45}	EN_{60}
A.	**FEED INTAKE AND WEIGHT GAIN**					
	Number of test animal	20	20	20	20	20
	Initial body weight (g)	5.12	5.24	5.15	5.21	5.22
	Live weight gain (g)	46.81	38.55	32.74	25.78	23.24
	Average weight gain (g/d)	0.48	0.40	0.33	0.27	0.24
	Percentage weight gain (%)	916.4	740.4	636.2	498.4	445.3
	Specific growth rate (%)	3.87	3.53	3.28	2.98	2.83
	Daily dry matter intake (mg/100g fish)	5978	5812	5729	5416	5410
	Digestible protein intake (mg/100g fish/d)	1826	1706	1665	1527	1065
B.	**APPARENT DIGESTIBILITY CO-EFFICIENT (%)**					
	Crude protein	89.19	78.26	67.11	62.75	58.97
	Crude lipid	90.78	87.23	84.19	80.77	71.12
	Gross energy	78.82	73.17	70.23	67.05	65.67
C.	**NITROGEN BALANCE (mg/100 g fish/d)**					
	Nitrogen intake	292.1	272.9	266.4	244.3	170.4
	Nitrogen absorbed	252.3	231.7	224.9	203.8	137.5
D.	**ENERGY BALANCE (Cal /100g fish/d)**					
	Gross energy intake	2245	2325	2384	2032	2161
	Energy absorbed	1939	1963	1941	1695	1743

Table - 3 : Average length, weight, condition factor and daily growth rate of *C. mrigala* fed on experimental diets for 60 days[1].

Diets	Initial			Final			
	Length (mm)	Weight (g)	c.f.	Length (mm)	Weight (g)	c.f. (g/day)	A.D.G.
EN_{00}	95.0±1.0	5.12±0.5	0.597	169.0±4.0	33.72±0.3	0.698	0.447[a]
EN_{20}	97.0±1.0	5.24±0.4	0.574	150.0±3.0	29.23±0.6	0.866	0.399[b]
EN_{30}	94.0±3.0	5.15±0.7	0.620	142.0±5.0	25.19±0.7	0.879	0.334[b]
EN_{45}	96.0±1.0	5.21±0.6	0.589	133.0±3.0	21.21±0.2	0.901	0.266[c]
EN_{60}	96.0±1.0	5.22±0.6	0.590	127.0±2.0	17.56±0.5	0.857	0.239[c]

1 Figures in the same rows having the same superscript are not significantly different ($P<0.05$).

Table - 4 : Percentage weight gain(%), FCR, PER and NPU (%) of *C. mrigala* fry maintained on different diets for 60 days.[1]

Diet	% weight gain	F C R	P E R	% N P U
EN_{00}	916.4[a]	1.62[a]	2.19[a]	24.57[b]
EN_{20}	737.6[b]	1.69[a]	2.00[a]	21.16[a]
EN_{30}	631.7[c]	1.86[a]	1.85[ab]	21.62[a]
EN_{45}	498.4[d]	2.05[b]	1.73[b]	20.16[a]
EN_{60}	444.1[d]	2.19[b]	2.32[a]	27.30[b]

[1] Figures in the same rows having the same superscripts are not significantly different ($P<0.05$).

Table 5 : The effects of different levels of *Nymphoides* meal on growth rate (wet weight gain in g) of *C. mrigala*[1]

Diets	Days on trial				
	0	15	30	45	60
	(mean±S.E.)			(mean±S.E.)	
EN_{00}	5.12[a]±0.5	14.39	20.72	37.78	51.93±0.3[b]
		(181.0)	(404.8)	(665.0)	(916.6)
EN_{20}	5.24[a]±0.4	13.18	22.17	32.27	43.79±0.6[d]
		(151.5)	(323.1)	(518.8)	(740.4)
EN_{30}	5.15[a]±0.7	12.78	19.79	29.36	37.89±0.7[d]
		(148.2)	(284.4)	(470.1)	(636.2)
EN_{45}	5.21[a]±0.6	12.23	17.30	21.29	30.99±0.2[c]
		(134.8)	(231.7)	(371.0)	(498.4)
EN_{60}	5.22[a]±0.6	11.73	15.99	23.32	28.46±0.5[c]
		(124.8)	(208.6)	(346.8)	(445.3)

[1] Figures in the same rows having the same superscript are not significantly different ($P<0.05$).

Figures in the parenthesis represent the % weight gain.

Table 6 : Gross carcass composition of experimental fish at the beginning and end of the experiment (values are expressed as %, wet weight basis)[1]

Components	Initial	Final				
		EN_{00}	EN_{20}	EN_{30}	EN_{45}	EN_{60}
Moisture	89.63[a]	78.27[b]	79.19[b]	80.01[b]	81.92[b]	82.46[b]
Dry matter	10.37[a]	21.73[bc]	20.81[bc]	19.99[bc]	18.08[b]	17.54[b]
Crude protein	4.07[a]	10.97[b]	10.56[b]	10.09[b]	9.73[b]	9.59[b]
Crude lipid	2.17[a]	3.92[b]	3.97[b]	4.05[b]	4.12[b]	4.16[b]
Ash	2.12[a]	2.30[a]	2.39[a]	2.47[a]	2.76[a]	2.82[a]
Gross energy (K cal/g)	0.51[a]	1.17[a]	1.13[a]	1.09[a]	0.99[a]	0.92[a]

[1]Figures in the same rows having the same superscript are not significantly different ($P<0.05$).

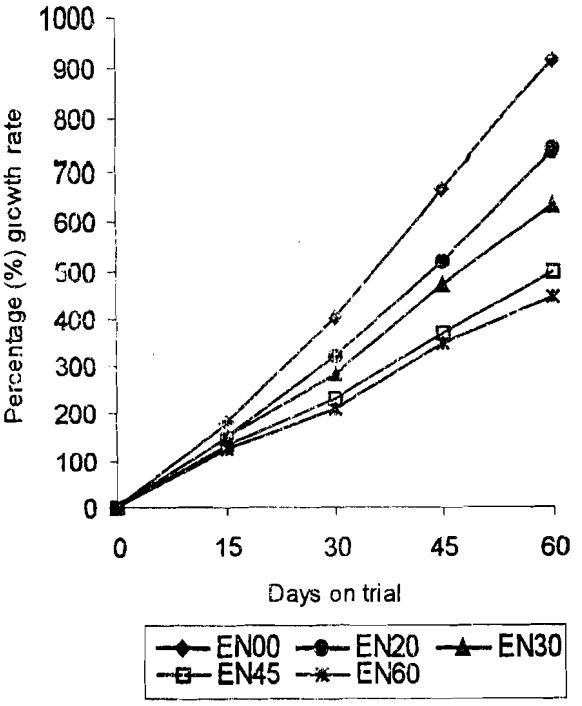

Fig 1. The effect of different levels of *Nymphoides* meal on % weight gain of *Cirrhinus mrigala*

ALLELOPATHIC EFFECT OF *MELALEUCA LEUCADENDRON* L. ON MUNG BEAN SEEDS

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Abstract

Melaleuca leucadendron L. plant was analysed to evaluate the existence of allelopathic effect using fully viable seeds of mung bean as the bioassay material. Different concentrations (1:2 and 1:4) of leaf extracts and leachates of *Melaleuca* reduced the percentage of seed germination as well as increased the T_{50} hours for all the different seed pretreatment hours. TTC stainability was significantly reduced in leaf extract and leaf leachate pretreated seed sample. Soluble carbohydrate level was rapidly increased in the leachates of seeds pretreated with leaf extracts and leachates of the *Melaleuca* plant. Protein levels as well as activities of dehydrogenase and catalase enzymes were significantly reduced in seed samples pretreated with leaf extracts and leachates. Inhibitory action was more prominent in leaf extracts than the leaf leachates.

Key words : allelopathy, enzymes, macromolecules, *Melaleuca leucadendron*, mung bean.

Introduction

Allelopathy refers to biochemical interaction among plants. It is the effect of one plant upon another occurring under natural conditions and exerted by chemical means other than nutritional ones. Allelopathy is different from competition and implies that the effect depends on a chemical constituent escaping into the environment. Since 1960, there has been a spurt in publications dealing with this phenomenon (1 – 7). It is an expression of the ecological phenomena which are normal constituents of the environment of the terrestrial plants (8, 9).

There are some common indices for assessing allelopathic action of plants or plant parts. These include, among others, seed germination behaviour (percentage and T_{50} of seed germination), field emergence capacity of seeds, seedling growth (root length, shoot length, leaf area) and metabolism. Moreover, the allelopathic potential of a plant particularly of exotic one, may turn it aggressive and invasive which in course of time can discourage and displace other co-existing biodiversity thriving the same habitat (10). With this background, the present investigation is an attempt to evaluate the allelopathic potential of *Melaleuca leucadendron,* an exotic tree of India growing abundantly in the coastal belt of Digha in

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West Bengal. For precise screening of allelopathic action of this plant some select physiological and biochemical parameters were analysed using mung bean seed as bioassay material.

Materials and methods

Fresh, mature and healthy 150 g leaves of *Melaleuca leucadendron* L. (Family – Myrtaceae), collected from the coastal belt of Digha, Purba Medinipur, were thoroughly homogenized using 150 ml distilled water. The homogenates were strained using a fine cloth and then centrifuged at 5000 *g* for 15 minutes. The supernatant was then made up to 300 ml using distilled water and this was considered as leaf extract of 1:2 (w/v) concentration and from this solution 1:4 concentration grade was made by using distilled water. Another lot of 150 g of leaf sample of this plant species was sun-dried and then immersed in 200 ml distilled water in 500 ml beaker for 72 hours and the leachate was then decanted in a separate beaker (500 ml). The total volume of the leachate was then made up to 300 ml using distilled water and this was taken as the leaf leachate of 1:2 (w/v) concentration and from this solution 1:4 concentration grade was made by using the distilled water. These two concentration grades each of extract and leachate were used for allelopathic analysis.

Fully viable 200 g mung bean (*Vigna radiata* L.) seeds were surface sterilized with 0.1% $HgCl_2$ solution for 90 seconds. The seeds were equally divided into five lots, and then separately presoaked in the leaf extracts and leachates in different concentrations (1:2 and 1:4) or distilled water for 3, 6, 9 and 12 hours. Thus the presoaked seed lots were thoroughly sun dried till the original moisture level was achieved. Thereafter, the seeds were kept in room temperature (30 ± 2°C) and thus allowed the seeds for experiments. Data on seed germination behaviour (percentage and T_{50} of seed germination), TTC-stainability, leaching of soluble carbohydrate and protein levels as well as catalase and dehydrogenase activities of seeds were analysed after 3, 6, 9 and 12 hours of seed pretreatment.

To analyse percentage germination, three groups of 100 seeds i.e. 300 seeds of each treatment were transferred to separate Petridishes containing filter paper moistened with 10 ml distilled water. Germination data were recorded up to 120 h of seed soaking following the International Rules for Seed Testing (11). The time for 50% germination (T_{50}) was determined following the method described by Coolbear *et al.* (12).

For analysing TTC stainability three 100-seed samples of dehusked mung bean seeds of ach treatment hours (3, 6, 9 and 12 hours) were allowed to imbibe in 0.5% TTC (2, 3, 5-triphenyl tetrazolium chloride) solution (w/v) in Petridishes for 16 hours in dark condition. Percentage TTC stainability were recorded taking samples from the embryonal axes of the mung bean seeds.

Soluble carbohydrate level was analysed from the extract and leachate -soaked seeds obtained after immersing 1 g seeds in 10 ml deionized distilled water for 16 hours as per the method of McCready *et al.* (13). Protein level was analysed from the seed kernels following the method of Lowry *et al.* (14).

For analysing dehydrogenase activity the TTC (2, 3, 5-triphenyl tetrazolium chloride)-stained (formazan formed) embryonal axes of the seeds of each treatment was extracted with 5 ml 2-methoxyethanol, and O.D. values of the solutions were recorded at 520 nm. This method was adopted after Rudrapal and Basu (15) with slight modifications.

Extraction and estimation of the enzyme catalase was done as per the method described by Snell and Snell (16) modified by Biswas and Choudhuri (17). For the assay of this enzyme the blank was taken as zero time control. The activity of this enzyme was expressed as $[(\Delta A \times Tv)/(t \times v)]$, where ΔA is the absorbance of the sample after incubation minus the absorbance of the zero time control, Tv is the total volume of the filtrate, t is the time (minutes) of incubation with the substrate and v is the volume of the filtrate taken for incubation (18).

All the data were statistically analysed at the treatment and replication levels; the least significant difference (LSD) was calculated at 95% confidence limits (19).

Results

Effect on percentage germination (Table 1). Data revealed that percentage seed germination gradually declined with the duration of seed pretreatment periods of each treatments. Percentage seed germination was inhibited by the leaf extracts and leachates of *Melaleuca* plant in mung bean seeds. This inhibitory effect was found to be drastic in seeds which experienced pretreatments for 12 hours and leaf extracts exerted slightly stronger inhibition than leaf leachates.

Effect on T_{50} values of germination (Table 2). Time required for 50% seed germination was found to be significantly high in the leaf extract and leaf leachate-pretreated samples. However, in all the cases 50% germination was achieved when data were recorded upto 12 hours of seed pretreatment.

Effect on TTC stainability (Table 3). TTC stainability was not affected by the leaf extracts and leaf leachates of *Melaleuca* except 12 hours pretreated seeds.

Effect on leaching of soluble carbohydrates (Table 4). Various hours of seed pretreatment enhanced leaching of soluble carbohydrate in mung bean seeds and the extent of leaching was found to be strictly pretreatment period dependent. Leaf extract and leachate pretreatment significantly induced rapid increase of soluble carbohydrate levels.

Effect on protein levels in seed kernels (Table 5). Seed pretreatments reduced protein levels in mung bean seed and the magnitude of reduction was found to be significantly high in seed samples pretreated with leaf extracts than leaf leachates of *Melaleuca* plant. Again, the inhibitory effects of the leaf extracts pretreatment was found to be high in seed lots which received 12 hours of pretreated seed.

Effect on dehydrogenase activity in seed kernels (Figure 1). Seed pretreatment period induced loss of dehydrogenase activity was gradually increased by seed pretreatment with leaf extracts and leaf leachates in the seed sample. Here also, the inhibitory action was best exerted by leaf extract pretreatment and the magnitude of inhibition was more significant in 12 hours pretreated seed sample.

Effect on catalase enzyme activity in seed kernels (Figure 2). As regards the changes of catalase enzyme under different pretreatment periods, activity of the enzyme declined progressively with the advancement of pretreatment period duration. The pretreating leaf extracts and leachates significantly increased the pretreatment periods induced loss of catalase activity.

Discussion

The present study shows that different hours of seed pretreatment of mung bean with different concentrations (1:2 and 1:4) of leaf extracts and leachates of *Melaleuca* reduced percentage germination (Table 1), increase T_{50} hours (Table 2) and reduced TTC stainability of seeds (Table 3), increased leaching of soluble carbohydrate (Table 4), decreased protein levels (Table 5) as well as dehydrogenase (Figure 1) and catalase (Figure 2) activities.

Reduced seed germinability is the important effect of allelopathic action of plants and such action is chiefly exerted by a number of inhibitors of diverse chemical nature (4, 20). In this investigation different concentrations (1:2 and 1:4) of leaf extracts and leachates-induced inhibition of percentage and T_{50} hours of seed germination is clear indicative of the allelopathic action of the test material. Relatively high allelopathic potential of leaf extract and leachate was recorded from its stronger germination inhibition capacity, significant increase T_{50} hours as well as significant reduction of TTC stainability of the seeds. Allelopathic action of *Melaleuca* plant can also be substantiated from the profuse leakage of soluble carbohydrate which is indirect indication of the damage of seed membrane. Membrane is the most important site of a seed which appears to be affected first by treatment with plant extracts having strong allelopathic action (5, 6). The results of the study is thus in conformity with some reported observations (4, 6, 7, 20 – 22).

Allelopathic potential of *Melaleuca* plant can further be corroborated from the present data on the leaf extracts and leachates-induced reduction of protein level as well as activities of dehydrogenase and catalase enzymes. Various inhibitors present in plants having allelopathic property reduce the overall metabolism of plants or plant parts, and particularly anabolic activities are reported to be strongly impaired (4 – 6). Results, therefore, point out that leaf extracts and leaf leachates of *Melaleuca* plant possess some chemicals (essential oil, cajeputol which is identical with eucalyptol) which efficiently render allelopathic action on the bioassay material (mung bean) of this study.

Thus, a conclusion can be made from this investigation using a number of physiological and biochemical indices that different concentrations (1:2 and 1:4) of leaf extract and leaf leachate of *Melaleuca* exert strong allelopathic effect on the test material. Longer periods of seed pretreatment (12 hours) experiment strengthen this effect. From the overall observations, leaf extract seems to be more effective than that of leachate with respect to exhibiting allelopathic action on the bioassay material of this experiment.

Table 1. Effect of seed pretreatment of different hours (3, 6, 9 and 12 hours) with leaf extracts and leachates of *Melaleuca* on percentage germination of mung bean seeds.

Treatments	*Germination (%)*			
	Seed pretreatment hours after intervals			
	3 hours	**6 hours**	**9 hours**	**12 hours**
Control	100	100	98.5	95.4
Leaf extract (1:2)	95.1	87.7	79.7	75.6
Leaf extract (1:4)	97.3	91.3	84.1	80.2
Leaf leachate (1:2)	96.4	90.5	81.0	78.3
Leaf leachate (1:4)	98.2	92.0	85.2	81.5
LSD (*P*=0.05)	NS	4.02	5.92	6.01

NS : Not significant.

Table 2. Effect of seed pretreatment of different hours (3, 6, 9 and 12 hours) with leaf extracts and leachates of *Melaleuca* on time (h) to 50% germination (T_{50}) of mung bean seeds.

Treatments	*T_{50} hours*			
	Seed pretreatment hours after intervals			
	3 hours	**6 hours**	**9 hours**	**12 hours**
Control	12.2	14.3	16.2	18.5
Leaf extract (1:2)	14.5	16.3	18.4	21.5
Leaf extract (1:4)	14.1	15.9	18.1	21.1
Leaf leachate (1:2)	13.7	16.0	18.3	20.9
Leaf leachate (1:4)	13.5	15.8	17.9	20.6
LSD (*P*=0.05)	1.02	1.25	1.31	1.65

Table 3. Effect of seed pretreatment of different hours (3, 6, 9 and 12 hours) with leaf extracts and leachates of *Melaleuca* on percentage TTC-stained of mung bean seeds.

Treatments	*TTC-stainability (%)*			
	Seed pretreatment hours after intervals			
	3 hours	**6 hours**	**9 hours**	**12 hours**
Control	100	100	100	100
Leaf extract (1:2)	98.3	96.8	95.1	92.0
Leaf extract (1:4)	99.0	97.0	95.3	93.0
Leaf leachate (1:2)	99.2	97.1	95.2	92.9
Leaf leachate (1:4)	99.6	97.3	95.4	94.2
LSD (*P*=0.05)	NS	NS	NS	0.85

NS: Not significant.

Table 4. Effect of seed pretreatment of different hours (3, 6, 9 and 12 hours) with leaf extracts and leachates of *Melaleuca* on leaching of soluble carbohydrate from mung bean seeds.

Treatments	*Soluble carbohydrate (mg/g/10 ml)*			
	Seed pretreatment hours after intervals			
	3 hours	**6 hours**	**9 hours**	**12 hours**
Control	2.10	3.01	4.06	4.77
Leaf extract (1:2)	2.42	3.81	4.89	6.07
Leaf extract (1:4)	2.40	3.44	4.53	5.95
Leaf leachate (1:2)	2.38	3.40	4.50	5.63
Leaf leachate (1:4)	2.36	3.28	4.45	5.20
LSD (*P*=0.05)	NS	0.26	0.37	0.42

NS: Not significant.

Table 5. Effect of seed pretreatment of different hours (3, 6, 9 and 12 hours) with leaf extracts and leachates of *Melaleuca* on protein level in kernels of mung bean seeds.

Treatments	*Protein (mg/g/wet weight)*			
	Seed pretreatment hours after intervals			
	3 hours	**6 hours**	**9 hours**	**12 hours**
Control	352.1	345.3	336.1	331.5
Leaf extract (1:2)	342.3	310.6	270.1	258.9
Leaf extract (1:4)	344.5	326.3	278.3	261.3
Leaf leachate (1:2)	346.0	342.7	288.1	266.1
Leaf leachate (1:4)	347.5	338.0	291.5	273.4
LSD (*P*=0.05)	NS	14.3	13.2	12.5

NS: Not significant.

Figure 1. Effect of seed pretreatment of different hours (3, 6, 9 and 12 hours) with leaf extracts and leachates of *Melaleuca* on the activity of total dehydrogenase enzyme in kernels of mung bean seeds.
Each bar is mean value of 3 replicates and the vertical lines on the bar represent the standard errors of the mean.

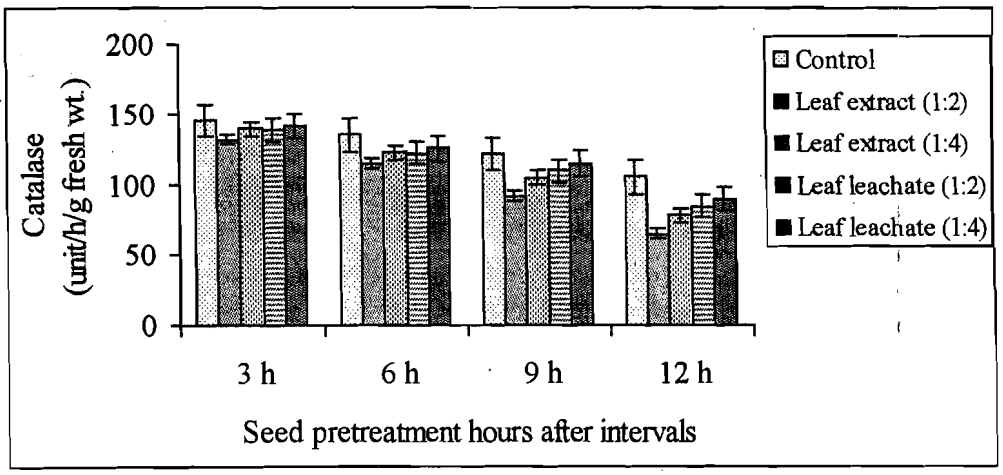
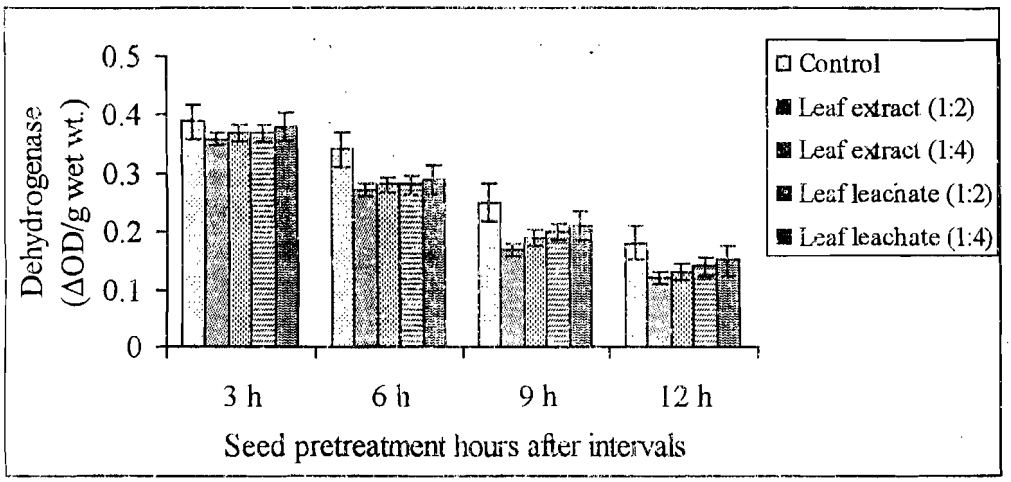


Figure 2. Effect of seed pretreatment of different hours (3, 6, 9 and 12 hours) with leaf extracts and leachates of *Melaleuca* on the activity of catalase enzyme in the kernels of mung bean seeds.

Each bar is mean value of 3 replicates and the vertical lines on the bar represent the standard errors of the mean.



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MYCORRHIZAL STATUS OF HIGHLY STRESSED AND LESS STRESSED SITES AT PURULIA

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Arbuscular mycorrhizal fungi (AMF) are widely distributed and are found in association with the root of more than 80% land plants (Smith and Smith, 1997). It shows higher level of root colonization in plants growing in soil of low fertility, especially deficient in P (Mosse, 1973). Low moisture levels reduce the diffusion rate of nutients to plants and decrease the availability of nutients. AM fungi help plants to resist water stress by absorbing water from lower water potential (Auge et al, 1994, Anderson et al, 1984) and retaining moisture in mycelial network (Alkarki, 1998) It helps plants to survive better in water stressed rain fed lateritic soil condition (Setua et al, 1999).

Purulia district of West Bengal is known as dry land having nutrient poor red lateritic acid soil. The following work was undertaken to get the preliminary information of vegetation and their AM fungal status on two sites; one under severe biotic pressure and other comparatively less stressed. Two samplings, one before rain and another after rains were taken for study.

The study was conducted at Neturia village in Purulia district (Latitude from 88° 0′ to 86°45′ East Longitude from 22°3′ to 22°45′ North). Quadrates of 50cm × 50cm were laid and total numbers of species were counted. Composite soil samples from both the sites before and after the rains were collected for analysis. VAM spores were extracted following Gerdemann and Nicolson (1963) and roots were examined for VAM infection (Phillips and Hayman, 1970).

At high stressed site, soil pH, Organic Carbon, Phosphorus and Potassium was much lower than less stressed site. (Table –1). These soil characteristics showed an increase after rains as nutrients, released fast due to increased decomposition of organic matter. The number of plant species and total number of plants were much higher in less-stressed site compared to highly stressed site. The species composition also differed. Mycorrhizal status was overall better in highly stressed site compared to less stressed site. (Table – 2). VAM spore population of soil was found nearly 3 times higher at highly stressed site before rain and four times after rain than less stressed site. The reduction in spore count observed after the rains was possibly due to the germination of the spores to infect fresh root. Partial

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decomposition and partial parasitic consumption of spores may also be a factor of reduction in spore number after rains. Level of infection percentage was much higher in high stressed site compared to less stressed site. After rain infection percentage was decreased approximately 3.8% to 20% in highly stressed and 10% to 43% in less stressed sites. The nutrient richness in less stressed site possibly caused poor mycorrhizal colonization, as excessive nutrients supply, particularly P tend to decrease mycorrhizal activity (Hayman 1970, Daft and Nicolson 1969, Khan, 1972, Readhead, 1975). The release of more nutrients during rains probably decreased the mycorrhizal colonization as was apparent at both the sites. The poor spore population on less stressed site also reflected the same. The high spore population and colonization percentage at highly stressed condition also supported the view that AM fungal symbiosis become more effective in dry and nutrient poor soils (Setua et al, 1999, Powel and Daniel, 1978). In less stressed soil, plants could absorb nutrients relatively easily and so it showed less dependence on mycorrhizae.

In dry, nutrient poor soil AM fungi provide effective mechanism to avoid water stress, absorbing water from lower water potential zone that roots can not absorb alone (Anderson et al 1984) and mycelial mats retain water in rhizosphere (Hamp et al 2000, Sen, 2000). Better mycorrhization in highly stressed site indicated high dependency of the species on mycorrhizal association. In natural ecosystem close positive correlation between plant cover and spore number was observed by Anderson et al (1984). In our study we found that stress has a positive correlation with VAM spore population.

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Table - 1. Changes in chemical characteristics of the soil at two sites before and after rains

Less stressed site				
Period pH	**% org. carbon**	**P_2O_5 (Kg/hq.)**	**K_2O (Kg/hq.)**	**Soil moisture %**
Before rain 6.2	0.75	37.6	114	3.09
After rain 6.5	0.76	39.6	204	8.69
High stressed site				
Before rain 5.2	0.46	30.5	80	2.56
After rain 5.8	0.54	37.8	84	8.45

Table - 2. Number of plants, mycorrhizal colonization and AMF spore under less and high stressed site before and after rains

Plant sp.	Before rain			After rain		
	Total no. of plants	Mycorr-hizal infection %	VAM spore popula-tion per 100g of soil	Total no. of plants	Mycorr-hizal infection %	VAM spore population per 100g of soil total
Less stressed site						
Aerva lanata	20	10		22	8	
Hemingraphis hirta	15	35		16	20	
Scoparia dulcis	15	40	146	20	32	75
Ageratum conyzoides	7	42		20	35	
Tridax procambens	2	20		5	18	
Oldenlandia corymbosa	6	25		9	20	
High stressed site						
Evolvulus sp.	4	52		6	48	
Spilanthes sp.	4	70		5	56	
Sida cordifolia	2	60	428	4	51	289
Eupatorium odoratum	1	76		2	70	
Parthenium sp.	2	78		4	67	

EFFECT OF INORGANIC CONSTITUENTS ON GROWTH AND TANNASE PRODUCTION BY *Bacillus Licheniformis* KBR6

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Summary

The influence of inorganic constituents on growth and tannase production by a non-pathogenic gram positive bacteria, *Bacillus licheniformis* KBR6 has been studied. Among the various inorganic constituents maximum amount of enzyme production (0.25 U/ml and 0.27 U/ml) was achieved in the fermentation medium supplemented with NH_4Cl and phosphate source like KH_2PO_4 respectively. Enzyme production was also enhanced in presence of divalent cations like Ca^{++} and Mg^{++}. In all the cases enzyme production is directly correlated with growth of the organism.

KEY WORDS : *Bacillus licheniformis, inorganic salts, tannase.*

Introduction

Tannase (Tannin acyl hydrolase; E.C.3.1.1.20) an industrially important enzyme hydrolyzes the ester linkages of tannic acid into glucose and gallic acid. It has wide applications in food, beverage, brewing, cosmetic and chemical industries (Lekha and Lonsane, 1997). Gallic acid, instant tea, acron wine, coffee flavored soft drinks, and high grade leather tannin is mainly prepared by using this enzyme. It is also used in clarification of beer, fruit juice and detannification of food as well as to clean-up highly polluting tannin from the effluent of the leather industry (Chae and Yu, 1983; Coggon and Sanderson, 1972). The hydrolytic product gallic acid (3, 4, 5 tri-hydroxy benzoic acid) has also tremendous industrial applications. Generally tannase producing microorganisms are isolated and cultivated in tannin rich medium where tannic acid act as inducer. In the present investigation optimum growth and tannase production by *Bacillus licheniformis* KBR6 was studied in presence of different inorganic constituents in the fermented media.

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Materials and Methods

Bacillus licheniformis KBR6 (IMI 379224), a non pathogenic tannase producing bacteria was used in this study and the stock culture was maintained in tannic acid agar slant at 4^0C. Enzyme production was carried out in the selective medium containing (g/l): tannic acid, 10; K_2HPO_4, 0.5; KH_2PO_4, 0.5; $MgSO_4$, 0.5; NH_4Cl, 3. The pH of sterilized media was adjusted to 5.0 using 0.5M NaOH. Cultivation of organism was done out in 250ml flask containing 50ml sterilized broth for 20h on rotary shaker (200rpm) at 35^0C. After 20h of growth the culture broth was centrifuged (5000 × g for 15min) and the supernatant was examined for tannase. Growth of the organism was determined at 620nm by turbidimetric method and correlated to cell dry weight.

The activity of extracellular tannase from *B. licheniformis* KBR6 was determined by the newly developed colorimetric method of Mondal *et al.* (2001). For an assay, 0.1ml of sample was mixed with 0.3ml of tannic acid substrate solution (1.0% w/v in 0.2M citrate buffer, pH-5.0), and incubated at 50^0C for 30min. The reaction was terminated by the addition of BSA solution (1mg/ml), which precipitates residual tannic acid. A control reaction with heat denatured enzyme was performed concomitantly. The tubes were then centrifuged (5000 × g, 10min.) and precipitate was dissolved in 2ml of SDS triethanolamine (1% w/v, SDS in 5% v/v, triethanolamine) solution. The absorbance was measured at 530nm after addition of 1ml of 0.13M aqueous solution of $FeCl_3$. One unit of tannase activity was defined as the amount of enzyme that is able to hydrolyze 1μ mol of ester linkage of tannic acid in 1min at specific conditions.

All the experiments were performed in triplicates and the results given here are the mean of the three.

Results and Discussion

Tannase is an inducible enzyme in microorganisms and tannic acid acts as a catabolic inducer as well as carbon source. The tannic acid containing enriched media generally devoid of organic constituents e.g. beef extract and peptone, as because tannic acid form complexes and become precipitated with these organic macromolecules. For this reason a semisynthetic media generally preferred where inorganic composition provide a vital role for growth and tannase production.

Effect of nitrogen source :

The presence of nitrogen is an essential prerequisite for any metabolic reaction. Enzyme production in presence of various inorganic nitrogen salts (0.03% w/v) was observed as well as represented in Table - 1. The order of tannase production in relation to inorganic salts can be arranged like: $NH_4Cl > NH_4HO_3 > (NH_4)_2SO_4 > NaNO_3$. Maximum tannase production

in NH_4Cl may be due to the presence of readily available nitrogen. Though sodium nitrate (0.25%) and ammonium nitrate (0.2%) have been found suitable for enzyme synthesis in *R.oryzae* (Hadi *et al.* 1994) and *A. japonicus* (Bradoo *et al.* 1997) respectively.

Effect of phosphate source :

Phosphates are very important bacterial nutrient for tannase synthesis. Effect of three different phosphate sources (0.05% w/v) viz. KH_2PO_4, K_2HPO_4 and $(NH_4)_2HPO_4$ on tannase production were examined (Table – 1). Among them both KH_2PO_4 and K_2HPO_4 were found to be effective in stimulating higher tannase yield by *B. licheniformis* KBR6. In comparison to control, 2.25 times more enzyme synthesis was observed in presence of KH_2PO_4. Optimization of phosphate concentration for tannase synthesis was not reported earlier, although most of the workers used different phosphate compound in the basal medium (Lekha and Lonsane, 1997). Phosphates are generally required in the microbial cell as energy substances, but Thirunavukkarasu and Priest (1980) mentioned their other role in extracellular enzyme synthesis. They assumed that phosphates may increase messenger RNA stability by inhibiting RNase activity and it may affect the cytoplasmic membrane in such a manner that membrane bound ribosomes are become better adapted for exoprotein translation.

Growth kinetics of the organism in relation to enzyme production was studied and better result was achieved in presence of KH_2PO_4.

Effect of metal ion :

Different cations have been added in tannic acid media to study their effects on growth and enzyme formation (Table – 1). It has been observed that presence of Ca^{++} and Mg^{++} ions in tannic acid media increased maximum tannase production in comparison to control. The stimulatory effect of these metal ions on growth and tannase production can be arranged in the following order: $CaCl_2 > MgCl_2 > PbCl_2 > BaCl_2$ & NaCl. Whereas other metal ions like Mn^{++}, Cu^{++} and Ag^{++} are inhibitory to bacterial growth and enzyme production. Both micro and macro elements act as elementary composition of cell, whereas particular ion has stimulatory effect to metabolic synthesis in specific group of microorganism (Schlegel, 1995). Tannase production by *A. niger* required traces of Fe^{3+}, Zn^{++} and Cu^{++} (Lippitsch, 1961).

Conclusion

The addition of inorganic constituents in the fermented medium increases the extracellular tannase synthesis by the organism. These results are promising because the strain produces tannase in inexpensive inorganic nutrients.

Acknowledgement

Authors are thankful to University Grant Commission, New Delhi for financial assistance and also wish to acknowledge Dr. S. Giri for his help.

Table – 1 : Effect of inorganic constituent on growth and tannase production.

Inorganic Sources		Concentration (g% w/v)	Growth (mg/ml)	Tannase (U/ml)
Nitrogen				
	NH_4Cl		0.95	0.25
	$(NH_4)_2SO_4$		0.67	0.18
	NH_4NO_3	0.3	0.92	0.22
	$NaNO_3$		0.45	0.13
	Control		0.28	0.14
Phosphate				
	KH_2PO_4		0.98	0.27
	K_2HPO_4	0.05	0.88	0.25
	$(NH_4)_2HPO_4$		0.67	0.18
	Control		0.30	0.12
Metal ions				
	$CaCl_2$		0.45	0.25
	$MgCl_2$		0.34	0.23
	$MnCl_2$		ND	ND
	$CuCl_2$		ND	ND
	$PbCl_2$	0.1	0.28	0.17
	$BaCl_2$		0.27	0.16
	$AgCl_2$		ND	ND
	NaCl		0.26	0.16
	Control		0.20	0.16

ND = Non detectable

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EFFECT OF FEEDING SOYBEAN MEAL ON THE GROWTH, CONVERSION EFFICIENCY AND CARCASS COMPOSITION OF INDIAN MAJOR CARP, *LABEO ROHITA* HAM.

Joydev Maity[1] and Shampa Patra

ABSTRACT

Effect of feeding soybean (*Glycine max*) meal was examined on the growth, conversion efficiency and carcass composition of *Labeo rohita* fingerling (average length 10.98 cm and weight 14.6±0.61 g) reared at the ambient water temperature 28 ± 1°C in an indoor flow through glass aquaria system. Fish were fed twice daily at 08 : 00 and 16 : 00 hours of the day and @ 6% body weight for 6 days in a week. Casein-Gelatin based iso-nitrogenous (38% crude protein) and iso-caloric (4.32 K Cal g^{-1} gross energy) semi purified test diets containing varying levels of soybean meal were (9.94g, 16.54g, 21.39g, 34.57g) formulated, of which S_1, S_2, S_3, S_4 contains raw soybean meal at different level and S_5, S_6, S_7, S_8 contains same composition but soybean meal was heat treated at 90°C for 10 minutes and SC as control.

The growth performance of *Labeo rohita* in terms of final mean weight, weight gain, weight gain %, specific growth rate significantly decreased with increasing level of raw soybean meal in the diets. The FCR value increased significantly ($p < 0.05$) for all the diets as compared to control whereas PER value showed reverse trend in all the treatments.

Acetylcholinesterase (AChE) activity significantly ($p < 0.05$) increased upto 50% inclusion of soybean meal in S_1 and S_2 although S_5 and S_6 showed opposite trend, however in the 75% inclusion level the AChE activity increased to a significant level till 45 days in S_3 and 30 days in S_7. But at 100% inclusion level the highest activity was recorded till 30 days in S_4 and 15 days in S_8. RNA/DNA level and protease activity in hepatopancreas, protein and lipid content in muscle showed similar trend as in AChE.

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It could be opined that, the limiting factor i.e. trypsin inhibitor present in the raw soybean reduces the growth rate. The results suggested that properly heat treated soybean meal could be an important alternative dietary protein source for IMC.

Key words **Soybean, *Labeo rohita*, Anti-nutritional factor, Trypsin inhibitor, Feed Conversion Ratio, Protein Efficiency Ratio**

INTRODUCTION

Fish meal is the major protein source in feeds for intensive fish farming. In the last few years, a considerable amount of research on partial or complete substitution of dietary fish meal using other plant protein sources has been conducted (Lequet and Kaushik, 1978; Pfeffer, 1982; Akiyama *et al.*, 1995; Boonyaratpalin *et al.*, 1998; Carter, 2000; Chen *et al.*, 1994; Davis *et al.*, 1995; Fontainhas-Fernendes, *et al.*, 1999; Gallagher, 1994; Lee and Jones, 1996; Twibell and Brown, 2000; Belal and Assem, 1995; Hoffman *et al.*, 1997; Webster *et al.*, 1995; Patra, 2000; Patra *et al.*, 2002a). Due to lower price and higher market availability of vegetable protein sources with high protein content, the inclusion of these feed stuffs in freshwater fish feed has increased substantially (Robinson and Li, 1994; Krogdahl and Holm, 1983; Krogdahl *et al.*, 1994).

The quality of a partially dietary protein source depends both on its digestibility and amino acid profile (Kaushik and Cowey, 1991). Apart from amino acid composition, which often unbalanced (Liener and Kakade, 1980; NRC, 1981; Kaushik and Lequet, 1984; Tacon and Cowey, 1985), endogenous antinutritional factors are the main factors limiting the use of high level of vegetable feed stuffs in fish feeds (Richardson *et al.*, 1985; Gatlin and Phillips, 1989; Satoh *et al.*, 1989; Olli and Krogdahl, 1995; Lopez *et al.*, 1999; Maity *et al.*, 2000; Maity and Patra, 2003; White *et al.*, 2000).

Among the plant proteins soybean meal is the most promising candidate for replacing partly or all of the fish meal proteins in fish diets. Soybean meal as a protein supplement has been used successfully in the diet of trout (Tacon *et al.*, 1983; Dabrowski *et al.*, 1989; Selden *et al.*, 2001; Rumsey *et al.*, 1994; Refstie *et al.*, 2000; Refstie *et al.*, 1997; Olli and Krogdahl, 1994; Oliva-Teles, *et al.*, 1994; Bureau *et al.*, 1998; Devies and Morris, 1997; Mitrenko, 1997), catfish (Wilson and Poe, 1985; Sadiku and Jauncey, 1998b; Falaye and Ahwieh, 1998; Jones *et al.*, 1996b), tilapia (Davis and Stickney, 1978; El-Sayed *et al.*, 2000; Shiayehu *et al.*, 1996; Wee and Shu, 1989; Wu *et al.*, 2000) and carp (Viola *et al.*, 1983; Viola *et al.*, 1984; Murai, *et al.*, 1986; Applerford and Anderson, 1997; Devi *et al.*, 1999; Hossain *et al.*, 1997; Jafri and Anwar, 1995; Nandeesha *et al.*, 1989).

Soybean meal can partially or totally replace animal protein sources in the diets of most cultivable teleost (Tacon, 1993). Recently, Koushik *et al.* (1995) were able to achieve total substitution of fish meal by a soy protein concentrate in the diet for rainbow trout. Results of studies conducted to evaluate the potential of different soybean products, however, show much variability. Almost all the earliest studies have been conducted with different

fishes other than IMC. Very little is known about the mechanisms of response in fish to dietary protease inhibitor in terms of pancreatic hypertrophy and intestinal proteases.

The main aims of this work was to study the substitution of fish meal by soybean meal with protein unit basis, and their effect on the growth performance, feed utilization, digestive enzymes of Indian major carp, *Labeo rohita.*

MATERIAL AND METHODS

Sample Collection and Processing

Soybean seed was collected from the market of Midnapore, West Bengal, India. The oven dried soybean was milled and packed in polythene bag and kept in the freezer at –2°C prior to use. The proximate composition of the soybean powder was estimated following AOAC (1990) standard analysis procedure.

Experimental diets

Five isonitrogenous (38% crude protein) diets (SC and S_1 - S_4) were formulated and containing increasing levels of dried raw soybean meals as replacement for fish meal at 0% (control), 25%, 50%, 75% and 100% (Table 1) and another four isonitrogenous (CP = 38%) diets (S_5 – S_8) were formulated with heat treated soybean meal (90°C for 10 minutes) using same ingredients for experimental trial (Table 2). All dietary ingredients were hand mixed and produced pellets in pelletilizer to form noodle like strands, which were broken into pellets of suitable size (2mm diameter) for diets of *Labeo rohita* fingerlings. The dry pieces of pelleted feed were then stored in a freezer at –2°C in sealed plastic bags until fed.

Analysis of dietary ingredients and prepared diets

The proximate analysis of dietary ingredients and diets were performed according to the procedures of the AOAC (1990). Diet performance was evaluated on experimental fish according to Olvera-Novoa *et al.* (1990).

$$\text{Weight gain (\%)} = 100 \left[\frac{\text{Final body weight - Initial body weight}}{\text{Initial body weight}} \right]$$

Specific Growth Rate (SGR% day^{-1})

$$= 100 \left[\frac{(\log_e \text{ final body weight} - \log_e \text{ initial body weight}}{\text{time, day}} \right]$$

$$\text{Feed Conversion Ratio (FCR)} = \left[\frac{\text{Dry weight of feed fed (g)}}{\text{Fish weight gain (g)}}\right]$$

$$\text{Protein Efficiency Ratio (PER)} = \left[\frac{\text{Fish weight gain (g)}}{\text{Protein fed (g, dry weight basis)}}\right]$$

Experimental Design

The feeding trial was conducted in specially designed glass aquaria of 130 liter capacity. *Labeo rohita* fingerlings (mean weight = 14.60 ± 0.61g) were obtained from rearing pond of Aquaculture Research Unit, Vidyasagar University Campus and acclimatize for two weeks in the laboratory condition with standard diet (38.0% crude protein) containing the mixture of fish meal, mustard oil cake, rice bran. The fingerlings were randomly distributed among the aquaria at a stocking density of 15 fish per aquaria. Fish were fed twice daily to satiation (6% body weight) at 8.00 and 16.00 hour daily, feeding allowance was adjusted each week on the basis of the average weight of the fish. The study was conducted for 60 days. Every 15 day interval fish were sacrificed for the estimation of enzyme, protein, lipid, DNA and RNA content.

Estimation of protein and lipid

The protein concentration of the enzyme extract and muscle was determined by the methods of Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard. Lipid of fish muscle was estimated following the methods of Folch *et al.* (1957) using chloroform-methanol mixture.

Estimation of Protease activity

Protease activity was estimated following the method of Snell and Snell (1971), which is the modified method of Bernfeld (1955).

Estimation of DNA and RNA

The DNA and RNA content of fish muscle were estimated according to the method of Munro and Fleck (1969) with some modifications. The calculation was done by the method of Strove and Makaravo (1989).

Protease inhibitor activity assay

Trypsin inhibitor activity was measured by the methods of Kakade *et al.* (1974) using BAPNA (Benzoyl-DL-Arginine-Paranitroanilide) as substrate and calculated the trypsin inhibitor activity according to the methods of Hamerstrands *et al.*, 1981.

Water Quality Analysis

The water quality was monitored periodically (every alternative day) following the methods of APHA (1989) till the end of the experiment and average them at every 10 days interval.

Statistical analysis

All calculations and statistical analysis were done on IBM P-III using statistical packages SPSS, STATISTICA, ASP. All data are expressed as Mean ± SEM (Standard error of mean) Regression analysis also done in the Excel package.

RESULTS

The test diets were found to be almost isonitrogenous and isocaloric by appropriate adjustment of fish meal and soybean meal nitrogen content (Table 1). Control diets (SC) contains fish meal without any replacement by soybean meal but diets S_1, S_2, S_3, S_4 fish meal replaced with 25%, 50%, 75% and 100% respectively by soybean meal. Trypsin inhibitor content in the diets gradually increased in different diets ($S_1 - S_4$) due to higher inclusion of soybean meal. In the diet S_4 i.e. 100% replacement of fish meal by soybean meal contains high level of trypsin inhibitor (72.10 mg g^{-1} feed).

Table 2 shows the same composition of different diets ($S_5 - S_8$) as shown in Table 1, but soybean meal was heated for 10 minutes at 90°C. There were no such significant difference ($p< 0.05$) of protein content were noted in the unheated ($S_1 - S_4$) and heat treated ($S_5 - S_8$) soybean diets. Trypsin inhibitor content decreased significantly in the diets ($S_5 - S_8$) as compared to unheated soybean meal containing diets.

During 60 days experimental period the water quality specially the pH, temperature, DO_2 and alkalinity etc. was estimated and presented in the Table 3. pH, temperature, DO_2 and alkalinity ranges from 6.8 – 7.2, 29-32°C, 6.040 – 9.042 ppm and 100.732 – 104.123 ppm respectively.

Cumulative growth performance of *Labeo rohita* fed the diets with 0 (SC), 25 (S_1), 50 (S_2), 75 (S_3) and 100% (S_4) of fish meal protein replaced by soybean protein are shown in Table 4a and Figure 1a during 60 days feeding trial. % weight gain, SGR, PER, feed intake value gradually decreased in the diets (($S_1 - S_4$) as compared to control. In control diet (SC) the % weight gain is 289.73, where as in the diet S_4 it is 135.81. FCR value gradually increased as compared to control fed the diets ($S_1 - S_4$). Mortality gradually increased at compared to control fed the diets ($S_1 - S_4$).

The growth performance of *Labeo rohita* was also reduced with the heat treated soybean diets ($S_5 - S_8$) during 60 days feeding trial and presented in the Table 4b and Figure 1b. Weight gain (%) gradually deceased fed the diets $S_5 - S_8$ (271.84, 270.05, 259.50 and 238.83) respectively as compared to control (289.73). SGR, PER, Feed intake value not significantly decreased when fish fed the diets ($S_5 - S_8$) as compared to control. Results shows that heated soybean meal in the different diets shows better growth performance as compared to unheated soybean meal during 60 days feeding trial.

During 60 days feeding trial, it was observed that the AChE activity increased with the increasing level of soybean meal in the diets and presented in Table 5a. In control diet AChE activity does not show similar trend.

The AChE activity gradually increased during of 15th, 30th, 45th and 60th days when fish fed with the S_1 and S_2 diets. AChE activity in S_3 diets increased up to 45th days (0 days

= 16.060 and 45 days = 18.019 μ mole $100g^{-1}$ tissue 30 $minutes^{-1}$) and then decreased during 60^{th} days (17.420 μ mole $100g^{-1}$ tissue 30 $minutes^{-1}$). AChE activity gradually increased up to 15 days fed the diet S_4 and then decreasing trend was notable during 30^{th}, 45^{th} and 60^{th} days.

AChE activity (μ mole $100g^{-1}$ tissue 30 $minutes^{-1}$) in the brain tissue of *C. mrigala* was also recorded during 60 days feeding trial fed the heat treated soybean containing diets ($S_5 - S_8$) are shown in the Table 5b. It was recorded that the AChE activity shows significant changes fed the diets ($S_1 - S_4$) and no such significant changes was recorded fed the heat treated diets as compared to control. Figure 2 shows the comparative activity of AChE of control (SC), S_4 and S_8 fed diets i.e. highest inclusion of soybean meal.

The protease activity in the hepatopancreas of *Labeo rohita* fed with the unheated diets ($S_1 - S_4$) and heat treated diets ($S_5 - S_8$) is presented in Table 6a and Table 6b respectively. Protease activity (μg glycine liberated $hour^{-1}$ mg^{-1} of protein) was increased gradually as compared to control up to 30 days fed the diets S_3 (initial = 97.04 and 104.02 at 30 days) and S_4 (initial = 98.02 and at 30^{th} day 106.39) but then decreasing trend was observed. When compared the unheated ($S_1 - S_4$) and heat treated ($S_5 - S_8$) soybean meal containing diets, the heat treated soybean diets ($S_5 - S_8$) does not shows significant changes in protease activity as compared to control. The comparative protease activity of hepatopancreas of *Labeo rohita* shown in the Figure 3 fed the diets SC, S_4, and S_8.

Muscle protein content in *Labeo rohita* was also recorded fed the diets $S_1 - S_4$ and $S_5 - S_8$ during 60 days feeding trial and presented in the Table 7a and Table 7b respectively. The protein content in the muscle gradually decreased fed the experimental diets ($S_1 - S_4$) as compared to control. There were significant increase in muscle protein content fed the heat treated soybean meal ($S_5 - S_8$) as compared to unheated soybean meal ($S_1 - S_4$) during 60 days feeding trial. The comparative protein content of the fish muscle shown in Figure 4 fed the diet SC, S_4, and S_8.

Muscle lipid content of *Labeo rohita* was estimated on 15^{th}, 30^{th}, 45^{th} and 60^{th} day of the feeding trial and heat treated diets ($S_1 - S_4$) and presented in Table 8a and Table 8b. Lipid content in muscle decreased with the higher inclusion of soybean meal in the diets. But heat treated soybean containing diets ($S_5 - S_8$) shows better lipid profile in muscle as compared to unheated soybean diets. Comparative lipid content in the muscle of *Labeo rohita* fed the diet SC, S_4, and S_8 during 60 days feeding trial shown in Figure 5.

Liver RNA/DNA ratio of *Labeo rohita* was estimated in every 15 days interval fed the unheated soybean containing diets ($S_1 - S_4$) and heat treated diets ($S_5 - S_8$) and resented in the Table 9a and Table 9b respectively. The RNA/DNA ratio was higher with the diet S_1 (3.59) and lower in the S_4 (1.58) during 60^{th} day. But heat treated soybean diets containing

shows better RNA/DNA ratio fed the diets S_8 (2.29) as the same replacement of unheated soybean containing diets S_4.

The result of regression analysis of protease enzyme and AChE activity was shown in the Table 10.

Table 1. Ingredients and proximate composition of experimental diets (SC and $S_1 - S_4$).

Ingredients	Diets (% fish meal replacement)				
	SC (0%, control)	S_1 (25%)	S_2 (50%)	S_3 (75%)	S_4 (100%)
Fish meal	34.80	24.36	18.57	12.17	-
Soybean flour	-	9.43	16.54	21.39	34.57
α-cellulose	4.30	8.35	9.35	14.63	18.73
Wheat flour	39.40	36.36	34.04	30.31	25.22
Mustard oil cake	10.00	10.00	10.00	10.00	10.00
Rice bran	5.00	5.00	5.00	5.00	5.00
Cod liver oil	2.50	2.50	2.50	2.50	2.50
Vitamin + Mineral*	1.00	1.00	1.00	1.00	1.00
Binder (gelatin)	3.00	3.00	3.00	3.00	3.00
Proximate composition (% Dry matter basis)					
Moisture (%)	8.00	8.90	8.20	8.85	8.65
Crude protein (%)	39.4	38.5	38.3	37.5	37.1
Crude lipid (%)	7.9	6.5	7.2	6.4	6.8
mg TI g^{-1} feed	0	18.69	36.37	56.39	72.10

* Vitamin and mineral mixture (Brand name : Ginsoma)

Each 0.33g contains Ginsing, 42.50mh, Thiamine and Methionine, 1.5mg, Vit. B_2 I.P., 2.5mg, vit. B_6 I. P., 1.0mg, vit. B_{12} I.P., 1.0mcg, Niacinamide I.P., 25mg, Calcium panthenate I. P., 5.0mg, Folic acid I. P., 0.3mg, vit. C I.P., 50.0mg, Zinc sulphate monohydrate, USP, 41.2mg, Calcium, 75.0mg, Phosphorus, 58.0mg, Iodine, 100mcg, Ferrous fumurate, 30mg.

Length of the pelette : 0.280±0.012 cm
Weight of each palette = 0.005g

Table 2. Ingredients and proximate composition of experimental diets containing heat treated soybean flour (SC and $S_5 - S_8$).

Ingredients	Diets (% fish meal replacement)				
	SC (0%, control)	S_5 (25%)	S_6 (50%)	S_7 (75%)	S_8 (100%)
Fish meal	34.80	24.36	18.57	12.17	-
Heat treated soybean flour	-	9.43	16.54	21.39	34.57
α-cellulose	4.30	8.35	9.35	14.63	18.73
Wheat flour	39.40	36.36	34.04	30.31	25.22
Mustard oil cake	10.00	10.00	10.00	10.00	10.00
Rice bran	5.00	5.00	5.00	5.00	5.00
Cod liver oil	2.50	2.50	2.50	2.50	2.50
Vitamin + Mineral *	1.00	1.00	1.00	1.00	1.00
Binder (gelatin)	3.00	3.00	3.00	3.00	3.00
Proximate composition (% Dry matter basis)					
Moisture	8.02	7.68	7.94	7.90	7.96
Crude protein	39.42	38.98	37.52	38.41	37.44
Crude lipid	7.8	6.5	6.8	6.4	6.2
mg TI g^{-1} feed	0	2.24	6.36	12.74	20.43
% of destruction of TI due to heat treatment	100	93	85	76	68

*Vitamin and mineral mixture (Brand name : Ginsoma)
Each 0.33g contains Ginsing, 42.50mh, Thiamine and Methionine, 1.5mg, Vit. B_2 I.P., 2.5mg, vit. B_6 I. P., 1.0mg, vit. B_{12} I.P., 1.0mcg, Niacinamide I.P., 25mg, Calcium panthenate I. P., 5.0mg, Folic acid I. P., 0.3mg, vit. C I.P., 50.0mg, Zinc sulphate monohydrate, USP, 41.2mg, Calcium, 75.0mg, Phosphorus, 58.0mg, Iodine, 100mcg, Ferrous fumurate, 30mg.

Length of the pelette : 0.280±0.012 cm
Weight of each palette = 0.005g

Table 3. Study of water quality parameters on the Aquaria during 60 days experimental trial fed the diets control (SC) and $S_1 - S_8$.

Experimental days		Parameters			
		pH	Temp. (°C)	DO_2 (ppm)	Alkalinity (ppm)
1 – 10 day	max.	7.1	31.0	9.042	102.957
	min.	7.0	29.0	7.426	101.021
11 – 20 day	max.	7.2	32.0	9.011	103.131
	min.	6.9	29.5	7.510	100.122
21 - 30 day	max.	7.3	30.5	8.994	103.176
	min.	6.9	29.5	6.749	100.974
31 - 40 day	max.	7.1	31.5	9.120	104.605
	min.	6.9	29.0	6.448	100.774
41 - 50 day	max.	7.0	32.0	9.092	104.123
	min.	6.9	29.0	6.461	100.972
51 - 60 day	max.	7.3	31.5	8.841	104.654
	min.	6.8	29.0	6.040	100.732

Table 4a. Cumulative growth performance and feed utilization of fish fed the experimental diets (control and S_1 – S_4) during 60 days trial.

Parameters	Experimental Diets				
	SC	S_1	S_2	S_3	S_4
Number of test animals	15	15	15	15	15
Average initial weight (g)	14.6 ± 0.51	15.0 ± 0.64	14.7 ± 0.42	14.4 ± 0.38	14.8 ± 0.61
Average final weight (g)	56.9 ± 1.10^a	48.7 ± 1.32^b	43.2 ± 0.98^c	35.4 ± 1.07^d	34.9 ± 0.98^d
Weight gain (%)	289.73	224.67	193.88	145.83	135.81
SGR (% day^{-1})	2.3	2.1	1.9	1.6	1.5
Feed intake (g day^{-1})	0.87	0.81	0.69	0.35	0.17
FCR	1.3	1.5	1.6	2.0	2.1
PER	2.0	1.7	1.6	1.3	1.2
Survival (%)	99	92	85	72	68

Results are means of three separate determinations (Mean ± SEM), Values with the same superscript in the same row are not significantly different ($p < 0.05$) from each other

Table 4b. Growth performance of *Labeo rohita* fed with heated Soybean meal diets (S_5 – S_8) during 60 days experimental period.

Parameters	Experimental Diets				
	SC	S_5	S_6	S_7	S_8
Number of test animals	15	15	15	15	15
Initial body weight (g)	14.60 ± 0.51	14.42 ± 0.34	14.66 ± 0.52	14.05 ± 0.42	14.21 ± 0.42
Final body weight (g)	56.90 ± 1.0^a	53.62 ± 0.8^b	54.25 ± 0.3^b	50.51 ± 0.3^c	48.29 ± 0.5^d
Weight gain (%)	289.73	271.84	270.05	259.50	238.83
SGR (% day^{-1})	2.30	2.23	2.14	2.13	2.03
FCR	1.30	1.60	2.05	2.20	2.60
PER	2.6	2.0	1.9	1.9	1.7
Survival (%)	99	97	94	86	82

Results are means of three separate determinations (Mean ± SEM), Values with the same superscript in the same row are not significantly different ($p < 0.05$) from each other.

Table 5a. Acetylcholinesterase activity (m mole $100g^{-1}$ tissue 30 $minutes^{-1}$) in the brain tissue of *Labeo rohita* fed the control (SC) and experimental diets (S_1, S_2, S_3, S_4) during 60 days feeding trial.

Experimental diets	Feeding days				
	0	15	30	45	60
SC	16.048 ± 0.20[a]	16.082 ± 0.74	15.983 ± 0.97	16.110 ± 0.62	16.012 ± 0.64[a]
S_1	16.068 ± 0.41[a]	16.230 ± 0.31	16.321 ± 0.40	17.645 ± 0.46	18.231 ±0.32[b]
S_2	16.520 ± 0.31[a]	16.478 ± 0.41	17.925 ± 0.30	18.052 ± 0.62	18.285 ±0.52[b]
S_3	16.060 ± 0.32[a]	17.521 ± 0.56	17.992 ± 0.52	18.019 ± 0.40	17.420 ± 0.62[c]
S_4	16.072 ± 0.34[a]	19.921 ± 0.76	18.756 ± 0.52	17.310 ± 0.59	16.973 ± 0.52[a]

Results are means of three separate determinations (Mean ± SEM), Values with the same superscript in the same column are not significantly different ($p < 0.05$) from each other.

Table 5b. Acetylcholinesterase activity (m mole $100g^{-1}$ tissue 30 $minutes^{-1}$) in the brain tissue of *Labeo rohita* fed the control (SC) and experimental diets (S_5, S_6, S_7, S_8) during 60 days feeding trial.

Experimental diets	Feeding days				
	0	15	30	45	60
SC	16.048 ± 0.20[a]	16.082 ± 0.74	15.983 ± 0.97	16.110 ± 0.62	16.012 ± 0.64[a]
S_5	16.120 ± 0.26[a]	16.076 ± 0.41	16.117 ± 0.15	16.201 ± 0.40	16.142 ±0.30[a]
S_6	16.063 ± 0.40[a]	16.980 ± 0.47	16.432 ± 0.17	16.242 ± 0.31	16.232 ± 0.33[a]
S_7	16.211 ± 0.23[a]	17.911 ± 0.62	17.015 ± 0.26	16.925 ± 0.59	16.098 ±0.29[a]
S_8	16.018 ± 0.17[a]	18.972 ± 0.34	17.078 ± 0.43	17.026 ± 0.32	17.006 ± 0.48[b]

Results are means of three separate determinations (Mean ± SEM), Values with the same superscript in the same column are not significantly different ($p < 0.05$) from each other.

Table 6a. Protease activity (mg glycine liberated hour^{-1} mg^{-1} of protein) in the hepatopancras of *Labeo rohita* fingerlings fed with different levels of Soybean meal containing different level of TI during 60 days feeding trial.

Experimental diets	Feeding days				
	0	15	30	45	60
SC	97.84 ± 0.32^a	98.30 ± 0.72	98.09 ± 0.21	98.11 ± 0.42	98.03 ± 0.76^a
S_1	98.40 ± 0.42^a	98.70 ± 0.19	99.47 ± 0.72	98.70 ± 0.15	98.43 ± 0.44^a
S_2	98.76 ±0.15^a	99.17 ± 0.60	100.02 ± 0.37	99.00 ± 0.42	98.75 ± 0.37^a
S_3	97.42 ±0.37^a	103.07 ± 0.72	104.02 ± 0.39	99.02 ±0.32	98.92 ± 0.41^a
S_4	98.02 ± 0.32^a	105.42 ±0.52	106.39 ± 0.62	102.65 ± 0.31	99.74 ± 0.30^b

Results are means of three separate determinations (Mean ± SEM), Values with the same superscript in the same column are not significantly different ($p < 0.05$) from each other.

Table 6b. Protease activity (mg glycine liberated hour^{-1} mg^{-1} of protein) in the hepatopancras of *Labeo rohita* fingerlings fed the control (AC) and heat treated soybean diets S_5, S_6, S_7, S_8 during 60 days feeding trial.

Experimental diets	Feeding days				
	0	15	30	45	60
SC	97.84 ± 0.32^a	98.30 ± 0.72	98.09 ± 0.21	98.11 ± 0.42	98.03 ± 0.76^a
S_5	97.99 ± 0.36^a	98.19 ± 0.72	98.42 ± 0.49	97.02 ± 0.14	98.42 ± 0.30^a
S_6	98.04 ± 0.41^a	99.31 ± 0.40	98.89 ±0.43	98.01 ± 0.42	98.15 ± 0.17^a
S_7	98.11 ±0.30^a	100.32 ±0.60	99.01 ± 0.60	98.24 ± 0.37	98.72 ± 0.61^a
S_8	98.21 ± 0.62^a	101.42 ±0.64	99.97 ± 0.74	98.72 ± 0.62	98.64 ± 0.62^a

Results are means of three separate determinations (Mean ± SEM), Values with the same superscript in the same column are not significantly different ($p < 0.05$) from each other.

Table 7a. Muscle protein (g $100g^{-1}$) of *Labeo rohita* fed different diets (control and $S_1 - S_4$) during 60 days feeding trial.

Experimental diets	Feeding days				
	0	15	30	45	60
SC	10.54 ± 0.97	10.76 ± 0.53	11.82 ± 0.77	12.34 ± 0.37	12.98 ± 0.62[a]
S_1	10.67 ± 0.73	10.74 ± 0.37	9.98 ± 0.63	9.32 ± 0.42	9.21 ± 0.32[b]
S_2	10.32 ± 0.42	10.64 ± 0.31	10.48 ± 0.21	9.01 ± 0.27	8.74 ± 0.45[c]
S_3	10.68 ± 0.90	10.32 ± 0.47	9.32 ± 0.24	8.79 ± 0.42	8.32 ±0.32[c]
S_4	10.10 ±0.48	10.01 ± 0.51	9.11 ± 0.31	8.68 ± 0.34	8.10 ± 0.13[d]

Results are means of three separate determinations (Mean ± SEM), Values with the same superscript in the same column are not significantly different ($p < 0.05$) from each other.

Table 7b. Muscle protein (g $100g^{-1}$) of *Labeo rohita* fed the diets (control and $S_5 - S_8$) during 60 days feeding trial.

Experimental diets	Feeding days				
	0	15	30	45	60
SC	10.54 ± 0.97	10.76 ± 0.53	11.82 ± 0.77	12.34 ± 0.37	12.98 ± 0.62[a]
S_5	10.62 ± 0.47	10.78 ± 0.51	9.99 ± 0.47	9.87 ± 0.37	9.59 ± 0.37[b]
S_6	10.72 ± 0.31	10.60 ± 0.54	10.54 ± 0.42	10.01 ± 0.34	9.68 ± 0.42[b]
S_7	10.71 ± 0.52	10.69 ± 0.32	10.32 ± 0.46	9.97 ± 0.51	9.42 ±0.30[b]
S_8	10.79 ± 0.41	10.29 ± 0.41	9.76 ± 0.37	9.82 ± 0.25	9.40 ± 0.39[b]

Results are means of three separate determinations (Mean ± SEM), Values with the same superscript in the same column are not significantly different ($p < 0.05$) from each other.

Table 8a. Muscle lipid (mg g^{-1}) content of *Labeo rohita* fed the diets control and $S_1 - S_4$ during 60 days feeding trial.

Experimental diets	Feeding days				
	0	15	30	45	60
SC	71.32 ± 0.21	76.02 ± 0.30	79.82 ± 0.27	82.35 ± 0.29	86.92 ± 0.37[a]
S_1	70.98 ± 0.38	75.29 ± 0.17	76.22 ±0.27	76.99 ±0.32	78.92 ± 0.20[b]
S_2	71.04 ± 0.31	70.46 ± 0.16	69.54 ±0.24	67.32 ± 0.35	68.07 ± 0.17[c]
S_3	71.22 ± 0.62	68.05 ± 0.19	64.30 ± 0.19	63.02 ± 0.38	62.04 ± 0.31[d]
S_4	70.79 ±0.68	64.52 ±0.34	61.02 ± 0.32	59.99 ± 0.42	58.72 ± 0.24[e]

Results are means of three separate determinations (Mean ± SEM), Values with the same superscript in the same column are not significantly different ($p < 0.05$) from each other.

Table 8b. Muscle lipid (mg g^{-1})content of *Labeo rohita* fed the diets control and S_5 – S_8 containing heat treated soybean meal during 60 days feeding trial.

Experimental diets	Feeding days				
	0	15	30	45	60
SC	71.32 ± 0.21	76.02 ± 0.30	79.82 ± 0.27	82.35 ± 0.29	86.92 ± 0.37[a]
S_5	71.02 ± 0.09	75.42 ± 0.32	76.91 ± 0.42	78.99 ± 0.44	79.04 ± 0.17[b]
S_6	71.48 ± 0.42	73.01 ± 0.41	71.46 ± 0.22	70.46 ±0.46	69.84 ±0.41[c]
S_7	70.92 ± 0.38	70.37 ±0.31	68.91 ± 0.47	67.82 ± 0.32	66.32 ±0.58[d]
S_8	70.72 ±0.49	67.02 ±0.29	64.38 ± 0.27	62.02 ± 0.31	64.39 ± 0.60[d]

Results are means of three separate determinations (Mean ± SEM), Values with the same superscript in the same column are not significantly different ($p < 0.05$) from each other.

Table 9a. RNA/DNA ratio in the liver tissue of *Labeo rohita* fingerlings fed with soybean meal containing different level of T1 during the experimental period (SC and S_1 – S_4).

Experimental diets	Feeding days				
	0	15	30	45	60
SC	2.76 ± 0.03	2.31 ± 0.03	2.97 ± 0.07	3.30 ± 0.04	3.59 ± 0.04[a]
S_1	2.97 ± 0.04	2.11 ± 0.04	2.44 ± 0.04	2.64 ±0.09	2.68 ± 0.04[b]
S_2	2.79 ± 0.09	2.01 ± 0.02	1.91 ±0.03	2.04 ± 0.05	2.10 ± 0.04[c]
S_3	2.82 ± 0.02	1.92 ± 0.06	1.80 ± 0.03	1.98 ± 0.08	1.64 ± 0.04[d]
S_4	2.91 ± 0.05	1.90 ± 0.07	1.76 ± 0.06	1.62 ± 0.04	1.58 ± 0.09[e]

Results are means of three separate determinations (Mean ± SEM), Values with the same superscript in the same column are not significantly different ($p < 0.05$) from each other.

Table 9b. RNA/DNA ratio in the liver tissue of *Labeo rohita* fingerlings fed with heat treated soybean meal containing different level of T1 during the experimental period (SC and S_5 – S_8).

Experimental Groups	Feeding days				
	0	15	30	45	60
SC	2.76 ± 0.03	2.31 ± 0.03	2.97 ± 0.07	3.30 ± 0.04	3.59 ± 0.05[a]
S_5	2.82 ± 0.09	2.99 ± 0.09	3.01 ± 0.02	3.20 ± 0.06	3.51 ± 0.2[b]
S_6	2.87 ± 0.04	2.97 ± 0.01	2.89 ± 0.04	2.84 ± 0.09	2.91 ± 0.07[c]
S_7	2.84 ± 0.06	2.69 ± 0.02	2.49 ± 0.03	2.16 ± 0.07	2.44 ±0.08[d]
S_8	2.76 ± 0.07	2.41 ± 0.02	2.32 ± 0.09	2.24 ±0.09	2.29 ± 0.06[d]

Results are means of three separate determinations (Mean ± SEM), Values with the same superscript in the same column are not significantly different ($p < 0.05$) from each other.

Table 10. Regression analysis for the protease (μg glycine liberated $hour^{-1}$ mg^{-1} of protein) and acetylcholinesterase (μ mole $100g^{-1}$ tissue 30 $minutes^{-1}$) activity of *Labeo rohita* fed on diets S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8

	Diet	Regression equation Y = mX + c (T-beta)	T-beta	R^2 value	F value	p value	t value
Acetylcholin-esterase activity	S_1	Y = 0.130X + 15.750	0.931	0.867	19.501	0.022	49.462
	S_2	Y = 0.510 X + 16.431	0.917	0.842	15.955	0.028	52.496
	S_3	Y = 0.322 X + 16.759	0.638	0.407	2.059	0.247	30.509
	S_4	Y = - 0.081 X + 17.968	-0.084	0.007	0.021	0.893	13.206
	S_5	Y = 0.017 X + 16.097	0.584	0.342	1.556	0.301	485.059
	S_6	Y = -0.040 X + 16.469	-0.179	0.033	0.099	0.773	52.713
	S_7	Y = -0.121 X + 17.074	-0.263	0.069	0.222	0.669	27.106
	S_8	Y = 0.003 X + 17.214	0.004	0.00001	0.00006	0.994	17.912
Protease activity	S_1	Y = 0.006 X + 98.728	0.022	0.0005	0.002	0.972	255.350
	S_2	Y = -0.019 X + 99.178	-0.058	0.004	0.009	0.926	212.631
	S_3	Y = -0.105 X + 100.700	-0.057	0.004	0.010	0.926	39.163
	S_4	Y = 0.067 X + 102.310	0.029	0.00008	0.003	0.962	31.938
	S_5	Y = -0.113 X + 98.316	-0.295	0.087	0.286	0.630	189.83
	S_6	Y = -0.252 X + 99.128	-0.739	0.547	3.619	0.153	305.485
	S_7	Y = 0.052 X + 98.638	0.077	0.006	0.018	0.902	103.571
	S_8	Y = -0.146 X + 99.646	-0.170	0.029	0.089	0.784	83.469

DISCUSSION

Good acceptance of all the experimental diets was of interest, since a low intake of feeds contains increasing level of some vegetable feed stuffs has been reported by several authors (Jackson *et al.*, 1982; Roselund, 1986; Jones *et al.*, 1996a).

Poorer nutrient utilization by *Labeo rohita* fed the diets containing higher level incorporation of raw soybean flour that those with lower level was evident. They had poorer SGR, FCR and PER values. This was as a result of trypsin inhibitor (TI) activity that increased in the diets with the increasing level of raw soybean (diets S_1 – S_4). The result of the present investigation indicates that soybean meal at lower inclusion level are good dietary protein source for carp fingerlings. The results show that the raw soybean flour contains higher level

of TI than the autoclaved soybean meal. Growth retardation and poor protein efficiency ratio was observed in channel catfish fingerlings fed soybean meal with high trypsin inhibitor activity (Wilson and Poe, 1985; Mambrini *et al.*, 1999; McGoogan and Gatlin, 1997). Similarly, growth response was poor in carp fed insufficiently heated soybean meal (Viola *et al.*, 1983). Results from the present experiment support the studies of Spinelli *et al.* (1979) who showed hat the growth of rainbow trout was reduced and high mortality (50% after 90 days of feeding) occurred in fish received a diet based on soybean meal.

Decrease in TI activity to tolerable level brought about by heat processing was observed to improve growth rate and PER of channel catfish (Wilson and Poe, 1985). Viola *et al.*, 1983, observed significance different growth response in carp fed diets containing adequately and slightly over heated soybean meal ($p< 0.05$). So, the present results demonstrated that high dietary levels of trypsin inhibitors might cause negative effects in *Labeo rohita.* It is possible from this study that antinutritional factor i. e. trypsin inhibitor might have impaired digestion and absorption of essential components of the diets containing soybean meal and causing depressed the growth rate of *Labeo rohita* fingerlings. Arndt *et al.* (1999) and Bransden and Carfor (1999) strongly support this results, i.e. heat treatment influence the nutritional quality of soybean.

The possible effects of antinutritional factor (ANF) present in soybean on IMC are not at all known (Escaffre *et al.*, 1997). Alexis *et al.* (1985) replaced 34% of the fish meal protein by protein from extracted soybean meal and this results improved growth rate and food utilization compared with fish fed a fish meal based diets. Kaushik *et al.* (1995) strongly emphasis that total replacement of fish meal by soybean meal create problem in fish growth and protein utilization in rainbow trout.

Trypsin inhibitors have been implicated in reducing protein digestibility and in pancreatic hypertrophy. It is well known the presence of trypsin inhibitors in the diet leads to the formation of the irreversible trypsin enzyme-trypsin enzyme complex, causing a trypsin drop in the intestine and a decrease in the diet protein digestibility (Liener, 1994; Elangoran and Shim, 2000; Sveier, 2001).

The increase in lipid deposition in muscle and liver, as well as the decrease in liver glycogen, have been reported by Sakamoto and Yone, 1978. This is in agreement with histological results obtained in the current study. Pancreatic hypertrophy was observed due to higher activity of pancreas to combat with TI in the soybean diet. Kidney also affected by the higher inclusion of TI based diet. Roboina *et al.* (1995) strongly supported our results. Refstie *et al.* (2001) strongly supported my reasult during the study of protein, lipid content in Atlantic salmon (*Salmo salar*) partial replacement the fish meal by soybean meal.

It is well established that the measurement of AChE activity is taken as a good indicator for toxicity study (Rainsford, 1978; Kumar, 1999; Kumar *et al.*, 1995). Several investigators have reported the changes in AChE activity as in ideal tool to examine neurophysiological changes in response to toxicants may be the TI in soybean also (Nemcsok *et al.*, 1985a).

In our present study AChE activity gradually increased with the higher inclusion of soybean in the diets but then decreasing trend was notices in the long term study. Similar observations also noted by Szabo *et al.* (1992) in *Cyprinus carpio*. Vijayalakshmi (1980) reported decreased level of AChE in brain, gill, liver and muscle of *Etroplus maculates* following an exposure to sumithion. The changes of brain AChE in vertebrates were reported to reduce the animal's survivality (Karozmar *et al.*, 1970; Montz and Kirkpatrick, 1985; Gupta *et al.*, 1998).

CONCLUSION

In our present investigation, it was observed that higher inclusion of soybean meal in the diet reduces the growth of *Labeo rohita*. Bur heat treatment improve the nutritional quality and enhance the growth in one hand and it is most cost effective to us. Soybean can be used as a fish ration but 100% replacement of fish meal is nor recommended.

ACKNOWLEDGEMENTS

This work was financially supported by All India Council for Technical Education (AICTE), New Delhi, India. The authors are deeply grateful to the members of Aquaculture Research Unit, Department of Zoology, Vidyasagar University, Midnapore, West Bengal, India for their untiring help.

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LEGENDS TO THE FIGURES

Figure - 1a. : Cumulative growth performance of *Labeo rohita* fed the diets S_1 – S_4 using soybean meal during 60 days feeding trial.

Figure – 1b. : Cumulative growth performance of *Labeo rohita* fed the diet S_5 – S_8 using heat treated soybean meal during 60 days feeding trial.

Figure – 2. : AChE activity of the brain tissue of *Labeo rohita* fed the soybean based diet (S_4) and heat treated soybean diets (S_8) using higher inclusion of soybean meal during 60 days feeding trial and compared with the control (SC).

Figure – 3. : Protease activity of the hepatopancreas of *Labeo rohita* fed the diet S_4 and S_8 using higher inclusion of soybean meal during 60 days feeding trial and compared with the control (SC).

Figure – 4. : Muscle protein profile of *Labeo rohita* fed the diet S_4 and S_8 using higher inclusion of soybean meal during 60 days feeding trial.

Figure – 5. : Muscle lipid profile of *Labeo rohita* fed the diet S_4 and S_8 using higher inclusion of soybean meal during 60 days feeding trial.

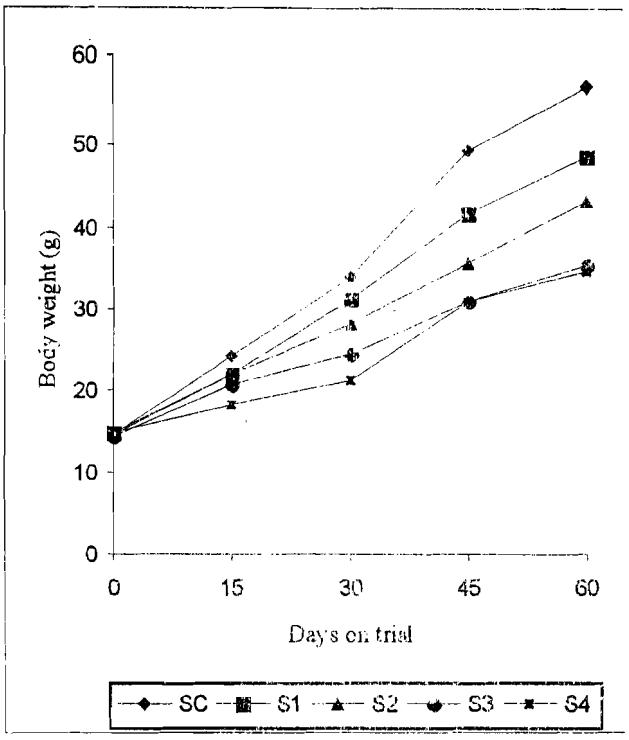

Figure – 1a

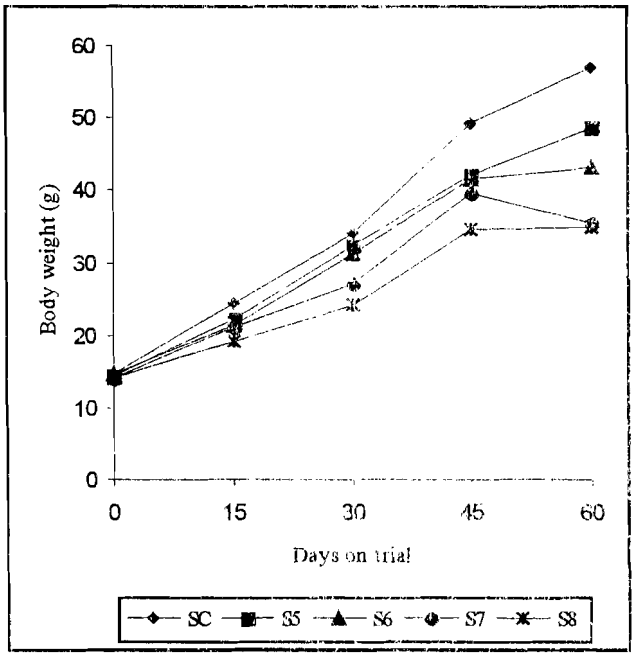

Figure – 1b

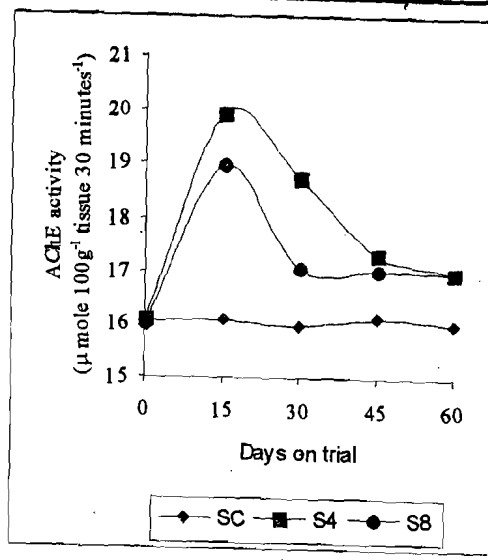

Figure – 2

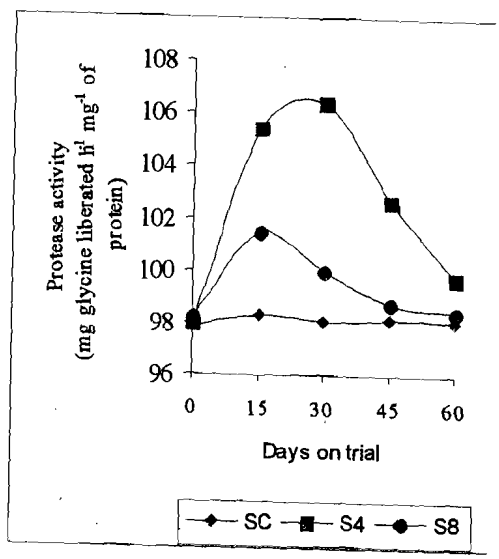

Figure – 3

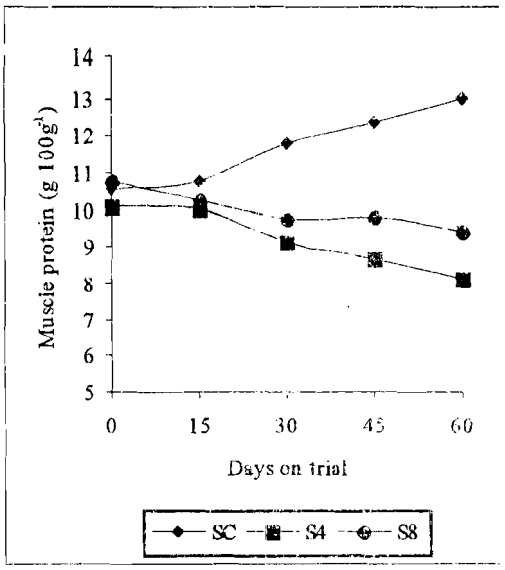

Figure - 4

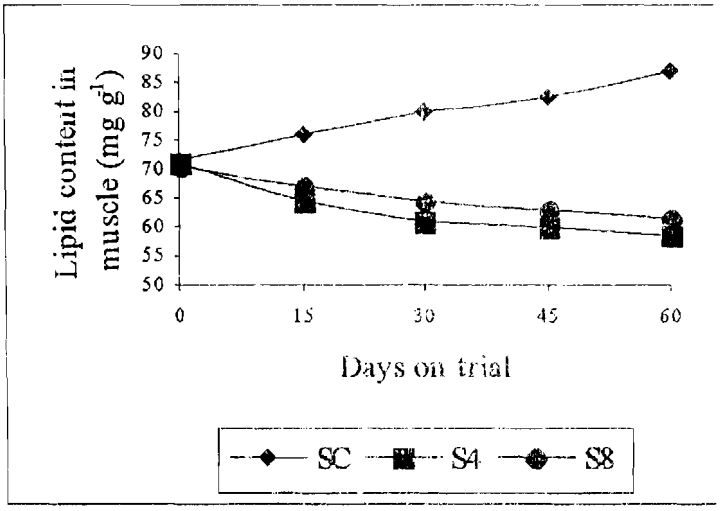

Figure – 5

TOTAL SOLUBLE PROTEIN PROFILE (SDS-PAGE AND PAGE) OF *Chara benthamii* BRAUN (CHAROPHYCEAE)

*Amal Kumar Mondal**

Abstract :

The total soluble protein of the *Chara benthamil* Braun was analyzed by Gel electrophoresis (PAGE and SDA-PAGE) to study its acidic and basic protein profile. SDS-PAGE revealed a total of 12 low protein bands between the molecular weight range of 25 kDa to 82.8 kDa and two high molecular weight bands above 116 kDa. The acidic protein profile showed six bands whereas the basic profile showed only four protein bands.

Key Words : *Chara benthamii,* gel electrophoresis, protein profile

Introduction :

The algae, which lack the terrestrial specilizations of bryophytes and tracheophytes, involve a heterogeneous assemblage of photosynthetic plants. Yet their role in applied phycology, holds immerse importance, such as the extensive use of seaweeds in human and animal diets and in industry, as well as their possible role in sewage treatment, in spece research and in the ecology of radioactive wastes. However, knowledge on the metabolic changes which occur during the life cycle of these lower groups of plants is still lacking, as lot of such problems still reamin unexplored, and if we are to interpret their biochemical diversity, we need a better understanding of the general biology of algae. With the development of new techniques in biochemistry and molecular biology, it will be easily possible to examine the role of intermediary pathways of metabolism and their significance in physiology. Among the various groups of algae, *Chara* L. belonging to the family Charophyceae and is considered to be the most advanced among the subgroup Charoideae as they are corticated (Morris, 1968), remains widely unexplored from the biochemical point of view.

C. *benthamii* Braun, is one such monoecious species, growing uploa height of about 35 cm and is dominant in the Howrah and Hooghly districts of West Bengal. C. *benthamii* is heavily incrusetea has slender stem, being corticated, diplastichous and 350-520 μ in diameter. The internodes are 1-3 times the length of the branchlets, the spine-cell are single, acute and projecting horizontally. Stipuiodes are well developed, elongated acute, projecting more or less horizontally, forming a single whori. The branchlets are 8-14, consisting of 4-5 segments and ecordcated. The bract-cells are 4-5 at the lowest nodes and 3 at the apex and are more

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or less straight and thick. There are 3 apical bract cells of different length with the anterior pair being 1-2 times the length of the ripe oogonium and the posterior pair on the first and second node and similar to the anterior pair. On the upper nodes they are half as long as the oogonium. The bracteoles are similar to the anterior bract-cells. The antheridia and oogonia usually develop at the lower three nodes. The antheridia are 390-490 μ in diameter, whereas the oogonia are up to 575-780μ in height, 150-160 μ wide at the base and erect. Oospores are golden-brown, 445-554μ in length and 310-400μ in width, with 7-8 conspicuous ridges.

The present paper reports the protein profile of *C. benthamii* by both Sodium dodecyl sulphate polyacrylamide gel electrophoresis and nondenaturing native polyacrylamide gel electrophoresis.

Experimental :

The plant material was collected in bulk from ponds from different places of West Bengal. The specimen was washed thoroughly with distilled water to remove mud and other debris and the extra water was soaked using a blotting paper. The specimen was then used for the study of protein profile by gel electrophoresis.

Protein was extracted with 0.2M Tris HCl buffer (pH 7.4) by homogenizing 500 mg of sample with 5-10 ml of extraction buffer at 4°C following the method of Sadasivam and Manickam (1996). It was then centrifuged at 15,000 ×g for 25 min. at 4°C. The supernatant was used for protein estimation by the method of Lowry et al. (1951). A calibrated solution of Bovine serum albumin was used as standard.

To study the protein profile, the protein sample after extraction was sbjected to SDS-polyacrylamide gel electrophoresis after boiling with equal amount of sample buffer [0.06 M Tris-HCl (pH 6.8), 1% SDS, 10% sucrose, 0.5% β-mercaptoethanol, 0.01% Bromophenol blue] at 100°C for 3 min and loading in the well of a 12% T. mini-gel (8×7 cm gel). SDS-PAGE was done following the method of Laemmli (1970). The gel was calibrated with marker proteins (M.W.range 14.4 kDa to 116 kDa) obtained from Genei, Bangalore. After electrophoresis, the gel was stained with 0.1% Coomassie Brillant Blue R250 and destained with methanol : acetic acid : water (4:1:5) mixture.

To protein sample was also subjected to non-denaturing gel electrophoresis to separate the acidic proteins from the mixture following the method of Ornstein (1964) and Davis (1964). 12.5% separating gel was prepared using 0.375M Tris HCl buffer (pH 8.9) and 4% stacking gel was prepared using 0.062 M Tris HCl buffer (pH 6.7). The gel was run using electrode bffer containing 0.05M Tris and 0.038M glycine (pH 8.2) after treating the proteins with sample buffer containing 2 ml of Tris HCl (pH 6.8) and 2 gm sucrose in 10 ml water. The gel was run at 50 volts (4mA) for 2 hours and 30 minutes, and, stained and destained as in case of SDS-PAGE.

To separate the basic proteins, the protein sample was subjected to PAGE following the method of Reisfeld et al. (1962). The separating gel (12.5%) was prepared by using separating gel buffer, pH 4.3 (0.6M KOH and 0.375M Glacial acetic acid) and 4% of the stacking gel

was prepared using gel buffer pH 6.8 (0.6 KOH and 0.62 M Glacial acetic acid). The protein sample was loaded into the gel after treating with sample buffer (0.06 M KOH, 0.062 M Glacial acetic acid, pH 6.8) containing 20% sucrose. The gel was run using electrode buffer of pH 4.5 (0.35 M β-alanine and 0.14 M Glacial acetic acid) at 50 volts (4 mA) for 2 hours and 30 minutes. After electrophoresis, the gel was stained and destained as mentioned in case of SDS-PAGE.

Results & Discussion :

The proteins of algae, like those of other plants, consists of many different molecular species and are perhaps predominantly enzymes possessing specific biological roles (Lewin, 1962). The total soluble protein content was found to constitute about 22.51% of the dry weight. The protein profile as studied by SDS-PAGE (Fig. 1B; Table 1) showed twelve bands which were designated as C1 to C12 in order of their decreasing molecular weights. Majority of the protein bands were clustered in the molecular weight range of 63 kDa and 83 kDa (C3 to C7) and between 40 kDa and 48 kDa (C8 to C11). There were two bands above 116 kDa (C1 and C2) and one low molecular weight protein band i.e. 25 kDa (C12). Some of the prominent protein bands included C3 (82.8 kDa), C6 (66.2 kDa), C10 (42.1 kDa) and C11 (40.7 kDa).

The protein profile as studied by native gel electrophoresis however showed interesting results (Fig. 1C; Table 2). The basic protein profile showed only four protein bands designated as CB1 to CB4. Two of the proteins were of high molecular weights i.e. two above 116.0 kDa (CB1 and CB2) and the other two protein bands CB3 and CB4 were of comparatively low molecular weights i.e. 77.3 kDa and 63.6 kDa. All the three proteins were also observed in SDS-PAGE.

The acidic protein profile however revealed six bands designated as CA1 to CA6. Of these CA2 (82.8 kDa), CA3 (66.2 kDa) and CA4 (55.6 kDa) (Fig. 1D ; Table 2) were not observed in SDS-Page. Probabily these basic proteins had broken down into their subunits during denaturing SDS-PAGE to give the bands C5 (71.0 kDa) and C9 (45.0 kDa) from CA1 (116.0 kDa) and the other two bands C8 (47.7 kDa) and C12 (25.0 kDa) from CA3 72.7 kDa) during SDS-PAGE.

Thus the quantitative and qualitative data of the biochemical constituents of algae can reflect the nutritional status and physiological condition of the plant and these studies can serve as a prelude for future studies on the genetic control of the biochemical steps involved in algal metabolism and photosynthesis. In an environment of low light intensities and frequent periods of desiccation, protein metabolism may be extensively modified. Arrested synthesis of protein molecules might be expected to result in an accumulation of amono acies and low molecular weight peptides. The nucleated portion of algae both RNA and protein are actively synthesized whereas in the enucleated portion of the cell, the RNA content remains unchanged although the soluble protein increases (Richter, 1959 a & b). Further the comperative protein profile of different species of *Chara* and numerical evaluation of the data can give an inner

view into their phylogenetic interrelationships and plant affinity.

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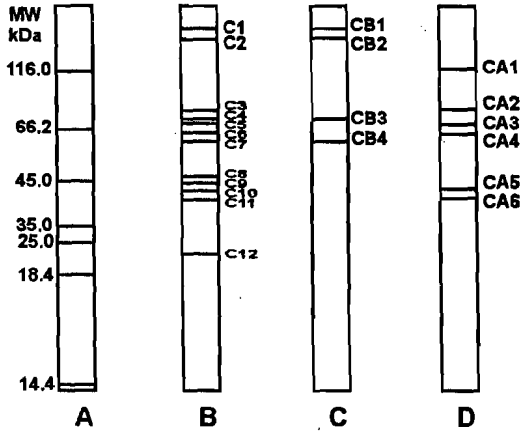
Table 1 : Molecular weight of the protein bands of *C. benthamii* as revealed by SDS-PAGE

Protein band	Molecular weight in kDa
C1	Above 116.0
C2	above 116.0
C3	82.8
C4	77.3
C5	71.0
C6	66.2
C7	63.6
C8	47.7
C9	45.0
C10	42.1
C11	40.7
C12	25.0

Table 2 : Molecular weight of the protein bands of *C. benthamii* as revealed by PAGE

Protein band	Molecular weight in kDa
CA1	116.0
CA2	82.8
CA3	72.7
CA4	66.2
CA5	42.1
CA6	40.7
Basic proteins	
CB1	above 116.0
CB2	above 116
CB3	77.3
CB4	63.6

Fig. 1



Legend to figure :

Fig. 1. Diagrammatic representation of the protein profile of *C. benthamii* (A) represents marker protein; (B) sample protein profile as revealed by SDS-PAGE; (C) represents basic protein profile (PAGE) and (D) represents acidic protein profile (PAGE).

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