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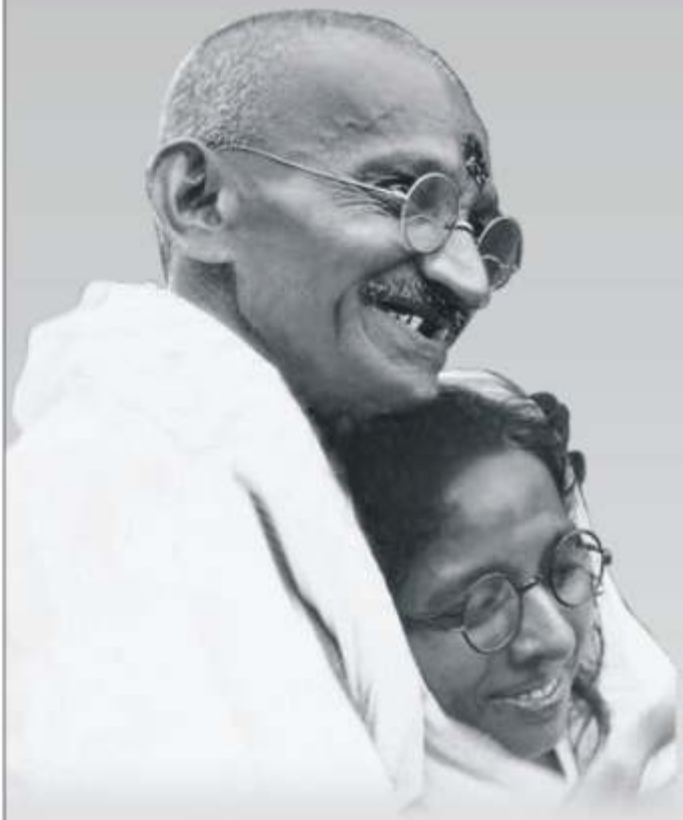

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Histological changes in the thyroid gland during the female reproductive cycle in *Hipposideros lankadiva (kelaart)*

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Abstract : The present investigation was undertaken to compare the histological changes in thyroid gland of female reproductive cycle of *Hipposideros lankadiva (kelaart).*Thyroid gland showed marked seasonal variation in weight, quantity of colloid and follicular epithelial height, suggesting that the thyroid gland remains inactive during quiescence and winter dormancy and it became active during the time of recrudescence and the remaining breeding behavior is similarly to the testicular cycle. Plasma thyroxin (T4) concentration showed a significant seasonal change with high concentration during the breeding and post-breeding period and low concentration during quiescence.

The observations confirm that during diapauses the thyroid gland show hypothyroid condition. Probably the stored colloid material is used by bat after the arousal from torpor as embryonic development gains momentum. It is suggested that the increase in the consumption of colloid material after arousal from torpor is due to hyper-metabolism which might be the requirement of feto-placental unit in the pregnant animal. Although, the hypothyroidism is associated with infertility, sterility and abnormal in bats. The present study records that it may also be related to embryonic diapause and slow growth of embryo in *H. lankadiva*.

Key words: *H. Lankadiva*, Thyroid gland, Embryonic diapause, Reproductive cycle.

Introduction

Hipposideros lankadiva (kelaart) is the tropical, non-hibernating, monoestrus, monotocous, hipposiderid bat. These bats are indigenous to the southwestern subcontinent of India. This is the only bat in this vast subcontinent representing the phenomenon of delayed development or embryonic diapause and also suggests a slow rate of embryonic differentiation after implantation resulting prolonged gestation period of 260 days (8.5 month) with the body weight of just 55gm as reported by Sapkal and Bhandarker (1984). Seraphim (2009 a &b) studied the endocrine interaction during different phases of the female reproductive cycle in *H. lankadiva (kelaart).* She noticed changes, both light and electron microscopic, in diapause stage. According to her, thyroid gland plays an important role in the maintenance of growth and reproduction. The hypothyroidism is believed to be closely associated with sterility, infertility and abortion (Mayant, 1964). In cattle, T3 deficiency results in silent estrous in female. Singh *et al.(2002)* reported seasonal changes in thyroid activity in the female sheath-tailed bat, *Taphozous longimanus* (Chiroptera: Emballonuridae). They found that thyroid gland showed marked seasonal variation in weight and secretory activities. It was inactive in quiescence from early to mid-winter dormancy and active during recrudescence and breeding period during late winter dormancy. They also reported that serum 3, 5,3'-triiodothyronine (T3) and thyroxine (T4) concentrations showed significant variation and closely coincide with thyroid activity. In the present study an attempt has been made to know endocrine interaction during different phases of the female reproductive cycle in bat, *Hipposideros lankadiva (kelaart).*

Materials and methods

Mature female *H. lankadiva* were collected from there roosting places in Chandrapur and Ballarshah (Maharastra) and Mandu (M.P.) from August up to May in such a way that all the important reproductive periods were represented. Live animals were brought to laboratory with minimum stress, anesthetized and killed by decapitation. Thyroid glands were dissected outweighed and fixed in alcoholic Bouins. After 24 hrs the glands were washed with 70% ethanol, dehydrated through graded series of ethanol, cleared in xylene and embedded in paraffin wax. The sections were cut at 5µ-75µ thick. The sections were stained with Ehrlick's hematoxylin and counter stained with eosin. The weight of the thyroid gland during different phases of the reproductive cycle was measured. Necessary statistical techniques were used.

Results

Thyroid gland of *H. lankadiva* is a bilobed structure. Measurement of the wt of thyroid glands during different phases of the reproductive cycle has been shown in the table 1. The present observations

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Table 1. Weight of the thyroid gland during different phases of the reproductive cycle

Reproductive state	Month	Weight of sample no			Mean Weight ± SEM
		I	II	III	
Estrus	August	5.6	7.8	7.2	6.80 ± 0.6582
Implant	Sept.	5.9	5.1	7.2	6.00 ± 0.6137
Diapause	Nov.	4.5	5.7	6.2	5.40 ± 0.5066
Arousal	Feb.	7.6	4.8	5.6	6.00 ± 0.8326
Lactation	June	5.4	5.0	6.6	5.60 ± 0.4830
Anestrus	July	5.8	6.4	5.9	6.00 ± 0.1870

suggest that the weight of the thyroid gland reaches its peak (6.80 ± 0.6582) during estrus the weight reduces during implantation (6.00 ± 0.6137) it is further reduced to (5.40 ± 0.5066) during in diapause period of the reproductive cycle. Again there is an increase in the wt of the gland during later part of pregnancy and lactation.

The follicles and parafollicular cells of the thyroid are embedded in the parenchyma. The histology of the thyroid gland during different phases of reproductive cycle is presented below.

Thyroid gland during estrus:

It is oval and triangular in shaped during this phase and contains follicles of varying sizes and shapes can be distinguished into 3 types depending upon their size and the type of epithelial cells lining.

1. A type follicle: (diameter 100µ - 150µ)

The thyroid comprised large numbers of follicles whose diameter ranges from 100µ - 150µ and is mainly located towards the periphery of the gland. The follicles are lined by sqamous epithelium. The follicles contain homogenous colloid. The cells cytoplasm is eosinophilic in nature, the nuclei are flattened. The follicles are less inquantity during estrous period.

2. B type follicles: (diameter 60µ - 100µ)

B type follicles have diameter ranging from 60µ - 100µ. These are medium sized and situated mainly in the inner portion of the gland and are lined by cuboidal epithelium. They are round in shape with centrally placed nuclei and the cytoplasm is basophilic in nature. The colloid material is homogenous.These are present in large numbers.

3. C type follicles: (diameter 30µ - 60µ)

Another type of follicles is designated as C type follicles. The diameter ranges from 30µ - 60µ. These follicles are small with narrow lumen occupying the interior portion of the gland and are lined by cuboidal and columnar epithelium that the nuclei take dark blue stain with hematoxylin thus showing basophilic nature. The colloid is homogenous and is also basophilic. These follicles became abundant during estrus phase.

Parafollicular cells:

These are arranged either singly or in group of 2 to 3 cells within the follicle and in the interfollicular connective tissue. These are close to the base of the epithelium and do not extend to the follicular cavity. These cells have large nuclei with faintly stained cytoplasm.

Thyroid gland during pregnancy:

In *H. lankadiva* after implantation female animal enters into torpor with less body activity and foraging movement is in the embryonic diapause stage. It start late August to early September. The pregnant females showed retarded growth of implanted blastocyst till the end of January. The thyroid gland during diapause presents following histological feature.

There is a large number of A type follicles and six to ten have acquired a diameter of about 167µ. These are situated at the periphery of the gland and are not observed in any of the previous stage. There large lumen is filled with homogenous eosinophilic colloid material. The B type which are medium size follicles are less in no as compared to A type and are lined by cuboidal epithelium. The C type follicles are less in number but are distributed throughout the gland. Parafollicular cells are found scattered in the interfollicular region.

Diapause state ends in the first week of February in *H. lankadiva* and thyroid gland of aroused animal shows interesting features. The oval gland shows presence of a minimum number of A type follicle while B and C types of follicles are abundant in no in aroused animals. During arousal stage the homogenous

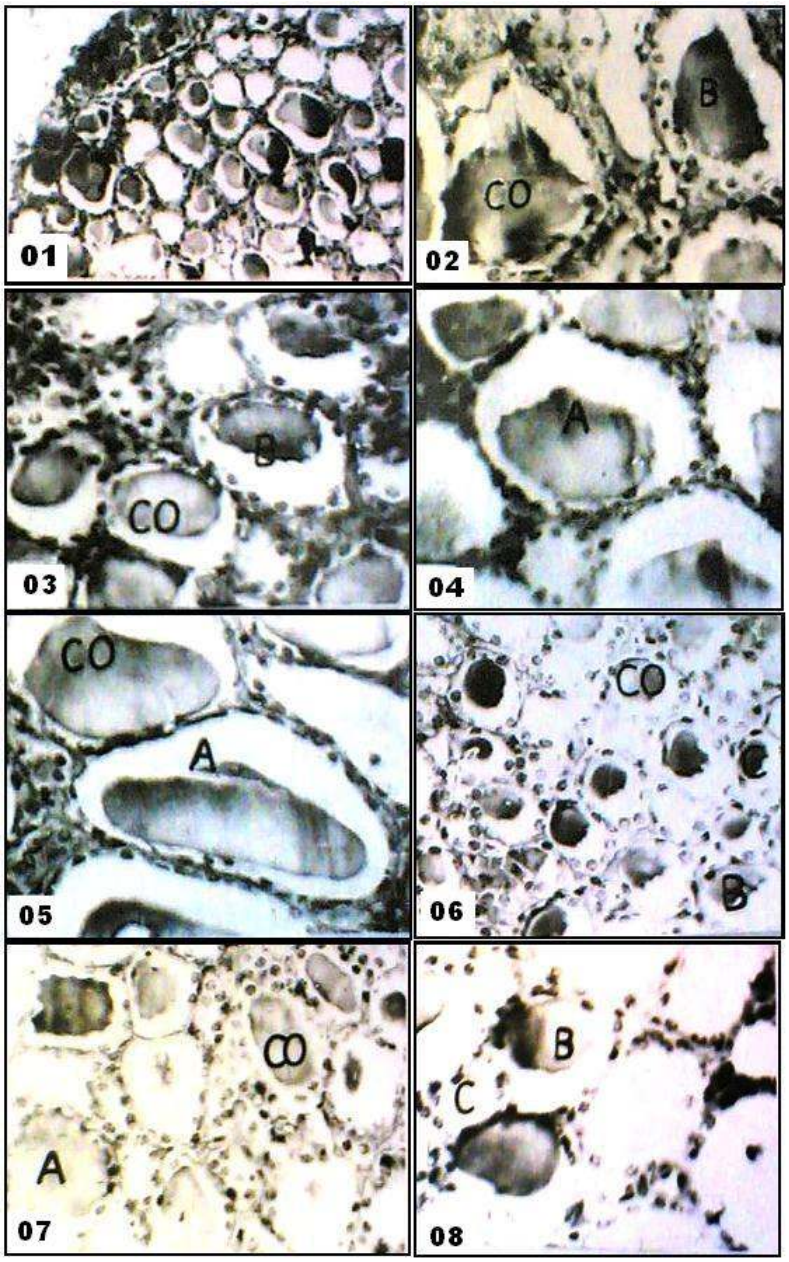

Fig. 1. Transverse section of thyroid gland of female *H. lankadiva* during estrus phase showing B and C type follicles. X 160.

Fig. 2. TS of thyroid gland of female *H. Lankadiva* during estrus phase showing B type follicles with cuboidal epithelium and homogenous colloid material. X450

Fig. 3. TS of thyroid gland of female *H. Lankadiva* during estrus phase during diapause showing B type follicles. X450

Fig 4. TS of thyroid gland of female H. Lankadiva during estrus phase during diapause showing A type follicles with low cuboidal follicular epithelium. X450.

Fig. 5. TS of thyroid gland of female *H. Lankadiva* during diapause showing A type follicles with largest diameter and homogenous colloid material (CO). X450.

Fig. 6. TS of thyroid gland of female during arousal phase showing B and C type follicles with cuboidal epithelium and vacuoilization in the colloid material (CO). X450.

Fig. 7. TS of thyroid gland of female *H. Lankadiva* during arousal showing A type follicles with cuboidal follicular epithelium vacuoles in the colloid. X450.

Fig. 8. TS of thyroid gland of female *H. Lankadiva* during lactation phase showing B type follicles with low cuboidal epithelium.X450.

follicular colloidal material shows vacuolation. The follicular epithelium is cuboidal in nature in B and C type follicules. Some of the follicles show depleted colloid material in their lumen. During late pregnancy there is further decrease in the number of very large follicles.

Thyroid gland during lactation:

Adult female *H. lankadiva* were observed in a state of lactation phase from June to July. During the oval shaped thyroid gland shows A type follicles with reduced number B and C type follicles are abundant.

Discussion

The insignificant variation in the wt. of the gland of *H. lankadiva* throughout the reproductive cycle is confirmation to the observation in bats as reported by earlier investigators (Burns *et al*., 1972; SINGH. & KRISHNA,1995 & 1966). Decrease in the wt. of the gland and the presence of the follicles of largest diameter is observed during torpor in pregnant female, *H.lankadiva*. This is due to the inactive state of the thyroid gland in which the stored colloid l has been consumed by the animal. Once it arouses from the torpor or at the end of the embryonic diapause the blastocyst starts developing. Chatefield and Lyman, (1950) and Holland *et al.* (1967) suggested that from arousal to hibernation or torpor is characterized by a transient period in which there is sharp rise in the rate of oxygen consumption and basal metabolic rate. Lardy and Kent (1963) stated that the thyroid hormones T -3 and T-4 regulate the cellular oxidation and metabolism. There is decrease in plasma thyroxin level (T-4) in *Macrotus californicus* after implantation and its lowest level is noticed in diapause (Burrow, 1972). A sharp rise after arousal is maintained till lactation.

An increase in the wt of the gland of *H. lankadiva* during arousal is observed. Histological, the aroused animals show medium and small sized follicles which contain with homogenous colloid material. Most of the follicles show vacuolization in the colloid material but some show complete depletion of the colloid material which indicates the active phase of the thyroid gland which continued till lactation.

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Morphology of Pyramidal neurons of Medial hippocampus in *E. scolopaceus* and *P. krameri*

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Abstract : Hippocampus in birds is divided into five fields namely: medial hippocampus, lateral hippocampus, parahippocampal area, central field of parahippocampus and crescent field. The pyramidal neurons are the main subtype of neuronal classes in the medial hippocampus of birds. An attempt has been made to study and compare the morphology of pyramidal neurons in the medial hippocampus of *E. scolopaceus* and *P. krameri.* It was observed that pyramidal neurons of *E. scolopaceus* were more advanced in terms of soma diameter and dendritic field in comparison to *P. krameri* showing more networking of pyramidal cells in *E. scolopaceus.*

Key words: *E. scolopaceus, P. krameri,* Medial hippocampus, Soma diameter, Dendritic field

Introduction

Three layered medial hippocampus (HCm), a ventromedial part of hippocampus in birds has been suggested to be equivalent to Ammon's horn in mammals (Ariëns Kappers *et al.,* 1936; Montagnese *et al.,* 1996). It is one among the five fields of hippocampus in birds and has been differentiated into three visible layers namely: suprapyramidal, pyramidal and infrapyramidal layer (Srivastava *et al.,* 2007). The cell layers have been designated so because of the abundance of pyramidal neurons in the HCm region of birds. Montagnese et al. (1996) reported bitufted pyramidal neurons to be the only projection neurons in HCm of male zebra finch whereas Srivastava *et al.* (2007) observed pyramidal-like and multipolar neurons in addition to pyramidal neurons in HCm of *E. amandava* and favored the view that pyramidal cells were the main type of projection neuron in HCm of *E. amandava* .

The objective of present study was to study and adduce the morphology of pyramidal neurons of HCm in two different birds viz. *Eudynamys scolopaceus* and *Psittacula krameri.*

Materials and Methods

Three adult female (each) *E. scolopaceus* and *P. krameri* used in this study were collected from Allahabad (25° 28' N, 81° 54' E). Golgi-Colonnier method (Blaesing et al., 2001) was employed for neuronal study. All the procedures were carried out according to institutional animal care guidelines.

Results

Pyramidal neurons were the most dominant type of neuron in HCm of *E. scolopaceus* and *P. krameri* (Fig. 1). The characteristics of pyramidal cells observed in the two birds are as follows:

1) *E. scolopaceus:* The medium sized triangular soma (diameter ranging from 19-25µm) gave rise to apical dendrite towards pia and two to three basal dendrites. The dendrites covered larger area (Table 1).

2) *P. krameri:* Thick apical dendrite of pyramidal neurons ran towards pia with finer basal dendrites radiating from the base of triangular soma. The soma diameter ranged from 18-20µm (Table 1).

The soma diameter and dendritic field calculated for female *E. scolopaceus* were greater than for female *P. krameri.*

Discussion

Regional specialization and differences in pyramidal cell structure may offer specific advantage for functioning of particular region i.e. HCm in present case. The soma diameter and dendritic field of pyramidal neurons in *E. scolopaceus* were observed to be larger in comparison to *P. krameri* (Table 1). The larger extent of dendritic arborization in *E. scolopaceus* indicates more networking of pyramidal cells allowing long-range connections in HCm of *E. scolopaceus.* The pyramidal neurons of *E. scolopaceus* and *P. krameri* share some common features (triangular soma, thick apical dendrite and finer basal skirt) with the neurons present in homologous structure of reptilian and mammalian telencephalon (Lacey, 1978; Guirdo *et al.,* 1998). The differences lie in the distribution pattern and extent of dendritic arborization of pyramidal neurons of hippocampus of birds, reptiles and mammals.

Acknowledgements

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Table 1: Characteristics of pyramidal neurons of medial hippocampus in *E. scolopaceus* and *P. krameri*.

Characteristics	***E. scolopaceus***	***P. krameri***
Soma diameter(in µm)	19-25 µm	18-20 µm
Dendritic Field(in µm)	75-139 x 66-121	24-95 x 19-40
Percentage of pyramidal neurons observed in HCm region	63.49%	58.13%

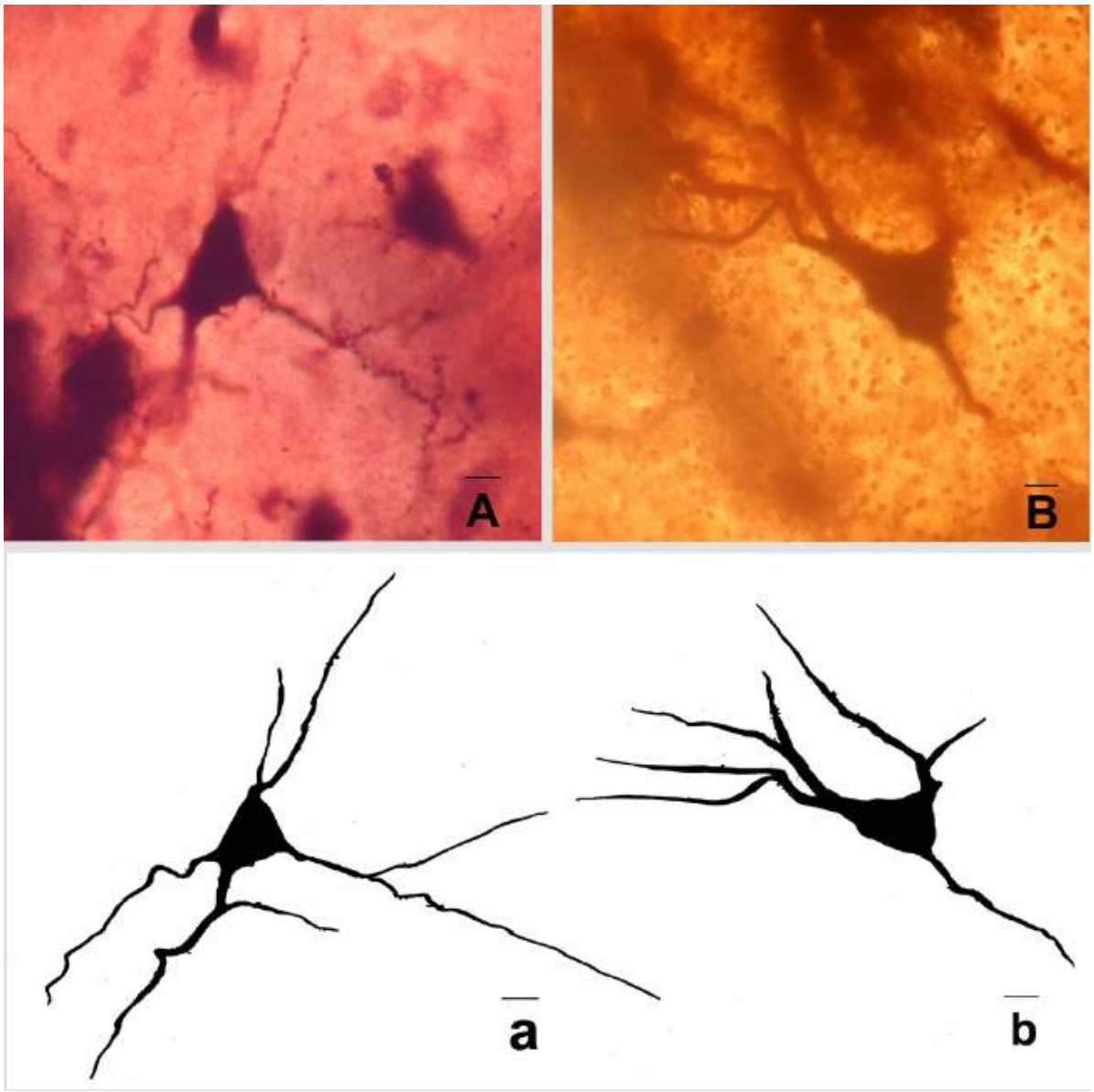

Fig. 1. Photomicrograph showing pyramidal neurons observed in medial hippocampus of (A) *E. scolopaceus*, (B) *P. krameri* and their respective camera lucida drawing. Scale bar = 10µm.

Allahabad, Allahabad for providing essential facilities for the present investigation.

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Recovery in Acid Aluminium Induced Toxicity in Gills with Silicon Dioxide in *Channa punctatus* (Bloch)

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Abstract : Water pH affects the solubility of Al metal. Gills in fishes serve vital functions like gaseous exchange and acid- base balance which is damaged due to metal toxicity. Under acidic condition at pH < 5 aluminium ($Al_2(SO_4)_3$) at sublethal dose (140 mg/l) brings morphological alterations in the gills including lamellar fusion, dealignment of microridged epithelial cells and hyperplasia of epithelial cells. These abnormalities can be attributed to response to prevent the entry of metal in the gills. The toxicity of aluminium on the gills of fish *Channa punctatus* (Bloch) can be reduced with the application of Silicon dioxide. Damage to the gill epithelium was discernibly lessened by the ameliorating effect of Silicon dioxide, which reduced the alterations in the gills effectively and in turn it increased survival in the fish.

Key Words: *Acid, Aluminium, Fish gill, Silicon dioxide, Amelioration*

Introduction

Water pH is an important chemical property for the survival of aquatic organisms because it affects the ability of fish and other aquatic organisms to regulate basic life- sustaining processes, primarily the exchanges of respiratory gases and salts with the water in which they live (Evans *et al*., 2005). The pH value of aquatic ecosystem affects the solubility of aluminium metal. Aluminium is the third most prevalent element which forms about 8% of the earth's crust. It a silvery white, ductile and malleable metal and belongs to group IIIA of the periodic table. It is extremely reactive and is not found as the free metal. Bauxite is the most important raw material for the production of aluminium, which contains up to 55% alumina (aluminium oxide). Bentonite, and zeolite are natural aluminium minerals which are used in water purification, sugar refining, brewing and paper industries. In the soil, in clay fraction aluminium comes through chemical weathering of rocks.

Both natural processes and anthropogenic sources contribute to the release of aluminium in the environment. It enters environmental media naturally through the weathering of rocks and minerals. Anthropogenic sources are air emissions, waste water effluents, and solid waste primarily associated with industrial processes such as smelting and mining etc. Aluminium becomes more soluble and more toxic to freshwater biota as pH of ambient water decreases below 5 (Gensemer and Playle, 1999). The acute toxicity of Al has been studied in different experiments by many authors (Gensemer and Playle, 1999; Berthon, 2002; Naskar *et al*., 2004; 2006; 2009). Aluminium acts as a gill toxicant to fish and cause ionoregulatory imbalance as well as haematological, respiratory disturbances (Neville and Campbell, 1988). Aluminium becomes soluble at low pH value <5 and are easily absorbed by fish and other aquatic organisms. Metal toxicity causes adverse effects on organism's activity, growth, metabolism, reproduction which are considered as sublethal effects(Wright and Welbourn,2002). When the pH of water is below 5.5, fish die or become seriously ill.

Silicon helps in the elimination of acute aluminium toxicity in fish when silicic acid reacts with aluminium to form hydroxialuminosilicates (HAS), one of the predominant forms of Al in acidified environment, which are extremely insoluble and play an important role in controlling the release of Al from soil to the aquatic environment (Exley *et al*., 1997; Exley *et al*., 2002). Hence the SEM study was undertaken to assess the ameliorating effect of Silicon dioxide in acid aluminum induced toxicity in gills of *C. punctatus* at sublethal concentration.

Material and Methods

Healthy fish *Channa punctatus* (Bloch) of approximate Weight 0.2±0.03 Kg Length 20.8+0.4 cm were procured and acclimated for 15 days under normal laboratory conditions(water temperature 25± l°C , pH

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7.1, Dissolved oxygen 6.2 mg/L ,total hardness 168 mg/L). After acclimation to laboratory conditions, fishes were divided in three groups , 20 in each and placed in separate glass aquaria. Fishes were fed *ad libitum* with *Tubifex tubifex* and *Daphnia* and were starved prior to experiment. Group I was maintained as control. Group II was exposed to Acidified water at pH < 5 with stock solutions of 1 M H_2SO_4 for period of 7 days. Group III was exposed to Acidified water along with sublethal dose of 140 mg/l of Aluminium Sulphate $Al_2(SO_4)_3.16H_2O$ (Qualigens) (LC_{50} is 220mg/l, Chakraborty *et al.* 2012) for 7 days. Group III fishes were treated with 150 mg of Silicon (metal) powder (Loba Chemie) for another 7 days to ascertain the ameliorating effect of Silicon in aluminium toxicity. The pH of ambient water of treated fish was maintained less than 5.0 with stock solution of 1M H_2SO_4.

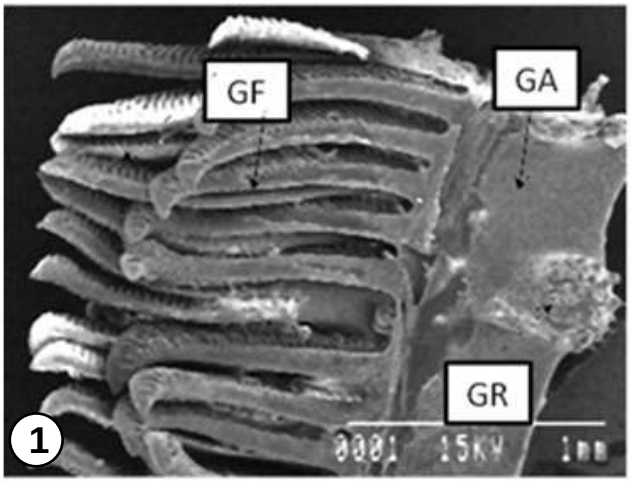
At the end of exposure period, gills from control and three experimentally grouped fishes (Acid treated, Al treated and silicon dioxide treated) were excised immediately and were washed repeatedly in normal saline for the removal of mucus. Gill tissues were fixed in 2.5% gluteraldehyde buffered with 0.1 Sodium cacodylate buffer (pH 7.4) for 24 hours at 4°C. Then they were washed for 2-3 times in washing buffer for 10-15 mins in each wash. Gill tissues were post fixed in 2% OsO_4 in buffer for 2 hours. Then gills were dehydrated in graded ethanol solution with final treatment in amyl acetate. The dehydrated tissues were critical point dried, mounted on stubs, gold coated and examined under the Scanning Electron microscope (Hitachi S-530).

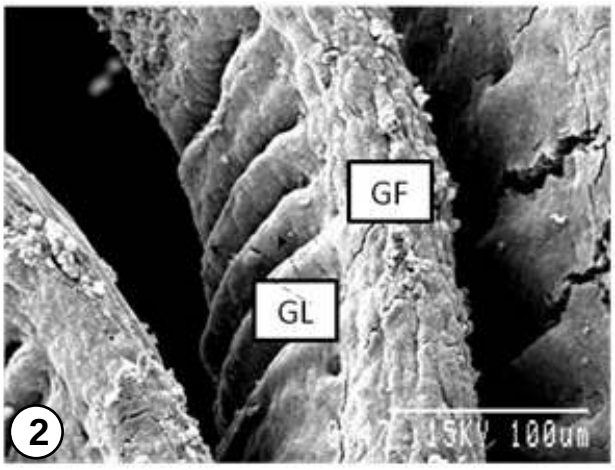
Results

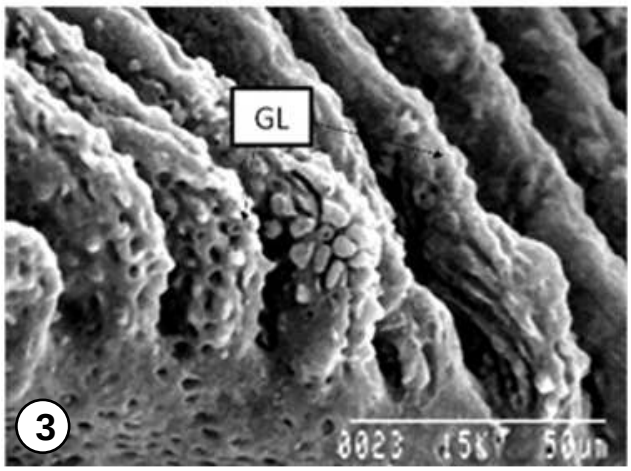
Fish mortality was absent in acid treated fish, while exposure to acid with aluminium caused death of fishes. Treatment with silicon as ameliorating agent, fish mortality reduced significantly. In SEM study of control gills, arrangement of gill filaments with free ends on the gill arch was observed. Secondary lamellae were arranged at equal space separated with each other. Microridged epithelial cells and mucous gland cells in secondary lamellae can be seen under higher magnification (Fig.1-6).

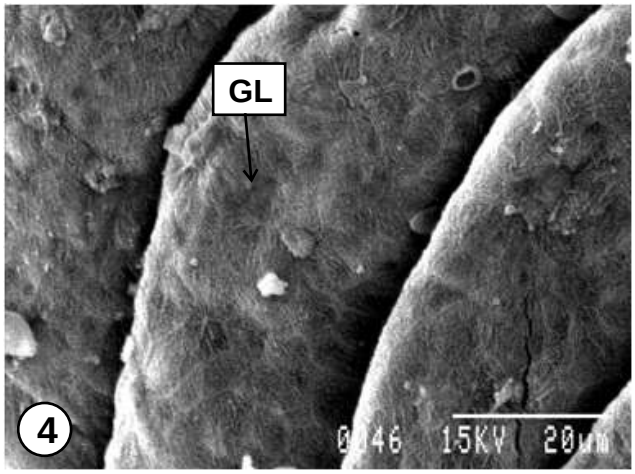
No discernable damage of gill epithelium was found under acidic condition. Normal alignment of microridges on epithelial cells was observed (Fig.7-8). Exposure to aluminium under acidic condition at pH<5 severe damage to gills along with excessive amount of mucus secretion was observed. Severe fusion of secondary lamellae which varies from partial to complete was seen. Swelling and clumping of secondary lamellae in the gills was due to gill epithelial hyperplasia (Fig.9-13). Microridges were dealigned in EC (Fig.14). Epithelial cells were fused with each other which altered the normal morphology (Fig 11).

Upon treatment with silicon as ameliorating agent, recovery in fish was observed. Structural damages to gill reduced with silicon. Fusion of lamellae was lacking in silicon treated gills. Normal gill architecture of epithelial cells and alignment of microridges were observed. Gills resumed the normal structure which increased the survival in fish (Fig 15-18)..






GL
3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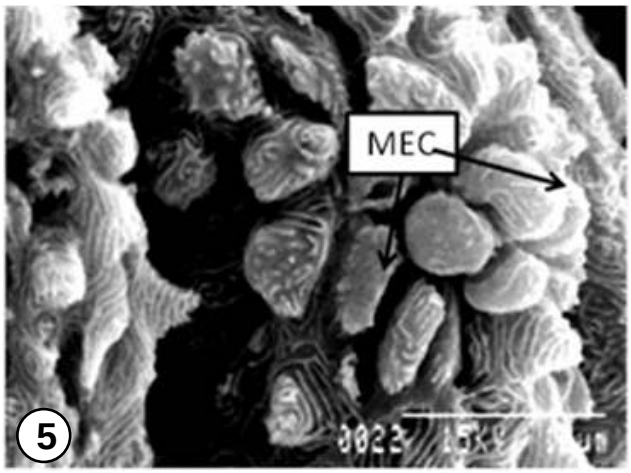
MEC
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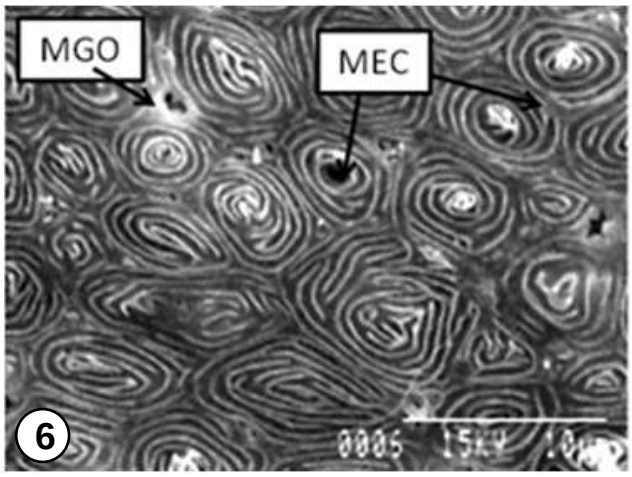
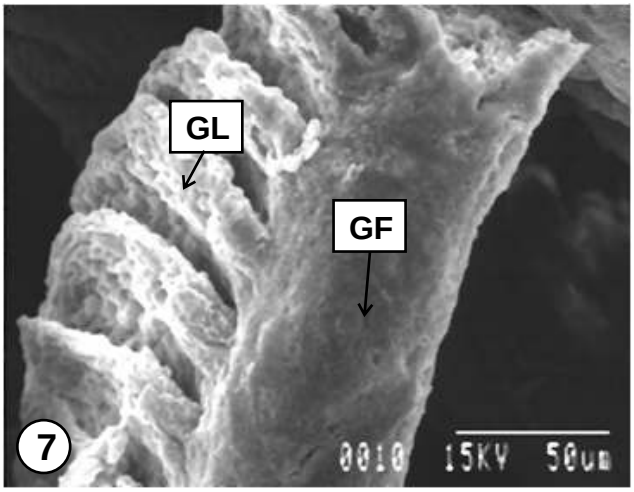

MGO
MEC
6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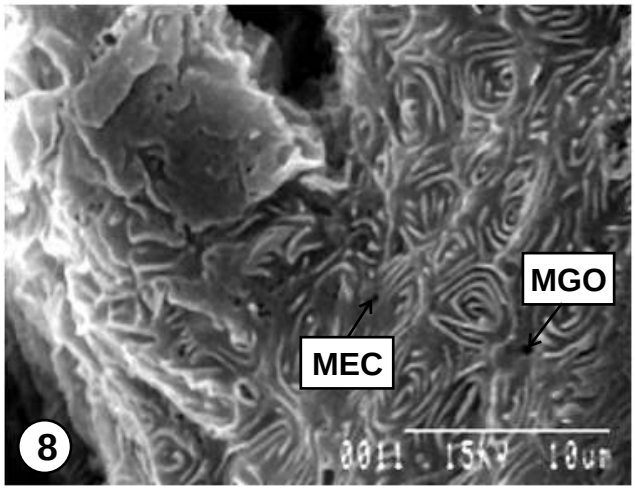
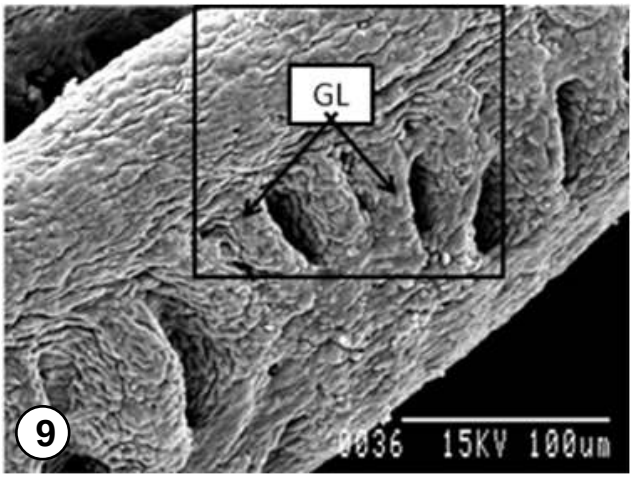

MGO
MEC
8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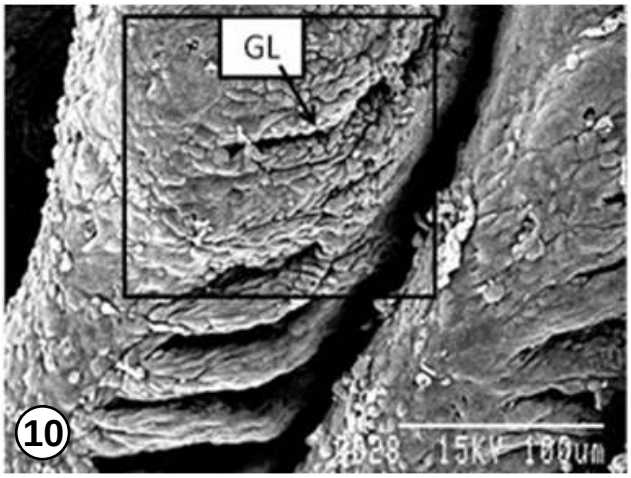
9
0036 15KV 100um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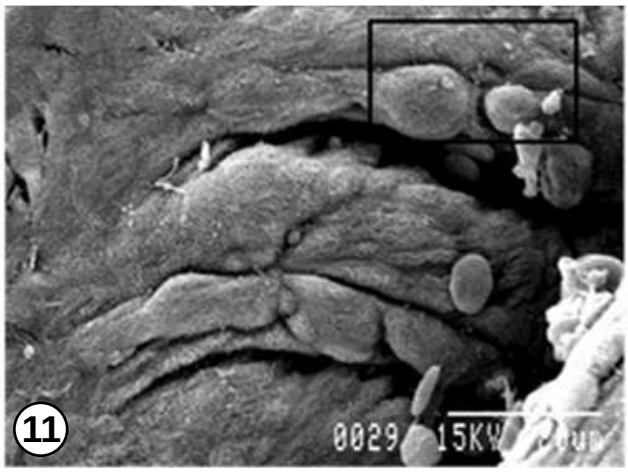
10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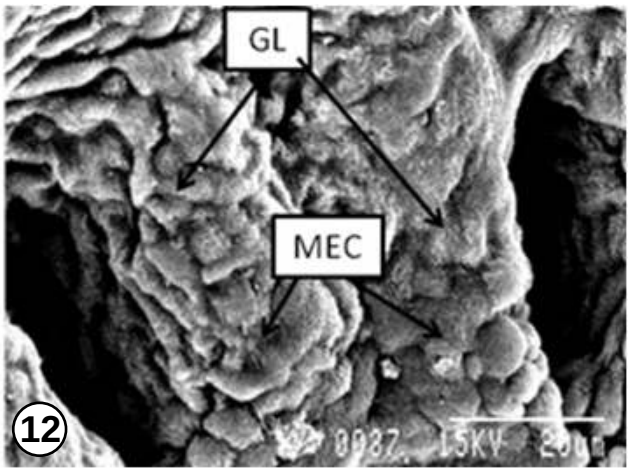
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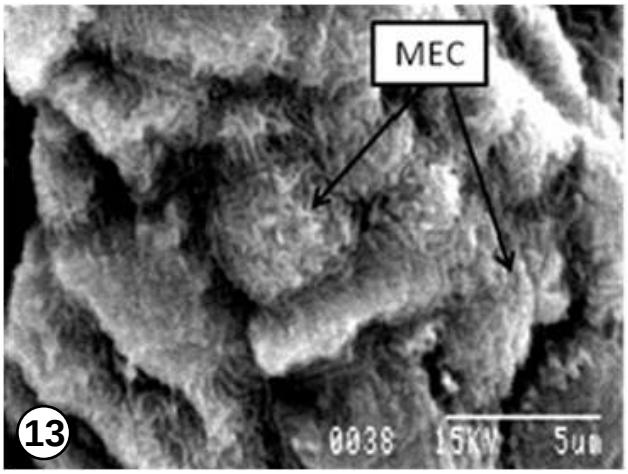
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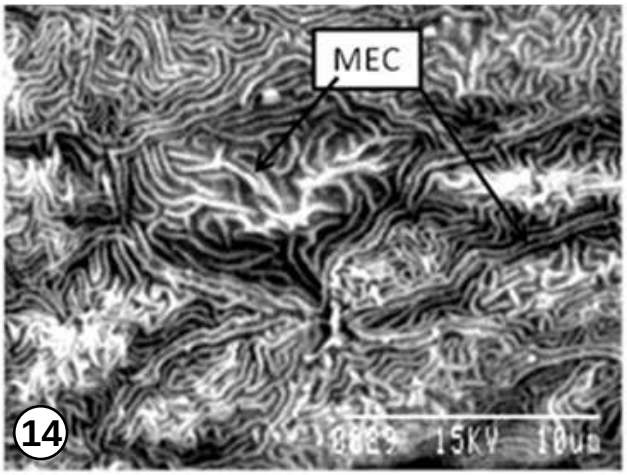
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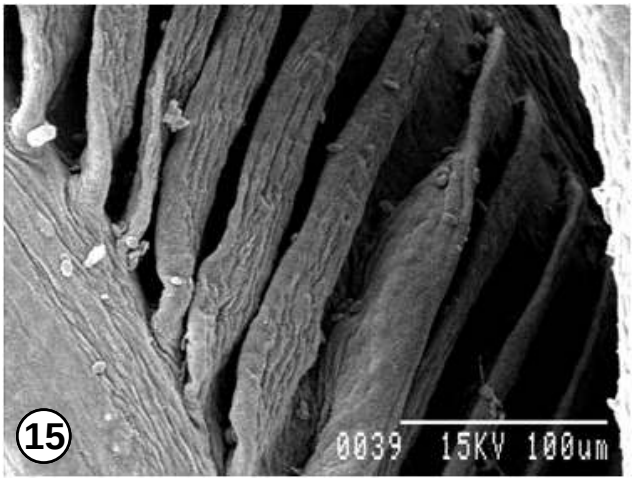
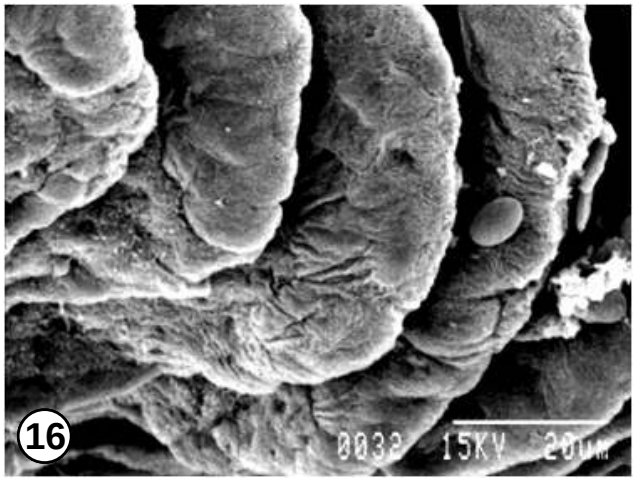
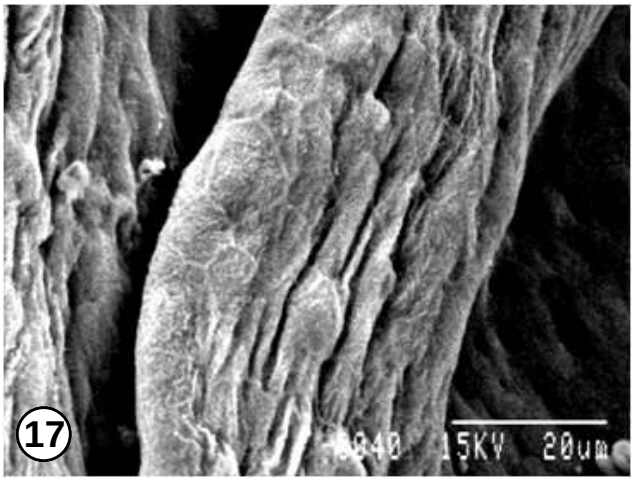
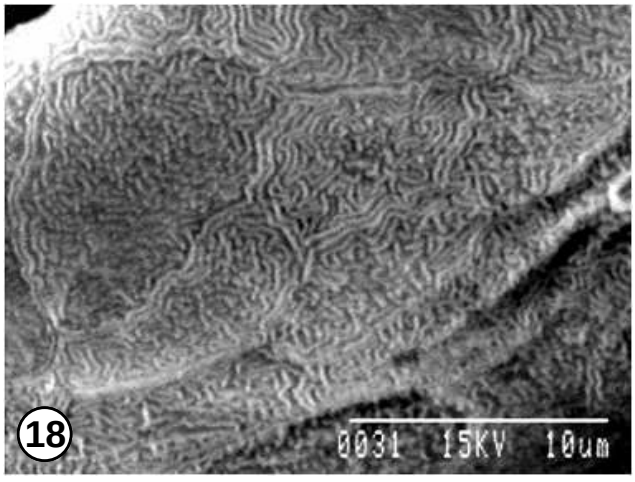
15KV 10um









Explanation to Figures

Scanning electron micrographs of gill of control fish, *C. punctatus* (Figure 1-6) Fig. 1 reveals normal architecture of Gill Filaments (GF) on Gill Arch(GA), Gill Racker (GR) is also present.

Fig. 2 shows arrangement of the gill Filament(GF) and Gill Lamellae(GL) under high magnification. Compact arrangement of epithelial cells is present in Gill Lamellae (GL).

Figs. 3, 4 and 5 showing Gill lamellae under higher magnification reveals Microridges in Epithelial Cells (MEC).

Fig. 6 showing Alignment of microridges on Microridge Epithelial Cell (MEC) and Mucous Gland Openings (MGO) is visible under higher magnification.

Fig.7 Scanning electron micrographs of gill of Acid treated fish, *C. punctatus* reveales no discernable damage to gill lamellae (GL; Microridges alignment are not disturbed in microridge epithelial cells(MEC).

Fig. 8.SEM Photomicrographs of gill of fish *C. punctatus* exposed to sublethal dose 140 mg/l Aluminium Sulphate. Severe fusion of gill lamellae(GL) partial and complete.

Figs. 9 and 10. Hyperplasia of epithelial cells in gill lamellae/

Figs. 11 and 12. Fusion of microridge epithelial cells (MEC) under higher magnification(Fig 13); Dealignment of microridges in epithelial cells.

Fig. 14. SEM Photomicrographs of gill of fish *C. punctatus* exposed to ameliorating agent 150mg/l of Silicon. Fig. 15 reveals absence of fusion in gill lamellae (GL); space between the gill lamellae are equal like in control condition.

Fig. 16. Epithelial cells of gill lamellae are returning to the normal architecture.

Figs.17&18. Alignments of microridges are resuming the normal arrangement of microridge epithelial cells.

Discussion

Relationships between pH and survival of fish in the laboratory and in selected natural systems have been studied since early times (Baker *et al.,* 1990). Acidification of soil may cause leaching of Al metal which affect water quality in the drainage area (Ahtiamen, 1992). Al mobilizes in its soluble form from soil to aquatic ecosystems and affects aquatic organisms and become the most important factor responsible for fish kills in acidified lakes (Walker *et al*., 2001).

The solubility of aluminium is highly pH dependant, increasing at both low and high pH values reflecting the amphoteric nature of the element. This relationship, coupled with the substantial reservoir of aluminium in soils and sediments, means that dissolved aluminium concentrations can be substantially higher in acidic or poorly buffered environments when subjected to sustained or periodic exposure to strong acid inputs. Under such circumstances aluminium may be transported from soil to surface waters. Acidic deposition, afforestation, the cessation of liming and sulphide oxidation all contribute to acidification and the release of previously bound aluminium. For most freshwater fish, aluminium toxicity is maximum at pH values around 5.5 (Palmer *et al.*,1988; Guibaud and Gauthier, 2003; Naskar *et al.,* 2009,), with little toxicity being exhibited above pH 6.5.The mobilization of Al in soluble forms from soil to the aquatic ecosystem is an important consequence of acidification of lakes and streams (Stutter *et al.,* 2001; Palmer and Driscoll, 2002; Driscoll *et al.,* 2003).

Gills act as an interface between fish body and its environment. It is an important organ for gaseous exchange, acid-base balance, ion regulation and ammonia excretion (Das *et al*, 2006). The gills are a multi-functional organ playing vital role in osmoregulation of fish (Hwang and Lee, 2008). This organ represents the main target-organ of pollutants due to its extensive surface area in contact with the external environment and the very thin barrier between the environmental water and internal milieu of fish (Dang *et al.,* 2000; Cerqueira and Fernandes, 2002). The mechanism of aluminium toxicity to fish has been attributed to the inability of fish to maintain their osmoregulatory balance and respiratory problems associated with coagulation of mucus on the gills, the former effect being associated with lower pH levels. Fish produces mucus to combat the aluminium in their gills. This mucus builds up and clogs the gills so that oxygen and salt transport is inhibited. Fishes are then unable to regulate their body salts and it results in osmoregulatory disturbance. The fusion of the lamellae together with the accumulation of Al on the gills in the exposure at pH<5 caused low oxygen uptake and asphyxiation of the fish. Clogging of the gills with mucus will raise the diffusional resistance to O_2 and reduces the water flow through the secondary lamellae which leads to asphyxia. Aluminium also precipitates in the gills and interferes with the transport of Oxygen, so that fish die of suffocation.

When Aluminium is in acidified ambient water the effect is concentrated mainly in the gills and the physiological processes related to this organ (Waring and Brown, 1995; Cole *et al.,* 2001; Teien *et al.,* 2006). Toxic levels of aluminum can have negative impacts on the fish gill epithelium, which is an important structure for gas exchange, ion regulation, acid-base balance, and the excretion of nitrogenous wastes (Evans, 1987). Respiratory effects are thought to be caused by the polymerization or precipitation of aluminum in acidic water as the water enters gills that are higher in pH (Gensemer and Playle 1999). The inorganic mononuclear aluminium complexes are more toxic than the organically-complex forms to aquatic organisms. Toxicity appears to be caused only by aluminium hydroxyl complexes, especially $Al(OH)^{2+}$ (Helliwell *et al.,* 1983). Exposure to Al induces gill damage due to increased mucus production, which alters osmoregulation and respiratory processes (Exley *et al,* 1997) and thereby causes hypoxia, hypercapnia, metabolic acidosis and finally respiratory failures (Allin and Wilson, 2000; Royset *et al.* 2005). In the gills, hypoxia has been associated with an adaptive increase in lamellar surface area in fishes (Chapman *et al.* 2000; Sollid *et al.*2003; van der Meer *et al.*. 2005) as well as gill epithelial hypertrophy and hyperplasia, goblet cell proliferation with increased mucus secretion, hemorrhage, edema and telangiectasis (Scott and Rogers, 1980).

Present investigation reports a substantial decrease in the extent of gill damage following treatment by Silicon dioxide in Al-Acid treated fish. It has been reported earlier that ligands such as humic, fulvic, and tannic acids, fluoride, phosphate, and silicate decrease toxicity by complexing aluminium (Helliwell *et al.*,1983). Complexity with Silicon dioxide tends to lower the Al toxicity. Under equilibrium condition, Silicon dioxide forms orthosilicic acid with water and Hydroxyaluminosilicates (HAS) are formed by the reaction of silicic acid with aluminium (Exley & Birchall,1992,1993) whereas Birchall (1995) has suggested that soluble silica is essential to living organisms because it binds endogenous aluminium and prevents its toxicity and reduces aluminium bioavailability, toxicity or both (Carlisle, 1987; Quartley *et al.,* 1993; Birchall *et al.,* 1989; Edwardson *et al.*,1993; Bellés *et al.*,1998; Exley *et al.*;1997). Oligomeric silica has much higher affinity for

aluminium than monomeric silica (Jugdohsingh,2000). Biological importance of Silicon has become increasingly recognized and the major importance of silicon is in limiting the bioavailability of aluminium (Birchall,1991). Modest levels (100µmol/l) of silicic acid in water can protect against aluminium toxicity in fish (Birchall *et al.*, 1989; Exley,1991). Aluminium has a close chemical affinity with silicon, with which it reacts to form hydroxyaluminosilicates (Birchall, 1992). Silicon has also been reported to play a preventive role both in accumulation of aluminium and in aluminium related disease in dialysis patients (Parry *et al*.1998).

Hence it can be concluded that in acid-aluminium toxicity induced fusion of the lamellae, hypertrophy and hyperplasia of the epithelial cells significantly increased the diffusion distance across the gills. The precipitation of Al on the gills may also have increased this diffusion distance which in its turn has a negative effect on oxygen uptake of the fish and causing mortality. Silicon dioxide has an ameliorating effect in Al toxicity by preventing the lamellar fusion and hypertrophy in the gills. It also helps to resume the normal alignment of microridges in epithelial cells.

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On-line Monitoring Plant Biomass for Large-scale Production of *Thymus Vulgaris*, a Medicinal and Culinary Herb

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Abstract : In the present study the mass scale culture of *Thymus vulgaris* L. was made in a Balloon-type bubble column bioreactor (BTBB). It is suggested that *T. vulgaris* can be used as a model system for the formulation of a mathematical model and will be used for on-line monitoring of biomass. The biomass accumulation was monitored within the reactor through measurements of electrical conductivity as the measure of the ion uptake and refractive index as a measure of the sugar uptake. The correlation of electrical conductivity or refractive index of the medium and the biomass has been formulated in the form of a mathematical model as proposed by Ramakrishnan *et al.* (1999). The biomass in terms of dry weight that accumulated after 25 days in the 5 L reactor (BTBB) was 3.151 gm/L. Destructive and on-line methods of biomass estimation gave similar results. The productivity of thymol, an active ingredient with distinct flavour and aroma in the shoots of *T. vulgaris* L was estimated to be 88.228 µg/L for each run in the bioreactor.

Keywords: *Thymus vulgaris*, On-line monitoring, Bioreactor (BTBB), Electrical conductivity, Refractive index, Mathematical model.

Introduction

Thymus vulgaris is a medicinal herb native to the Mediterranean region. The plant can be cultivated at temperature between 12- 15 degree Celsius with mild moisture by shoot cuttings or from seeds. Hence it can be cultivated in India only in the hilly regions. The plant is known for its aroma present maximally in the leaves, stem and purple flowers. The plant is also used for culinary purposes. The whole herb is used in the treatment of digestive disorders, sore throats, fevers, etc., as reported by Selmi and Sadok (2008). The medicinal properties of the herb are due to the essential oil present in the epidermal oil glands. The active ingredients that are present in the oil are monoterpenes and other derivatives. Reda *et al.* (2005) confirmed that the thymol is the major constituent in the essential oil of *T. vulgaris*. On account of its rich source of thymol, the propagation using tissue culture may be employed for a large scale production of the plant. Regeneration of shoots from meristem tips are often used as initial explants for large-scale micropropagation of *Thymus vulgaris* as suggestred by Vila *et al.*, 2002; Prehn *et al.*, 2003.

Ramakrishnan *et al.* (1999) studied the monitoring biomass in root culture systems. They stated that the ability to predict fresh weight is important since it is proportional to the biomass volume fraction that determines mass transfer and other transport characteristics.

Different bioreactor designs are available providing the optimum environment for effective cell growth as well as secondary metabolite production (Eibl and Eibl, 2008). But Balloon-type bubble column bioreactor (BTBB) is more effective for on-line monitoring of plant biomass. Electrical conductivity measurements have been used by many researchers as an indirect method of biomass estimation in on-line monitoring of plant cultures in bioprocess engineering studies for its accuracy and efficiency (Ryu *et al.*, 1994; Thanh *et al.*, 2005). The biomass accumulation can be monitored within the reactors through measurements of electrical conductivity as the measure of the ion uptake and refractive index as a measure of the sugar uptake.

Materials and Methods

In the present study, the aseptic shoot cultures of *T vulgaris* was grown in MS media with 30g/L sucrose, then culture was placed in the 5 L bioreactor. To estimate the biomass that is accumulating during a bioreactor run, two media parameters viz., electrical conductivity (a measure of ionic strength) and refractive index (a measure of sugar content) were estimated. Later, the mathematical equation was used to estimate the biomass accumulated in the reactor. The correlation of biomass and electrical conductivity as well as refractive index has been shown in the work. In this research endevour, methods of on-line estimation of two critical reactor properties have been addressed, namely dry weight indicative of biosynthetic capacity of the shoot cultures of *T vulgaris* L. and fresh weight indicative of actual physical space occupied by the shoot cultures in reactors which has a direct impact on the mass transfer

requirements in a reactor. The correlation of electrical conductivity or refractive index of the medium with the biomass has been formulated in the form of a mathematical model which will enable us to predict the biomass that would be obtained after a run in a Balloon Type Bubble column bioreactor (BTBB).

Shake flask studies: The optimized tissue density for shoot cultures of *T. vulgaris* L were aseptically cultured in sterile modified MS (Murashige and Skoog, 1962) medium with 30 g/L of sucrose.

Optimizing inoculum density: It was decided by growing cultures of *T vulgaris* in 250 mL tissue culture bottles with 30 mL sterile modified MS medium supplemented with 30 g/L sucrose. The inoculum densities in these experiments were varied from 1-10 g FW/L and the duration of growth period was 30 days (corresponding to the log phase of growth). All the experiments were done in triplicates.

Time course profile using shake flasks: To determine the time course profile using the shake flasks, the bottles were kept on a gyratory shaker and harvested in triplicates at intervals of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 days. Harvested bottles were subjected to fresh weight, dry weight and entrained liquid volume measurements. The fresh weight was estimated by removing the excess medium from the shoots using a blotting paper while the dry weight was estimated after drying the shoots for 48 hours. The freely drained liquid volume was used to estimate liquid medium properties such as electrical conductivity (Model EQ-667 conductivity meter; Equip-tronics, Mumbai, India), refractive index (Temperature compensated ERMA INC hand refractometer, Tokyo, Japan) and pH (Model-PHAN, Labindia Instruments Pvt Ltd, Navi Mumbai, India). The fresh weight and dry weight were determined on a digital weighing balance (Model-AUW220D, Shimadzu Corporation, Kyoto, Japan).

Balloon type bubble column bioreactor (BTBB): Correlation factor obtained from the shake flask studies were used for on-line monitoring of biomass in a 5 L Balloon-type bubble column bioreactor (BTBB) made of borosilicate glass. A sintered glass located at the base of the reactor body was used for aeration and the air flow was adjusted to 3.0 L/min during the cultivation to allow sufficient mixing. The bioreactor has a sampling port at the bottom that allows for the removal of medium aliquots for on-line monitoring. The lid of the reactor is made to fit tightly into the mouth of the reactor and it has two opening which is fitted with tubing made of silicon. One of them serves as an air vent, while the other opening aids as an inoculation port for fresh medium, antifoam, acid or alkali to adjust pH, etc, hence it is fitted with a bacteria proof filter. The bioreactor unit was sterilized by autoclaving at 121 °C and 15 psi for 30 min. The sterile reactor unit was then mounted in a laminar air flow cabinet. Two liters of sterile modified MS medium supplemented with 30 g/L of sucrose was poured into the bioreactor. It was then inoculated with actively growing aseptic shoot explants of *Thymus vulgaris* L at the optimum density that was standardized during the shake flask studies. The reactor was maintained at a temperature of 26 ± 2 °C with a photoperiod of 12 hrs with photon flux density of 70μmolm^{-2}s^{-1}.

On-line monitoring of biomass in the bioreactor: The day of inoculation or the 0 day sampling was done by aseptically aliquoting 2 mL of medium from the bioreactor containing the explant using the side outlet. The medium conductivity, refractive index and pH were measured by the instruments mentioned above. At intervals of 5 days, 2 mL aliquots were sampled for measurement of electrical conductivity, refractive index and pH. Later these data were used to predict the biomass of the explant in the bioreactor.

Results and Discussion

Shake flask studies

The inoculum density selected was 5g/L showing specific growth rate as 0.313 day^{-1}. Inoculation density has been known to affect the growth performances of plants. At very low densities, plants do not grow normally, because of loss of essential substances by diffusion from the cell into the external medium is in confirmation to the observations of George and Sherrington (1984). At higher densities, growth inhibition of plants is known to occur due to production of toxic metabolites. Hence it is necessary to identify an optimal inoculum density to conduct bioreactor studies based on batch growth experiments conducted at different inoculum densities.

Shake flask studies to identify potential on line monitoring technique

The shake flask studies give valuable insight into various aspect of plant tissue culturing using aseptic conditions. This is so because the shake flask studies are the benchmark against which reactors are compared and strategies formulated to get the best outcome from a large scale bioreactor (McKelvey *et al.,* 1993). A number of parameters that were analyzed during the on-line monitoring of shake flask studies of shoot cultures have been compiled in Fig.1. These parameters include electrical conductivity, refractive index and fresh weight of the biomass. These parameters are significant as they help in correlating as well as monitoring the shoot growth in larger volume reactors. The graphical

representation of the growth (Fig.1) shows a pattern of smooth curve that is referred to as sigmoid growth curve for both fresh and dry weight measurements. A growth curve was established to determine the growth characteristics and growth pattern of the shoot cultures. The 'lag' phase was from day 0 to day 10 during which very little growth was observed. The 'log' phase shows a dramatic increase in shoot growth which occurred during day 10 to day 25. During the 'log' phase of growth, the growth rate was calculated and found to be 1.041 g/day with a generation time of approximately 23.064 hrs per gram of the shoot cultures.

Fresh weight and dry weight correlation

The decrease in electrical conductivity and refractive index in the growth medium is consistent with biomass accumulation in shaking flasks. Electrical conductivity is an indicator of the inorganic components in plant tissue culture medium, while refractive index is a measure of the total sugar content in the liquid medium.

A scatter plot of the biomass accumulation in terms of δFW, (where δ indicates difference in final and initial) versus EC based ion uptake, δ(EC V) and refractive index based sugar uptake, δ(RI V) show linear correlations with FW accumulation (Fig. 2 and 3). For *T. vulgaris* L shoot cultures, the ionic yield was 0.0299 g FW per (mS. mL) of EC V while the carbon yield was 0.0565g FW per (g. mL) of RI V. The ability to predict fresh weight is particularly important since this is proportional to the biomass volume fraction which determines mass transfer and other culture transport characteristics. The observation of the present study is in conformity to the findings of Ramakrishnan *et al.* (1999).

A similar scatter plot of the biomass accumulation in terms of δDW versus EC based ion uptake, δ(EC V) and refractive index based sugar uptake, δ(RI V) shows linear correlations with DW accumulation (Figs. 4 and 5). These correlations are not only plots of biomass accumulation versus measured nutrient concentrations (that is electrical conductivity and refractive index) but are also a measure of mass uptake. A linear correlation between DW accumulation and nutrient uptake implies that the ionic yield and carbon yield are constant. For *T. vulgaris* L shoot cultures, the ionic yield is 0.0025 g DW per (mS. mL) of EC V while the carbon yield is 0.0047g DW per (g. mL) of RI V.

Model implementation

Mathematical models of biological processes are often used for hypothesis testing and process optimization. Physical interpretation of results to obtain

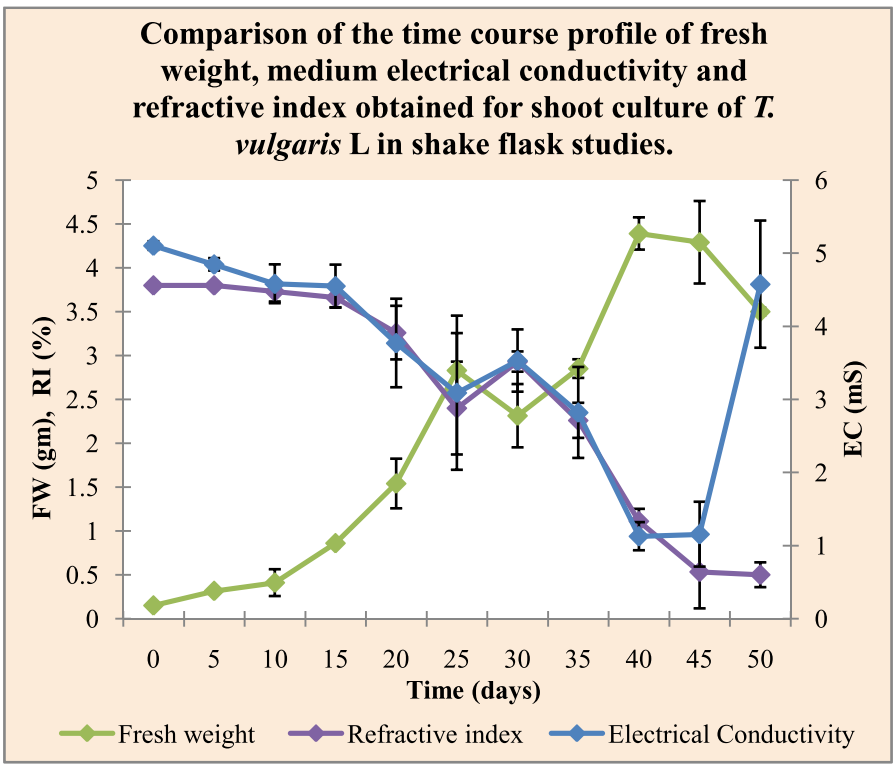

Fig. 1

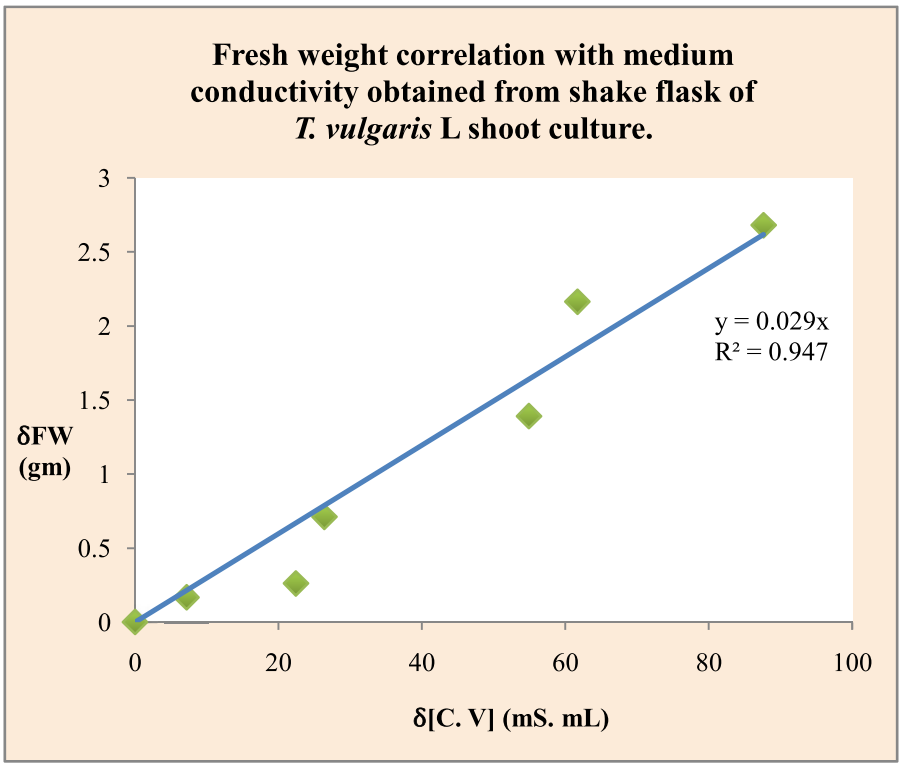
Fresh weight correlation with medium conductivity obtained from shake flask of *T. vulgaris* L shoot culture.
δFW (gm)
δ[C. V] (mS. mL)
y = 0.029x
R² = 0.947
0
0.5
1
1.5
2
2.5
3
0
20
40
60
80
100

Fig. 2

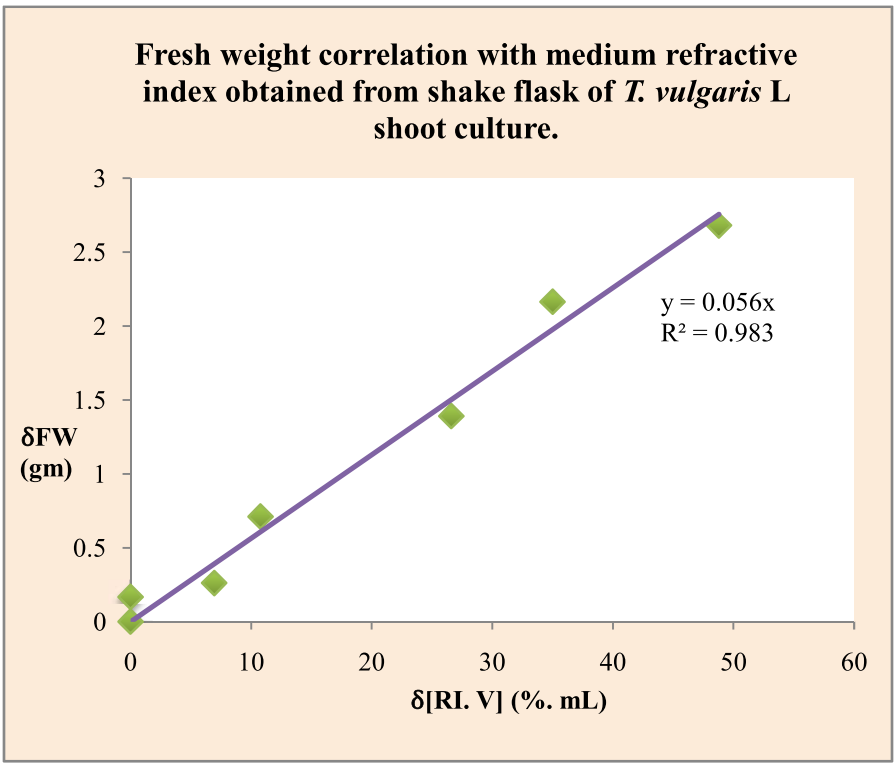
Fresh weight correlation with medium refractive index obtained from shake flask of *T. vulgaris* L shoot culture.
δFW (gm)
δ[RI. V] (%. mL)
y = 0.056x
R² = 0.983
0
0.5
1
1.5
2
2.5
3
0
10
20
30
40
50
60

Fig. 3

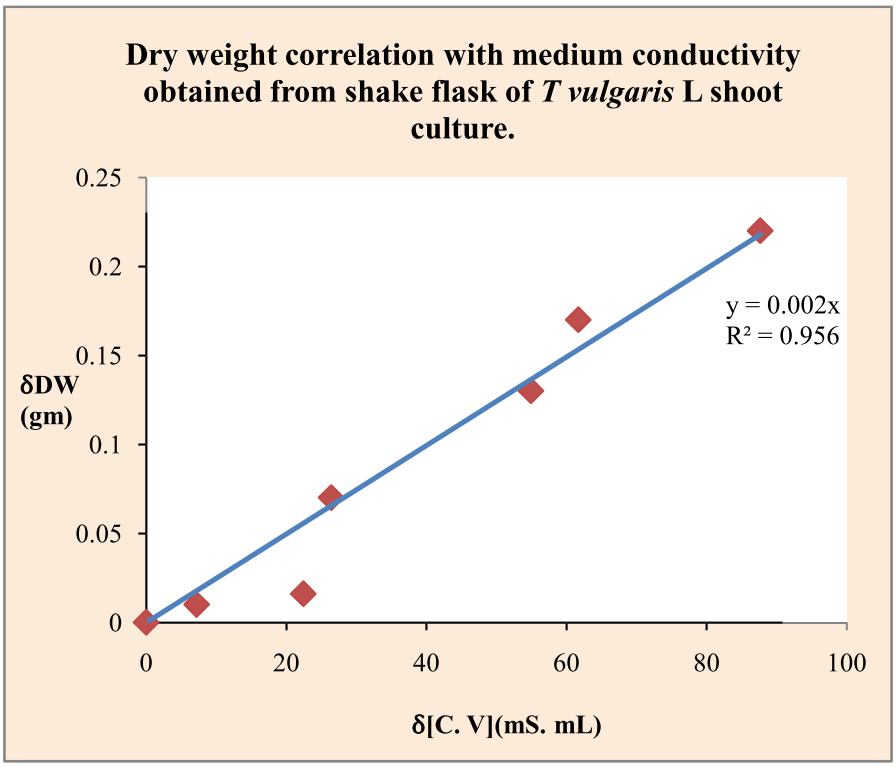
Dry weight correlation with medium conductivity obtained from shake flask of T vulgaris L shoot culture.
0.25
0.2
0.15
0.1
0.05
0
δDW (gm)
y = 0.002x
R² = 0.956
0
20
40
60
80
100
δ[C. V](mS. mL)

Fig. 4

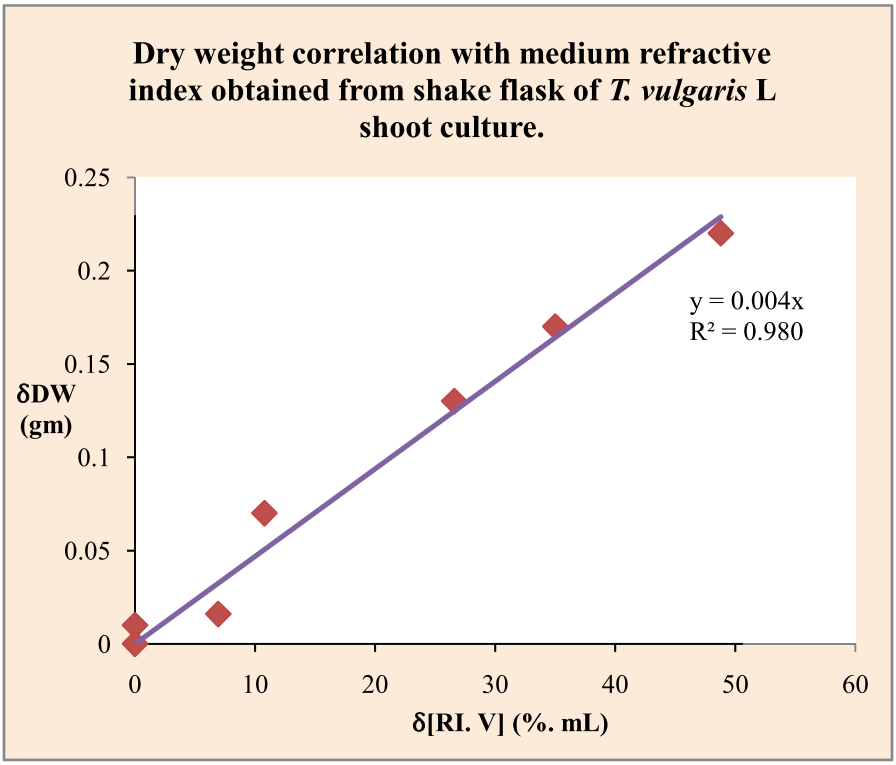
Dry weight correlation with medium refractive index obtained from shake flask of T. vulgaris L shoot culture.
0.25
0.2
0.15
0.1
0.05
0
δDW (gm)
y = 0.004x
R² = 0.980
0
10
20
30
40
50
60
δ[RI. V] (%. mL)

Fig. 5

greater insight into process behavior is possible when structured models that consider several parts of the system separately are employed. The information obtained is very useful for large scale bioreactor cultivation of plant tissues (Ramakrishnan *et al.*, 1999).

Large scale aseptic processing of plant shoot cultures requires on-line monitoring of biomass based on correlation because it is not possible to remove representative aliquots of biomass to verify growth. The reactor growth performance can be expressed as either dry or fresh weight accumulation where the dry weight is the indicator of biosynthetic capacity and the fresh weight is an indicator of physical space occupied by the shoot culture. The typical form of representation of a mathematical model is a simple proportionality between the decline in the medium conductivity and the increase in biomass, which is expressed as under:

$$(DW - DW_{\circ}) = K(C_{\circ} - C) \text{ or } (FW - FW_{\circ}) = K(C_{\circ} - C)$$

(DW = dry weight in grams, $DW_{\circ}$ = initial dry weight in grams, FW = fresh weight in grams, $FW_{\circ}$ = initial fresh weight in grams, C = conductivity in mS, K = proportionality constant (g/mS. mL), $C_{\circ}$ = initial conductivity in mS

A similar correlation based on sugar uptake can also be applied for shoot cultures.

The model was then validated with time course data from the shake flask studies. The empirical correlation was implemented in a mass balance model utilizing conductivity and refractive index. The time course profile of biomass (g DW/L) obtained from shake flask studies for shoot cultures of *T. vulgaris* L grown for 30 days by destructive harvest method and the biomass estimates using conductivity correlation is depicted in Fig. 6. A similar profile was noticed for the refractive index correlation (Fig 7). As is evident from the Figs. 6, 7, 8 and 9, the observed and observed biomass showed increase as a function of time and exhibited similar trend. Therefore, for the scale up studies these empirical correlations were implemented. The proposed model has been validated with time course information (DW and FW) from shake flasks and corroborated with data obtained from the 5 L bioreactors.

On-line monitoring of biomass in BTBB

Fig. 10 depicts the on-line profile of electrical conductivity and refractive index of the medium from the BTBB. The negative slope indicates consumption of ions and sugars by the shoot cultures. Figs. 11 and 12 depicts time course estimates of biomass. Both the graphs show a similar trend in growth.

The growth curve of the shoot cultures failed to show a 'lag' phase which is generally expected, rather the growth pattern is linear and shows only the 'log' phase from day 0 to day 20. The reason for the shoot

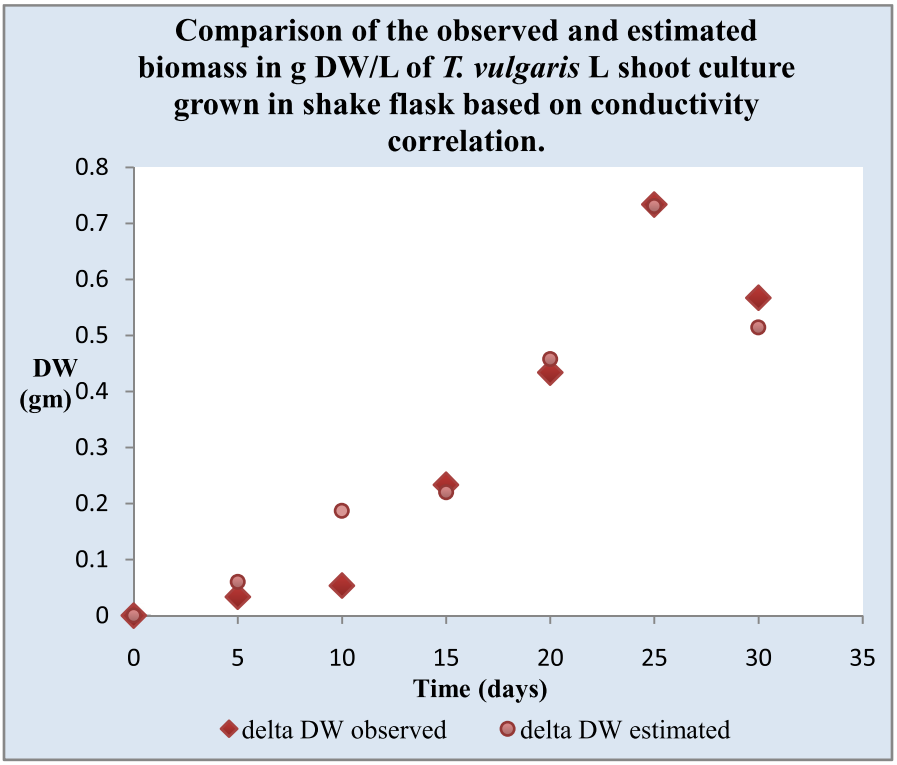

Fig. 6

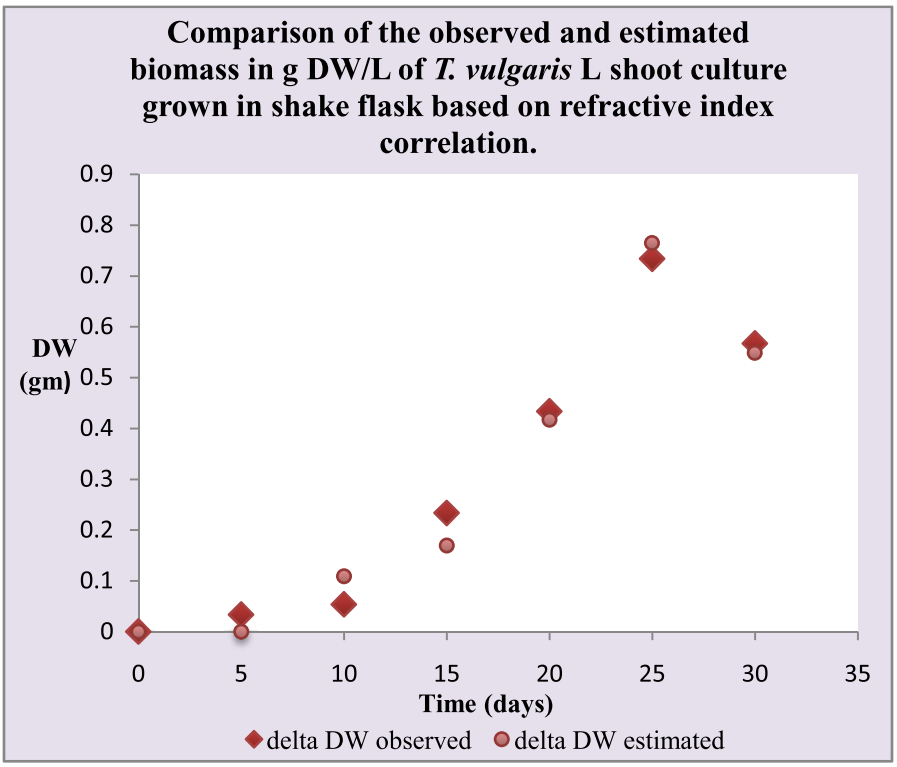
Comparison of the observed and estimated biomass in g DW/L of T. vulgaris L shoot culture grown in shake flask based on refractive index correlation.
DW (gm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
Time (days)
delta DW observed
delta DW estimated

Fig. 7

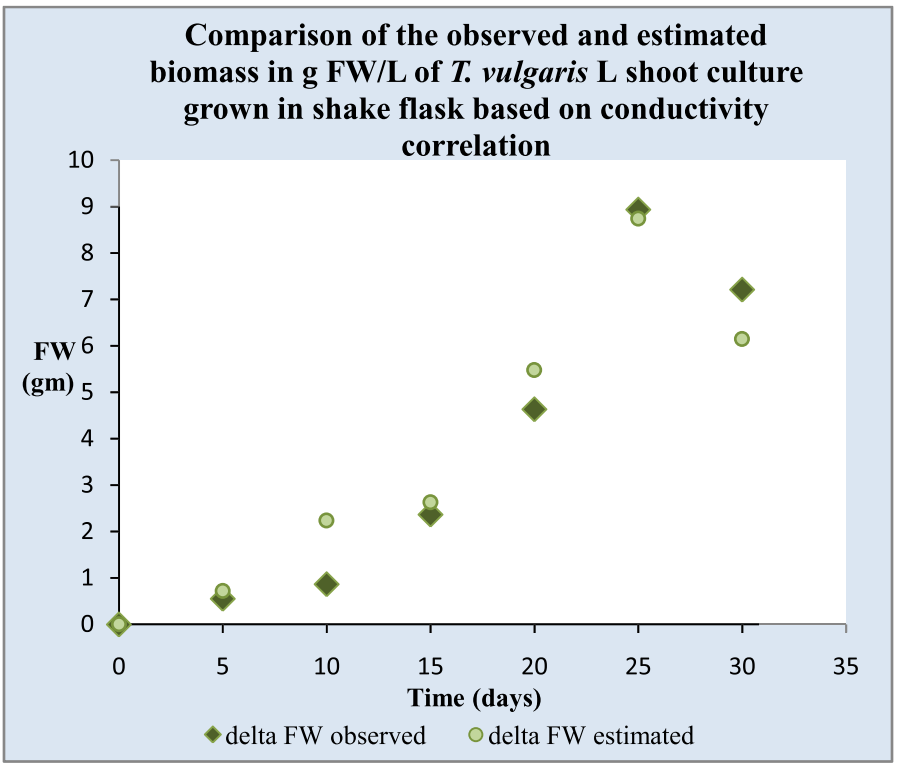
Comparison of the observed and estimated biomass in g FW/L of T. vulgaris L shoot culture grown in shake flask based on conductivity correlation
FW (gm)
10
9
8
7
6
5
4
3
2
1
0
0
5
10
15
20
25
30
35
Time (days)
delta FW observed
delta FW estimated

Fig. 8

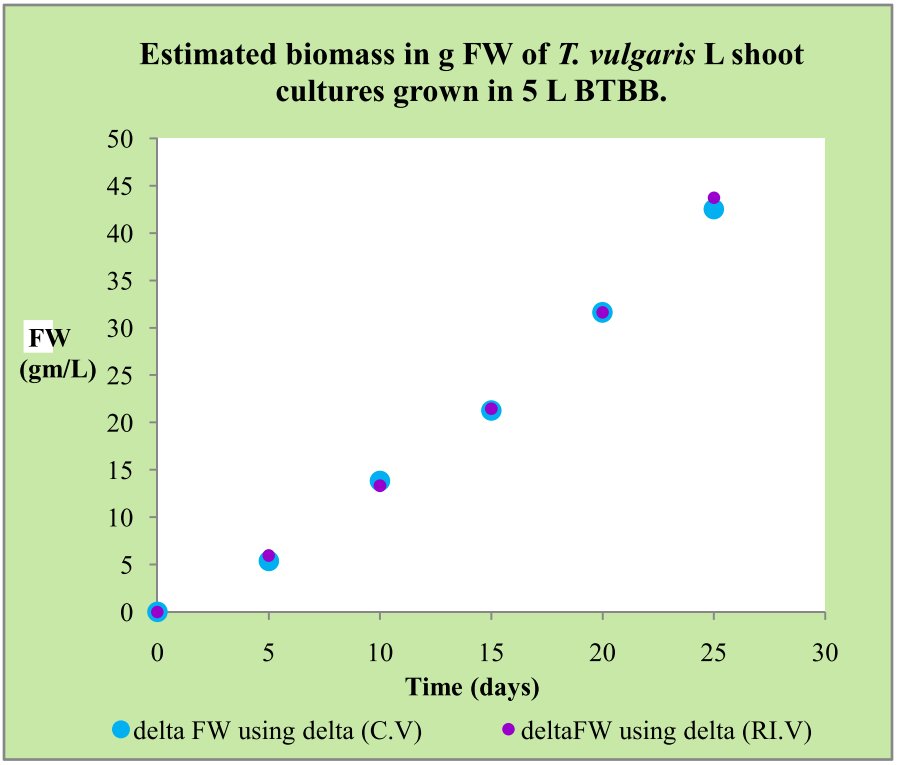
Estimated biomass in g FW of T. vulgaris L shoot cultures grown in 5 L BTBB.
FW (gm/L)
50
45
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
Time (days)
delta FW using delta (C.V)
deltaFW using delta (RI.V)

Fig. 11

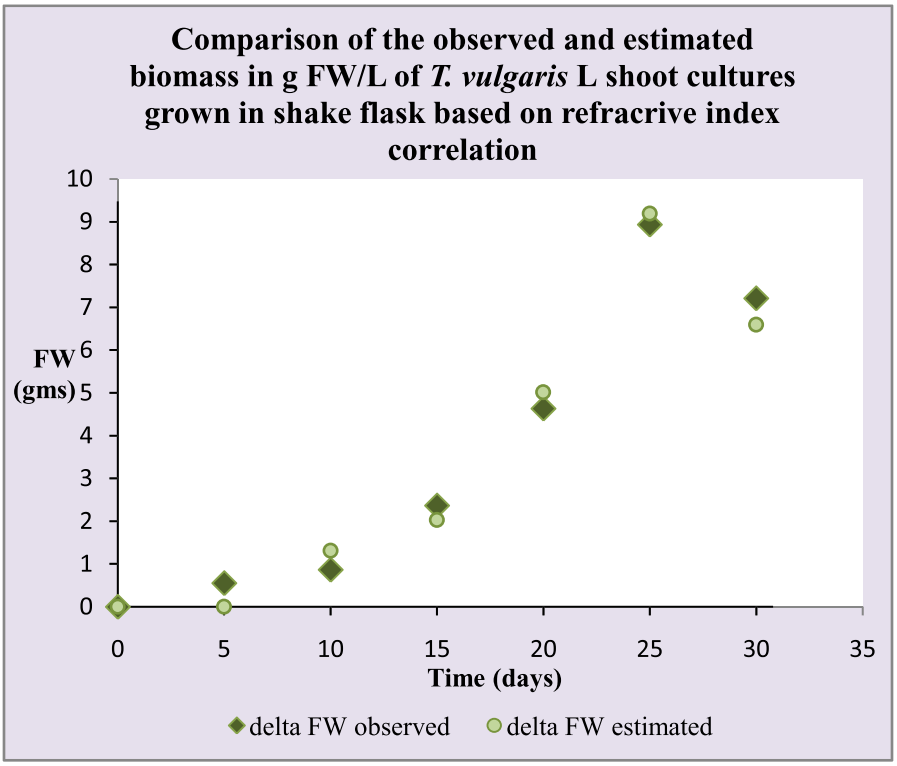
Comparison of the observed and estimated biomass in g FW/L of T. vulgaris L shoot cultures grown in shake flask based on refracrive index correlation
FW (gms)
10
9
8
7
6
5
4
3
2
1
0
0
5
10
15
20
25
30
35
Time (days)
delta FW observed
delta FW estimated

Fig. 9

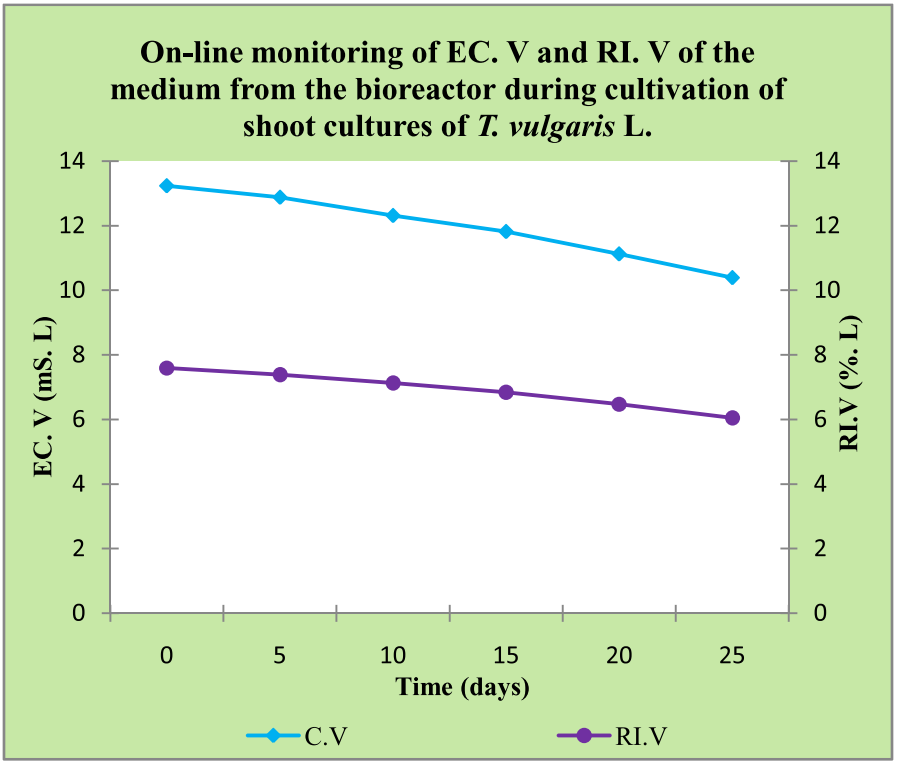
On-line monitoring of EC. V and RI. V of the medium from the bioreactor during cultivation of shoot cultures of T. vulgaris L.
EC. V (mS. L)
RI.V (%. L)
Time (days)
C.V
RI.V

Fig. 10

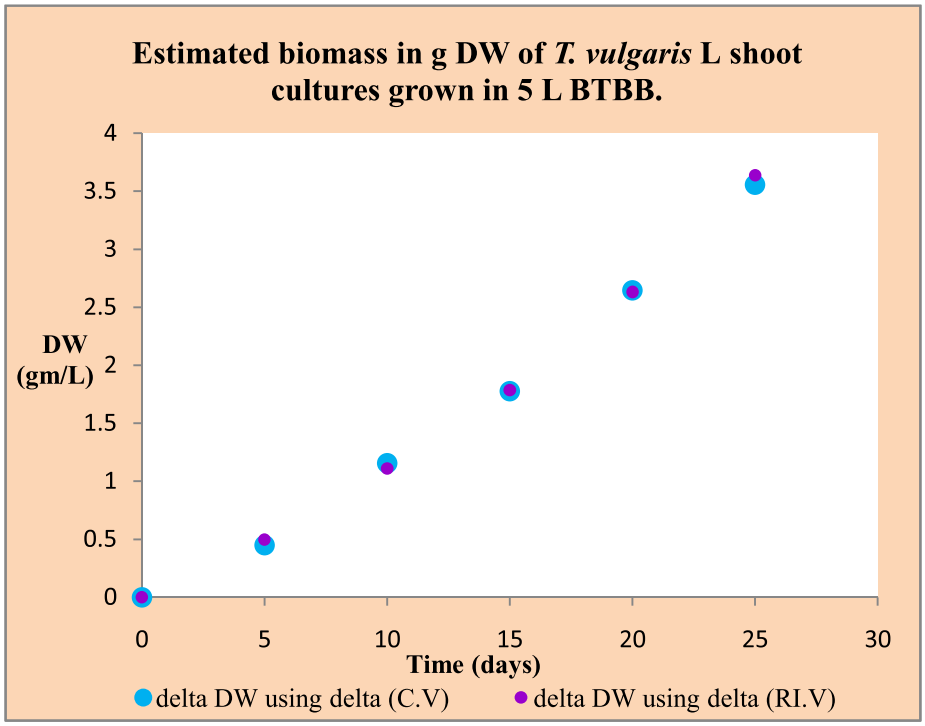
Estimated biomass in g DW of T. vulgaris L shoot cultures grown in 5 L BTBB.
DW (gm/L)
Time (days)
delta DW using delta (C.V)
delta DW using delta (RI.V)

Fig. 12

cultures showing this kind of growth pattern may be due to the stage of growth at which they were seeded into the bioreactor. The shoot cultures were in the 'log' phase of growth in 250 mL tissue culture bottles. They were then transferred into the bioreactor with ample nutrients in the form of ions as well as sugar in the form of sucrose. Moreover, in the bioreactor they are being constantly supplied with sterile air at a constant rate as compared to the shake flasks where aeration is not adequate.

After the 25 day growth of the shoot cultures in the 5 L bioreactor, they were collected and drained off the excess medium. The shoot explants were then weighed and compared to the estimated value. The shoot explants were then dried between sheets of blotting paper at room temperature for 48 hours. The dry weight was also then compared to the estimated values. Table 1 shows the comparison of estimated and actual biomass in terms of fresh weight and dry weight.

The scale-up aspect shows that air driven balloon type bubble column bioreactors are a good option for large scale cultivation of shoot cultures. The refractive index (indicator of sugar content) and electrical conductivity (indicator of ionic content) have been shown to correlate with dry weight as well as fresh weight accumulation of the shoot cultures. The thymol productivity in the shoots of *T. vulgaris* L for each run of the bioreactor was estimated to be 88.228 µg/L.

Table 1 : Comparison of estimated biomass and the actual biomass obtained in the 5 L balloon type bubble column bioreactor.

***T. vulgaris* L shoot culture**	**Observed biomass**	**Estimated biomass**	
		Using EC correlation	**Using RI correlation**
Fresh weight (g/ L)	40.317	42.53	43.731
Dry weight (g/L)	3.151	3.556	3.638

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First record of metacercaria of a rare reptilian trematode *Kaurma* Chatterji, 1936 from a gastropod *Pila globosa* Swainson, near district Barabanki.

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Abstract : Chatterji, 1936 established the genus *Kaurma* Chatterji, 1936 with *E. longicirra* as its type specis, for the adult trematodes, collected from the intestine of a freshwater turtle *Emyda scutata* Peters, 1868 of Rangoon and placed it under the family Allocreadiidae Stossich, 1904 and the subfamily Allocreadiinae Looss, 1899. During course of present investigations on the trematodes of snails of district Barabanki, several specimens (37) of *Pila globosa* Swainson examined, the gut of only four was found infected with the metacercarial stage of trematodes. A careful and thorough examination revealed them to be of the genus *Kaurma* Chatterji, 1936 as they bear all the characters of the adult, except the eggs. Gastropods, in general act as intermediate hosts in the life cycle of trematodes but records of metacercariae or adults in *Pila globosa* are not many in India [Anjaneyulu (1967)**,** Murty (1970) and Pandey (1973)]**.** The present contribution is second in its series by the present authors.

Key words: *Kaurma,* metacercaria, *Pila globosa*

Introduction

Pila globosa, commonly known as apple snail, an amphibious gastropod, is commonly found in ponds, pools, lakes, marshes, paddy fields, streams and rivers, having dense vegetation of Northern India and has also been reported from brackish waters. Its distribution ranges from Assam, Bihar, Himachal Pradesh, Jharkhand, Maharashtra, Madhya Pradesh, Meghalaya (Garo hills South), Orissa, Rajasthan, Uttar Pradesh, West Bengal but surprisingly not recorded in Punjab (Ramakrishna and Dey, 2007). It is a voracious feeder, consuming aquatic vegetation like *Pistia* and *Vallisnaria.* It serves as a food resource of villagers and its shell is also used in traditional medicines.

Material and Methods

The snails were collected from 'Naya Tal' covering an area of about one acre in Garhi, near district Barabanki. They were brought to the laboratory in small earthen pots and maintained in small water pools. They were fed with *Vellisnaria* and *Pistia* leaves and dissected at convenience. Almost all the body organs like foot, pulmonary sac, organs of pallial complex and gills were carefully examine under binocular microscope, in small petridishes containing normal saline. Larvae, thus collected, were first studied alive and subsequently flattened, with the help of a cover slip on slide, fixed overnight in 70% alcohol, stained with Aceto-alum Carmine, dehydrated in ascending grades of alcohol, cleared in Xylol and mounted in Canada Balsam. Figures were drawn with the help of drawing tube, attached to a Phase Contrast microscope (Olympus CX-41) and measurements (in millimeters) were taken with the help of an occulometer.

Description

Aspinose body is oval, 2.82 -3.10 mm x 0.88-1.53 mm. Oral sucker is spherical, sub-terminal 0.32-0.38mm x 0.27-0.37mm. Ventral Sucker is 0.28-0.46 mm x 0.27-0.45 mm, larger than oral sucker, situated in the middle of body. Prepharynx is indistinct, muscular pharynx measures 0.14-0.20mm X 0.15-0.23mm. Oesophagus is absent. Intestinal bifurcation, in some specimens, is partially overlapped by pharynx. Intestinal caecae are long, uniform in width and extend up to posterior extremity of body. Testes are tandem, transversely elongated, situated in the posterior third of body, 0.24-0.66mm x 0.13-0.22mm - 0.17-0.41mm x 0.14 - 0.29 mm respectively. Vas defernces runs anteriorly and opens in cirrus sac. Cirrus sac is saccular, broad in middle and narrow anteriorly and posteriorly, 0.11-0.24 mm x 0.06-0.11mm, anterior to ventral sucker slightly bending towards right side of body. It encloses a coiled vesicula seminalis, pars prostatica, ductus ejaculatorious and a cirrus. Genital pore is inter-caecal, towards right side of body. Ovary is globular, 0.11-0.15 mm x 0.11 - 0.13 mm, slightly towardly right of median line, situated posteriorly close to ventral sucker. Laurer's canal is not seen. Ootype is somewhat larger than ovary, 0.10-.15mm x 0.07-0.17 mm. Uterus is short,visible only in between anterior testis and ventral sucker. It arises from ootype, runs anteriorly in a zigzag manner and forms a muscular metraterm to open into genital atrium through female genital aperture. Vitellaria consists of large follicles and are distributed in extracaecal and intracaecal fields, from anterior border of intestinal bifurcation to posterior end of body, merging posteriorly. Eggs are absent. Excretory bladder is "V" shaped and excretory pore opens outside at

posterior end of body.

Discussion

Chatterji, 1936 established the genus *Kaurma* Chatterji, 1936 with *E. longicirra* as its type specis, for the adult trematodes, collected from the intestine of a freshwater turtle *Emyda scutata* Peters, 1868 of Rangoon and placed it under the family Allocreadiidae Stossich, 1904 and the subfamily Allocreadiinae Looss, 1899. It is characterized by oval aspinose body, well developed suckers, oral sucker smaller than ventral sucker, oesophagus absent, pharynx well developed, intestinal caeca reaching posterior end of body, testes in posterior half of body, tandem, lobed, cirrus sac anterior to ventral sucker, having coiled vesicula seminalis, pars prostatica and ductus ejaculatorious, long and muscular cirrus, ovary pretesticular extreme close to ventral sucker, receptaculum seminis present, metraterm well developed, vitellaria between pharynx and hind end of body and confluent behind testes, pre-testicular uterus. Since the metacercaria under study has almost all the characters of *Kaurma* except eggs, in all probability it belongs to the genus. Surprisingly since then, neither the larva nor the adult of this parasite has been reported in India by Indian workers till date though certain species have been reported from different regions in South East Asia (Yamaguti, 1958). Report of metacercarial stage from a snail, is quite significant in this region. Though adults could not be obtained, it is most likely that the fresh water turtles feed on infected snails and the larvae develop to become adults in final host. Specific diagnosis is not possible till we get the experimentally developed adults.

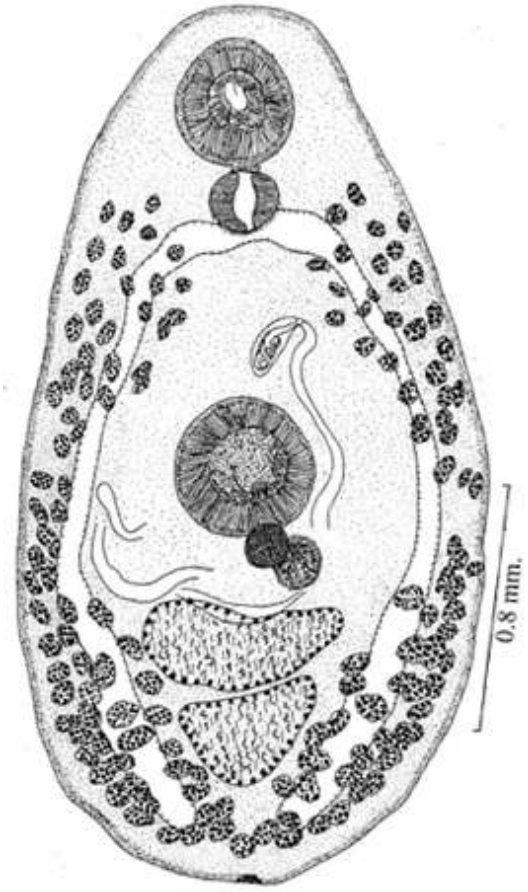

Fig. 1. Metacercaria of *Kaurma* Chatterji 1936

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Histoenzymological distribution of acetylcholinesterase in the Corpus cerebelli of two Indian air breathing teleosts.

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Abstract : The present study describes the distribution of acetylcholinesterase enzyme (AChE) in the various layers of corpus cerebella of brain of two Indian air breathing teleost, *Heteropneustes fossilis* and *Channa punctatus*. The distribution pattern of enzyme showed uneven distribution of enzyme in all the three layers of corpus cerebelli. The outermost molecular layer (ML) in *Heteropneustes fossilis* showed fainted activity for AChE, while the Purkinje cells (PC) present in the intermediate layer exhibit strong cholinesterase positive reaction in their cell bodies. The inner granular layer (GL) also demonstrated intense reaction to AChE. However, the molecular layer (ML) and the inner granular layer of corpus cerebelli of *Channa punctatus* also showed strong intense reaction in comparison to *Heteropneustes fossilis*. The Purkinje cells in the intermediate layer exhibit mild or no activity. It is suggested that the variability of AChE distribution may perhaps deals with noncholinergic role in addition to its main cholinergic role.

Key Words : Acetylcholinesterase, Purkinje cell, Granular layer, Cerebellum, Brain.

Introduction

Acetylcholinesterase (AChE) is a hydrolytic enzyme belonging to the family of type B carboxylesterase which hydrolyses the neurotransmitter acetylcholine in to choline and acetate at the neuromuscular junction (Appleyard and Johnson, 1992; Soreq & Seidmann, 2001; Kumar and Tembhre, 2010). Medically the acetylcholinesterase is an important for the diagnosis and treatment of neurodegenerative diseases such as cardiac and Alzheimer's diseases (Praveen & Kumar, 2005). The role of acetylcholinestrase is also used a biomarker in environmental biomonitoring by Lionetto *et al.* (2010)

AChE also plays some non-cholinergic function apart from its main cholinergic role. It plays a role in cell- adhesion, cell differentiation and neurogenesis (Silman & Sussman, 2005; Downes & Granto, 2004; Chub *et al*., 1980, 1982; Genever *et al*., 1999). Several studies on brain displaying AD lesions have shown changes in the expression and distribution of AChE (Talesa, 2001). These wide roles of AChE provide adequate base in functionally correlating it with its variable histochemical distribution.

The histochemical distribution of cholinesterases in the avian and mammalian brains has been well documented in the literature (Whittaker, 1953; Shute & Lewis, 1963; Krnjevic & Silver, 1964; Cookson *et.al*., 1996 ; Ishii & Friede, 1967; Bhatt & Tewari, 1978; Giris, 1980). The histology of the cholinergic nerves in the reptilian brains was studied by numerous investigators (Sethi & Tewari, 1976, 1977; Srivastava & Tripathi, 2007; Tripathi & Srivastava, 2007; Maurya and Srivastava, 2012. The distribution of AChE in fish brain has been studies by Contestebile & Zanoni (1975), Contestebile (1975) and Sood & Sinha (1983). Demble *et al*. (2000) studied the concentration effects of exposure of selected insecticideson the brain AChE in the common carp, *Cyprinus carpio L*.

Materials and Methods

Five adult males of *Heteropneustes fossilis* (Length 16 ± 18 cm, weight 35 ± 40 gm) and *Channa punctatus* (length 15 ± 17 cm, weight 45 ± 50 gm) were collected from the natural habitat of Ranchi district and acclimatized for laboratory. All the experiments were carried out according to ethical guidelines of Ranchi University, Ranchi.

Animals were anesthetized with 0.2% 2-phenoxy ethanol. Fishes were perfused transcordially with 500 ml solution of 0.5% paraformaldehyde and 1.5% gluteraldehyde in 0.1 M phosphate buffer (pH 7.4). Brain was dissected out and post fixed in the same solution for six hours. Brain was then given 2-3 changes in 15% sucrose solution in 0.1 M phosphate buffer and stored in the same solution for 1- 3 days. Brain was sliced at 30 ?m thickness on cryocut at 22°C. Serial sections were then processed for AChE staining described by Hedreen *et al.* (1985). Suitable controls were also maintained.

Results

Cerebellum of teleosts consists of three parts, the vestibulolateralis lobe, the corpus cerebelli and valvula cerebelli. Corpus cerebelli lies on the top of rostral rhombencephalon comprised the wide outer molecular layer (ML), middle thin intermediate layer and inner granular layer (GL) (Figs. 1 - 2).

The histological preparations of the corpus cerebelli of *Heteropneustes fossilis* shows that the outermost thick molecular layer (ML) showed fainted activity for AChE (Fig. 1). The irregularly arranged Purkinje cells (PC) were either ovoid or pyriform in shape were placed in the in the intermediate layer. The cell bodies exhibit deep strong reaction. The inner granular layer (GL) also showed intense reaction for AChE (Table - 1). In the corpus cerebelli of *Channa punctatus*, the molecular layer (ML) and inner granular layer both take deeper reaction along. The Purkinje cells present in the intermediate layer showed mild or no AChE activity (Fig. 2, Table - 1).

Discussion

Wulliman M.F. (1998) and Kumar and Tembhre (2010) described the anatomy and histology of the brain of fishes. In the present study,the cerebellum of these fishes also consists of three parts, the vestibulolateralis lobe, the corpus cerebelli and valvula cerebelli. Corpus cerebelli lies on the top of rostral rhombencephalon comprised the wide outer molecular layer (ML), middle thin intermediate layer and inner granular layer.

AChE activity in the different layers of corpus cerebelli demonstrated variability in both the species examined The presence of AChE in Purkinje cells in different fish species have been reported in *Salmo* ,*Poecilia* , *Carassius*, *Phoxinus*, *Porichtys* , *Acipenser*, *Scyliorhinus* and *Danio* (Contestebile & Zanoni (1975); Contestebile *et al.*, 1977, 2004; Ekstrom, 1987; Brantley and Bass, 1988; Adrio *et al*., 2000; Anadon *et al*., 2000; Perez *et al*., 2000). In the present investigation the cholinergic Pukinje cells were not observed in the cerebellum of teleosts as noticed by Clemente *et al*. (2004). AChE positive granule cells were also observed in several fish species (Centestebile and Zannoni, 1975; Contestebile *et al*., 1977;) and in other vertebrates (Contestebile and Tabanelli, 1977; Willani et al. 1977; Kusunoki et al. 1987; Robertson & Roman, 1989). However. The results of the present investigations are in conformity to the above referred authors who have reported cholinergic Purkinje cells. The cerebellum has been described as one region where AChE exists beyond the requirements or in the absence of cholinergic

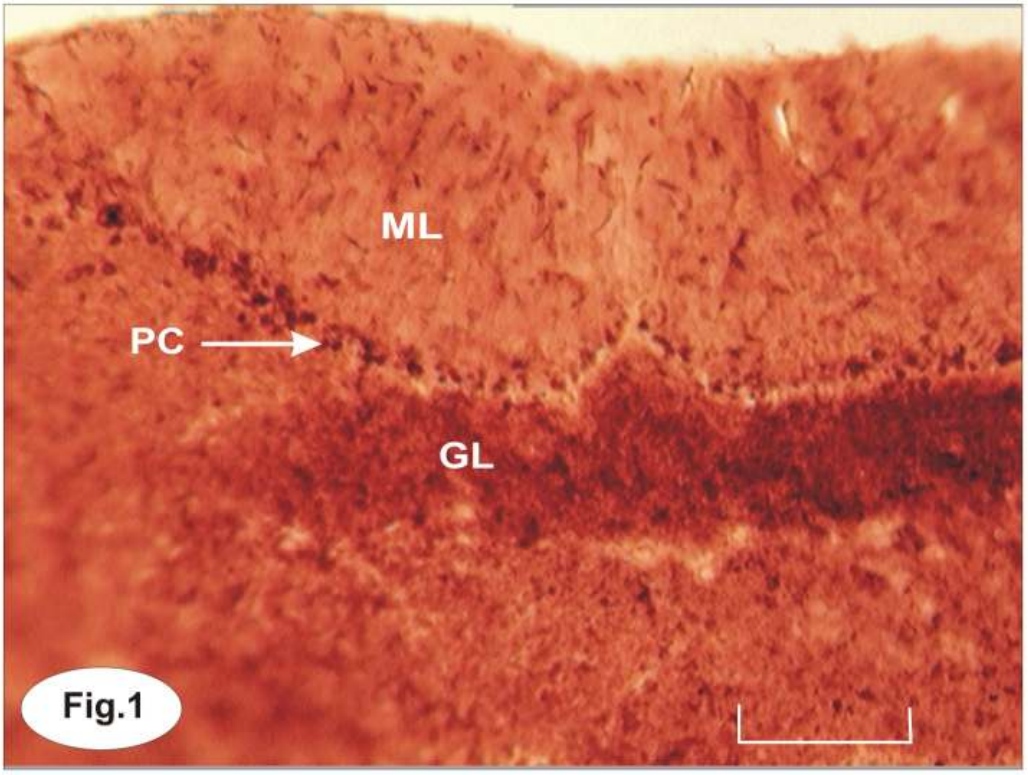

Fig. 1 : 30 micron transverse cryocut section passing through carpus cerebelli of *Heteropneustes fossilis* showing AChE activity in different layers (10 X) (Scale Bar - 100 micron).

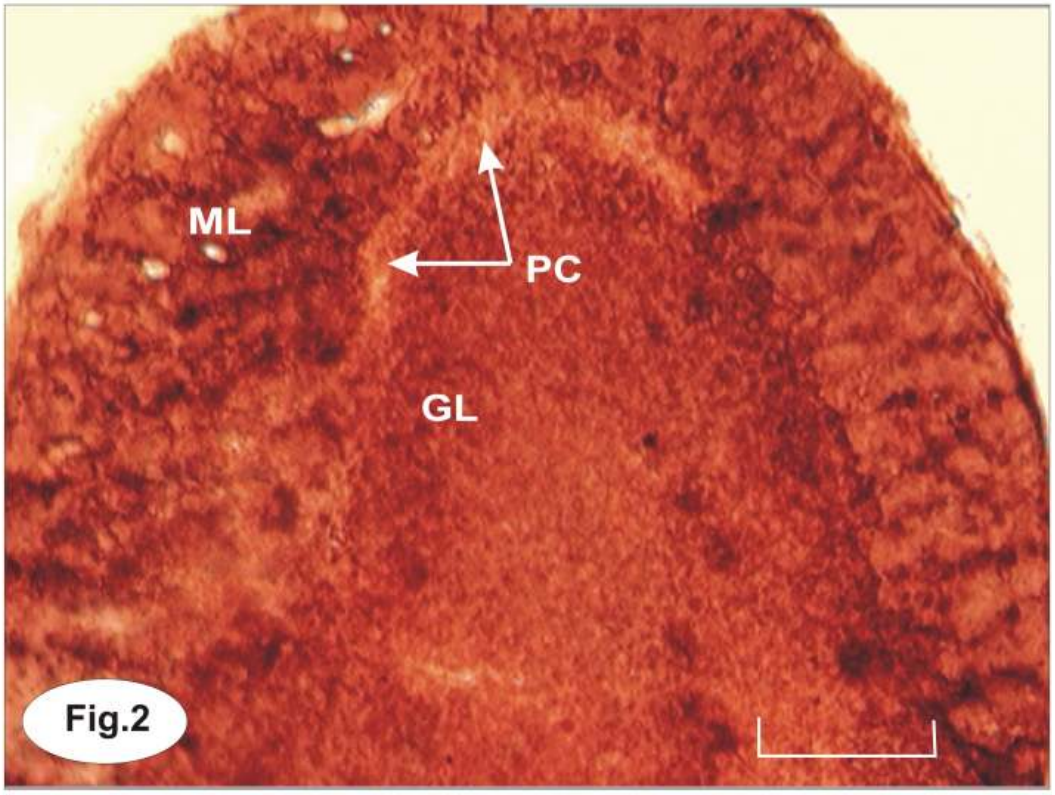

Fig. 2 : 30 micron transverse cryocut section passing through carpus cerebelli of *Channa punctatus* showing AChE activity in different layers (10 X) (Scale Bar - 100micron).

Table 1

Sl. No.	Layers	Abbreviations	AChE Activity	
			Heteropneustes	***Channa***
1.	Molecular Layer	ML	+—	+++
2.	Intermediate Layer of Purkinje Cell	PC	+++	+—
3.	Granular Layer	GL	++	++

Notation : Very Intense : +++, Intense : ++,
Moderate/mild : +—. Negative : ——

transmission in mammals. It may be presumed therefore that Purkinje cells have similar role in the presently studied fishes, because it is also reported earlier that fish granule cells use the excitatory neurotransmitter glutamate in their synapses with purkinje cells.

The cytoarchitectonic properties of the teleostean cerebellar cortex and its input-output characterstics are so similar to other vertebrates that it probably subserves functions in motor learning and coordination as well (Wullimann, 1998).

However, the presence of AChE in the different layers of corpus cerebelli may be attributed to its non-cholinergic roles which have been explored recently. AChE plays role in synaptogenesis, morphometric processes, cell differentiation along nervous system (Silman, & Sussman, 2005). Furthermore AChE hydrolyses substance P, met - leu-enkephalin, and neuropeptides as well (Chub et al., 1980, 1982).

Thus the essence of discussion is that the different layers of corpus cerebelli in the presently

studied fishes as a whole reflect the configuration of the neurons exhibiting AChE activity in their parikarya, plasma membrane & synapses with its non classical roles. And in totality these layers are helping in the transmission of non cholinergic nerve impulses and playing significant role in physiological and metabolic processes.

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Antifeeding and Insecticidal Potentials of Verbenaceous Botanicals against grubs of *Henosepilachna vigintioctopunctata* Fabr. (Coleoptera: Coccinelidae)

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ABSTRACT : In present study, the insecticidal and antifeedant activity of alcohol extracts of leaves, seed and bark of four verbenaceous plants have been evaluated on the 3rd instar grubs of *Henosepilachna vigintioctopunctata* Fabr. The plants used in this investigation are *Clerodendron siphonanthus*, *Lantana camara* Linn, *Lippia geminate* HBK and *Vitex negundo* Linn. The antifeedant activity was assessed through the feeding protection bioassay. Based on the EC_{50} values, the extracts of *Vitex* seed and *Lantana* unripe fruit showed significant protection power against insects. The EC_{50} values were 0.015 and 0.027 respectively which are statistically significant. *Vitex* seeds extract showed high toxicity within 48 hours with mean mortality rate as 80.86 % where as *L. camara* unripe fruits, *L. camara* leaves and *Clerodendron* leaves produced 78.26 %, 68.83% and 67.90% grub mortality respectively. The toxic percentage of the effects of extract of roots and seeds of *C. siphonanthus*, leaves of *V. negundo* , bark of *V. negundo* and geminating leaves of *L. geminate* were observed as 65.70, 65.50, 64.66, 64.16, 48.93 and 35.90 respectively within the same period of 48 hrs. It is suggested that these plants can be used as bio-control as insecticides.

Key Words: Biocontrol, Instar gurb, Antifeedant

Introduction

India is basically an agro-based country and more than 80% of Indian population still depends on agriculture and Indian economy is largely determined by agricultural production. It is unanimously agreed that insect-pests are the main factors causing damage to crops adversely affects agricultural production. The monetary loss due to feeding by larvae and adult insects alone contributes to billion dollars per annum. Among the Coleopteran, *H. vigintioctopunctata* is the key pest that causes severe damage to crops and brings about significant loss yielding (Chandel *et al.*, 1987; Thakur and Mehta., 2004). Abbaszadeh (2011) very recently suggested that the aqueous fraction of *C. infortunatum* act as insecticidal and antifeedant on *H. armegra.* Chandel (2012) described toxicological compatibility of known biopasticide, *Azadirachta indica* and *Acorus calamus* against mustard aphid, *L. erysimi.*

A considerable concern has been raised about the adverse effects of pesticides affecting environment and also resistance development against pesticide. Hence, there is imperative need for development of safe alternative plant protections by botanical insecticides and antifeedants which have least side effects (Tewari and Moorthy, 1985; Verma *et al.* 1986; Rao *et al.*, 1990; Arivudainambi and Nachiappan, 1993; Meshram and Kulkarni, 1996; Faknath and Kawal , 1993; Dekha *et al.*, 1998).The use of plants for medicinal and insecticidal purposes dates back to antiquity (Sahayaraj, 1998; Vekaria and Patel, 2000; Dwivedi and Garg, 2003; Dubey *et al.*, 2004). Recent studies have focused on natural plant products as alternatives for insect-pest control.

Material and Methods

Grubs of *H. vigintioctopunctata* were collected from agricultural fields in the vicinity of Kanpur. The grubs were taken to the laboratory, placed individually and reared in groups of 20 grubs in containers. Containers were punched to permit air flow. Each group was fed for 48 hr with fresh leaves of brinjal.

The plant materials used in the present investigation were collected mainly from wasteland and wild areas while a few plants were collected from cultivated fields. The collected materials were dried in shade, made into powder and the extracts were prepared with the help of extraction apparatus using petroleum /alcohol as solvent. Four verbenaceous plant extracts viz., *C. siphonanthus* (seed, root and leaves), *L. camara* leaves and bark), *L. geminate* (leaves and bark) and *V. negundo* (leaves, seed and bark) were used for their biological efficacy against grubs and adults of *H. vigintioctopunctata*.

Antifeeding Test**:** Brinjal leaves of five centimeter square were cut and dipped in the extracts of different concentrations for two minutes. They were dried under clip and left under electric fan for about ten minutes to make a film of the extracts on the leaves for each set of treatment, one was kept as control in which,

the leaf pieces were dipped in Benzene + emulsified water only. The treated pieces were kept in Petri dishes on moist filter paper and two third instars grubs of *H. vigintioctopunctata* were released in each Petri dish to feed for 4 hours. Three replicates per treatments were maintained. The area of leaf consumed by two grubs in each replication was measured and results were compared with control.

The data of leaf area consumed by two grubs *of H. vigintioctopunctata* in each replication was bulked in these values and the percentage of leaf area protected over control was calculated. The protection was estimated over damage. The concentrations were converted into log concentrations (100 X). The data were subjected to the Probit analysis. The EC_{50} value in respect to each extract was calculated. The fitness of test was tested by comparing table at respective degree of freedom (df). The variance rate was calculated and the fiducial limits were worked out. Finally, the regression columns were drawn with the regression equation (Abbott, 1925).

Insecticidal Test: The 24 hr starved, third instars grubs were used for experimental purpose. The insecticidal test of the plant extracts were performed by dry-film technique. One ml. of solution was sprayed on the Petri-dish. Each concentration was tested in three replications and was kept as control (Benzene + emulsified water). To record the mortality, the sprayed Petri-dishes were gently shaken under an electric fan till the herbal extracts evaporated, leaving behind a uniform dry film of extract on the glass surface. Inside each pair of Petri dish, ten numbers of 24 hrs starved third instars grubs were released and allowed to remain there up to two hours. After this, they were transferred to the fresh Petri dish containing fresh food for feeding. Mean mortality per cent of grubs was observed after 6, 12 and 24 hrs. Laboratory tests were conducted under controlled conditions (27 ±2° C temperatures and 75± 5 % humidity).

Results and Discussions

Table 1 and figure 1 revealed the calculation of log concentration, Probit protection, regression graph of antifeedant test on *H. vigintioctopunctata* Fabr. The EC_{50} value is depicted in table 1. On the basis of EC_{50} values, it clearly indicates that extracts of *Vitex* seed exhibit potent antifeedent activity and shows promising protection towards the grubs of *H. vigintioctopunctata*. **T**he results of the present investigations regarding the feeding deterrents are in conformity with the finding of the earlier investigators (Tripathi *et al.,* 1990; Prakash *et al.,* 1990*;* Ra*o et al.*,1990; Yano and Kamimura, 1993; Meshram *et al.*,1994; Huang and Zhou, 1995; Huang and Okamura, 1995; Yasui and Kato, 1998; Joshi and Lockwood, 2000; Juan and Sans, 2000, Pandey and Khan, 2000; Ogendo *et al.* 2003 Kannathasan *et al*., 2007; Perez *et al.,* 2010). Sindhu and Singh (1975) reported that kerne of *Azadirachta indicahas* antifeeding and insecticidal properties. Recently, Chandel *et al.* (2011) studied the compatibility of *Azadirachta indica* against painted bug, *B. cruciferarum* and mustard aphid, *L. erysimi* and held that they good biopesticde for controllong antifeeding and insecticidal agents. The results of the present investigation revealed that verbenaceous plants can be

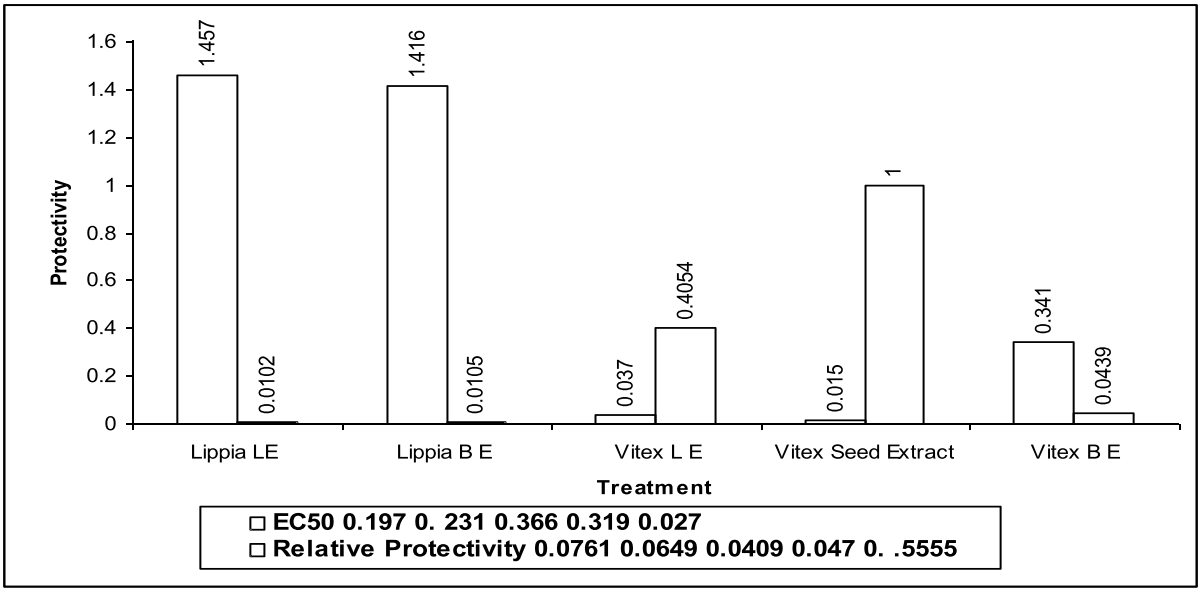

Fig. 1. Calculation of log conc. / Probit Protection regression graph.

Table 1: Calculation of log conc. / Probit Protection Regression graph
(Summary of Antifeedant test on *H. vigintioctopunctata* Fabr.)

Plant Extracts	Het.	X^2	Regression Equation	EC_{50}	Fiducial Limit
Clerodendron Leaves Extract	3	0.75	Y = 0.77x + 3.96	0.197	M_1= 1.7441 m_2 = 0.9358
Clerodendron Seed Extract	3	0.88	Y = 0.84x + 3.84	0. 231	M_1= 1.7533 m_2 = 1.0066
Clerodendron Root Extract	3	0.78	Y = 0.69x + 2.38	0.366	M_1= 1.5864 m_2 = 1.1389
Lantana Leaves Extract	3	0.17	Y = 1.00x + 3.49	0.319	M_1 = 1.8136 m_2 = 1.2063
Lantana Unripe fruites Extract	3	0.42	Y = 0.53x + 1.41	0.027	M_1 = 1.0234 m_2 = 1.0202
Lippia Leaves Extract	3	1.52	Y = 2.10x + 1.36	1.457	M_1= 1.5144 m_2 = 0.4061
Lippia Bark Extract	3	1.17	Y = 2.72x + 0.58	1.416	M_1= 1.6003 m_2 = 0.0336
Vitex Leaves Extract	3	1.34	Y = 0.73x + 4.56	0.037	M1= 1.1678 m2 = 0.0321
Vitex Seed Extract	3	0.58	Y = 0.61x + 4.84	0.015	M_1 = 1.6293 m_2 = 1.0876
Vitex Bark Extract	3	1.48	Y = 0.58x + 0.32	0.341	M_1 = 1.7262 m_2 = 1.4272

In case of X^2 was found non significant heterogeneous at P=0.05, Y=Probit Kill, X=Log Concentration X 10^2
D.F.=Degree of Freedom, $E.C._{50}$= Concentration Calculated at given 50% Protection

used as effective pesticide as important as *Azadirachta.* Ventura and Ito (2000) reported a large number of plants having antifeedint properties against a number of different agricultural pests. Suindararajan and Kumuthakalaralli (2001) evaluated *Gnidia glauca* and *Toddalia aseatica* extracts against *H. armigera* larvae and reported that both extracts showed the high antifeeding action to the larvae.

Kumari *et al.*(2003) described antifeedant and growth inhibitory effects of some neo-clerodane diterpenoids isolated from *Clerodendron* species (Verbenaceae).They have isolated a compounds clerodendrin B, 3-epicaryoptin, 15-hydroxyepicaryoptin and held clerodin as effective antifeedants at 10 ¼g/cm^3 (30 ¼g/g) with diet against *E. vitella* and at 10 ¼g/cm^2 of leaf against *S. litura.* Dwivedi and Bhati (2006) reported the antifeeding response of acetone extracts from four plant viz, *R. communis, E. officinalis T. erecta* and *Z. aungustifolia and claimed that* 100.00, 82.68, 75.48 percent and 77.98 percent protection from *C. chinensis.*

The table 2-3 and 4 and figure 2-5 reveals that the plant extract of *Vitex* seed extract and *Lantana* unripe fruit extracts produced the maximum mortality. It killed

Table 2 : Mean mortality percentage of *H. vigintioctopunctata* Fabr. in case of different combination under laboratory conditions :

Treatment (Plant extracts)	**Con. (%)**	**Mean Mortality percent After**					
		6 hrs.		**12 hrs.**		**24 hrs.**	
		T_1	**T.B.V.$_1$**	**T_2**	**T.B.V.$_2$**	**T_3**	**T.B.V.$_3$**
Clerodendron leaves	0.5	43.08	46.6	46.92	53.4	50.77	60.0
Clerodendron leaves	1.0	66.15	83.3	68.85	87.0	71.56	90.0
Clerodendron leaves	2.0	83.85	98.9	90.00	100.0	90.00	100.0
Clerodendron seed	0.5	41.15	43.3	46.92	53.4	52.80	63.5
Clerodendron seed	0.5	54.78	66.7	56.79	70.0	61.22	76.7
Clerodendron Seed	1.0	83.85	98.9	90.00	100.0	90.00	100.0
Clerodendron root	0.5	48.85	56.7	48.85	56.7	54.78	66.7
Clerodendron root	1.0	54.78	66.7	56.79	70.0	63.44	80.0
Clerodendron root	2.0	83.85	98.8	90.00	100.0	90.00	100.0
Lantana leaves	0.5	48.85	56.7	52.78	63.4	61.22	76.2
Lantana leaves	1.0	56.79	70.0	63.93	80.7	68.85	87.0
Lantana leaves	2.0	68.85	87.0	90.00	100.0	90.00	100.0
Lantana unripe fruits	0.5	52.78	63.4	61.22	76.8	83.85	98.8
Lantana unripe fruits	1.0	68.85	87.0	83.85	98.8	90.00	100.00
Lantana unripe fruits	2.0	83.85	98.8	90.00	100.0	90.00	100.0
Lippia leaves	0.5	18.44	10.0	26.56	20.0	26.56	20.0
Lippia leaves	1.0	26.56	20.0	33.21	30.0	39.23	40.0
Lippia leaves	2.0	45.00	50.0	50.77	60.0	63.14	80.0
Lippia bark	0.5	26.56	20.0	40.0	40.0	45.00	43.3
Lippia bark	1.0	45.00	50.0	50.77	60.0	56.79	70.0
Lippia bark	2.0	50.77	60.0	63.14	80.0	63.14	80.0
Vitex leaves	0.5	45.00	50.0	50.77	60.0	56.79	70.0
Vitex leaves	1.0	50.77	60.0	63.14	80.0	71.56	90.0
Vitex leaves	2.0	71.56	90.0	90.00	83.7	90.00	98.9
Vitex seed	0.5	63.14	79.6	71.56	90.0	71.56	90.0
Vitex seed	1.0	71.56	90.0	90.00	100.0	90.00	100.0
Vitex seed	2.0	90.00	100.0	90.00	100.0	90.00	100.0
Vitex bark	0.5	39.23	40.0	45.00	50.0	63.14	80.0
Vitex bark	1.0	56.79	70.0	63.14	80.0	71.56	95.5
Vitex bark	2.0	63.14	80.0	90.00	100.0	90.00	100.0
Control		0.00	00.0	18.44	10.0	18.44	10.0

(T_1, T_2, T_3 = Treatments and TBV.$_1$, TBV.$_2$, TBV.$_3$= Transformed Back Values)

C.D. for the treatment combination means = 0.147

C.D. for treatment x period means = 0.048

Table 3 : Mean mortality % of *H. vigintioctopunctata* in various plant extracts and periods.

Treatment (Plant extracts)	**Mean mortality percent after**						**Mean % Mortality**	
	6 hrs.		**12 hrs.**		**24 hrs.**			
	T_1	TBV_1	T_2	TBV_2	T_3	TBV_3	G.T.	TBV
Clerodendron leaves	64.36	81.3	68.59	86.7	70.77	89.1	67.90	85.9
Clerodendron seed	59.92	74.9	64.57	81.6	68.00	88.0	64.16	81.0
Clerodendron root	62.49	78.7	65.21	82.4	69.40	87.6	65.70	83.1
Lantana leaves	64.26	81.1	68.90	87.1	73.35	91.8	68.83	86.9
Lantana unripe fruits	68.49	88.5	78.35	95.9	87.95	99.8	78.26	95.9
Lippia leaves	30.00	25.0	36.84	36.0	42.97	46.6	35.90	34.4
Lippia bark	40.79	42.7	51.04	60.5	54.97	67.0	48.93	56.8
Vitex leaves	55.77	68.4	67.97	85.9	72.78	91.2	65.50	82.8
Vitex seed	74.90	93.2	83.85	98.9	83.85	98.9	80.86	97.5
Vitex bark	53.05	63.9	66.04	83.5	74.90	93.2	64.66	81.7
Control	0.00	0.00	18.44	10.00	18.44	10.00	12.26	4.25

($T_{1,}$ $T_{2,}$ $T_{3=}$ Treatments and T.B.V.$_{1,}$ T.B.V.$_{2,}$ T.B.V.$_{5=}$ Transformed Back Values)

C.D. for treatment x period means = 0.075

C.D. for treatment means(plant extract) = 0.032

C.D. for treatment means (control) = 0.160

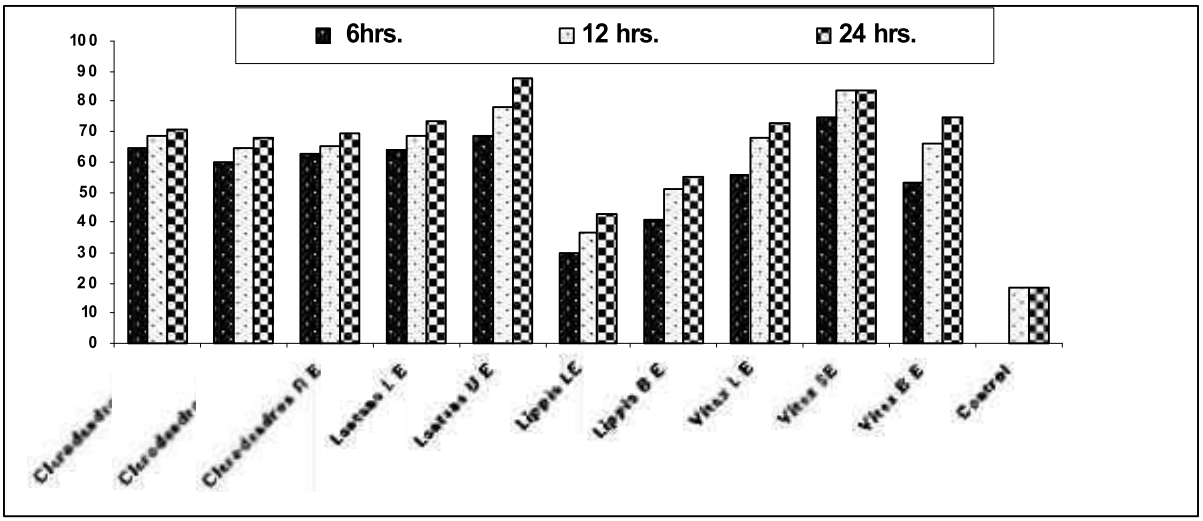

Fig. 2. Mean mortality % of *H. vigintioctopunctata* in different periods irrespective treatments

Table 4 : Mean mortality % of *H. vigintioctopunctata*. in concentrations irrespective of periods in laboratory.

Conc.	Mean mortality percent after						Mean mortality %	
	6 hrs.		12 hrs.		24 hrs.			
	T_1	TBV_1	T_2	TBV_2	T_3	TBV_3	G.T.	TBV
0.5	42.70	46.0	48.98	56.9	56.64	69.8	49.44	57.7
1.0	55.83	68.5	62.94	79.3	68.42	86.5	62.39	78.5
2.0	72.47	90.9	83.39	98.6	84.62	9912	80.16	97.1

(T_1, T_2, T_3 = Treatments and $T.B.V._1$, $T.B.V._2$, $T.B.V._5$= Transformed Back Values)

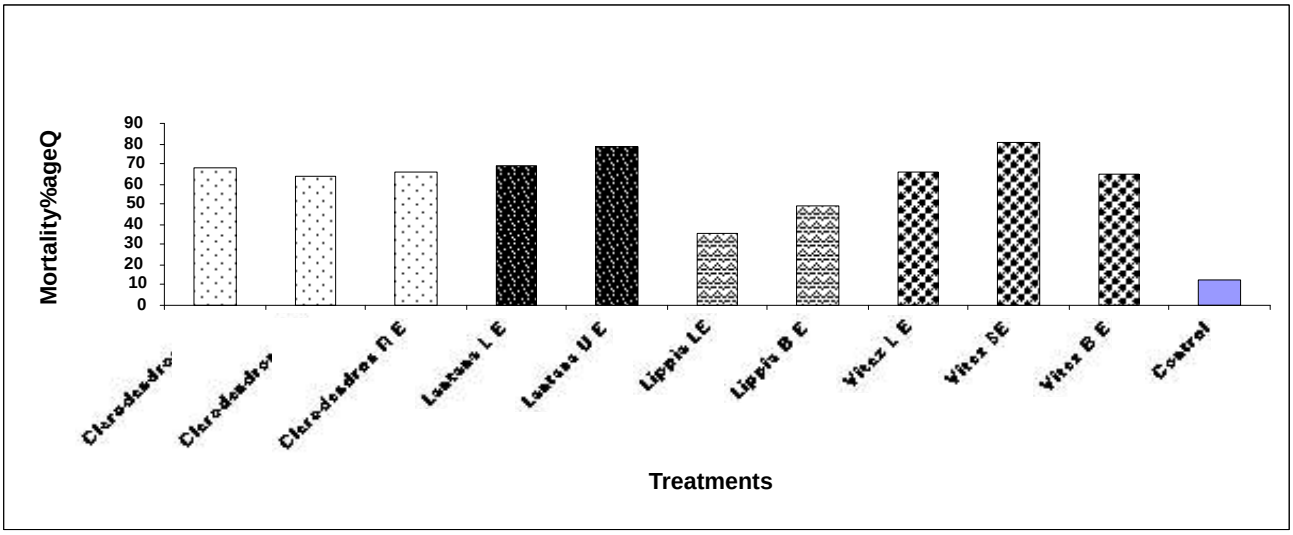

Fig. 3. Mean mortality % of *H. vigintioctopunctata* after 24hrs periods.

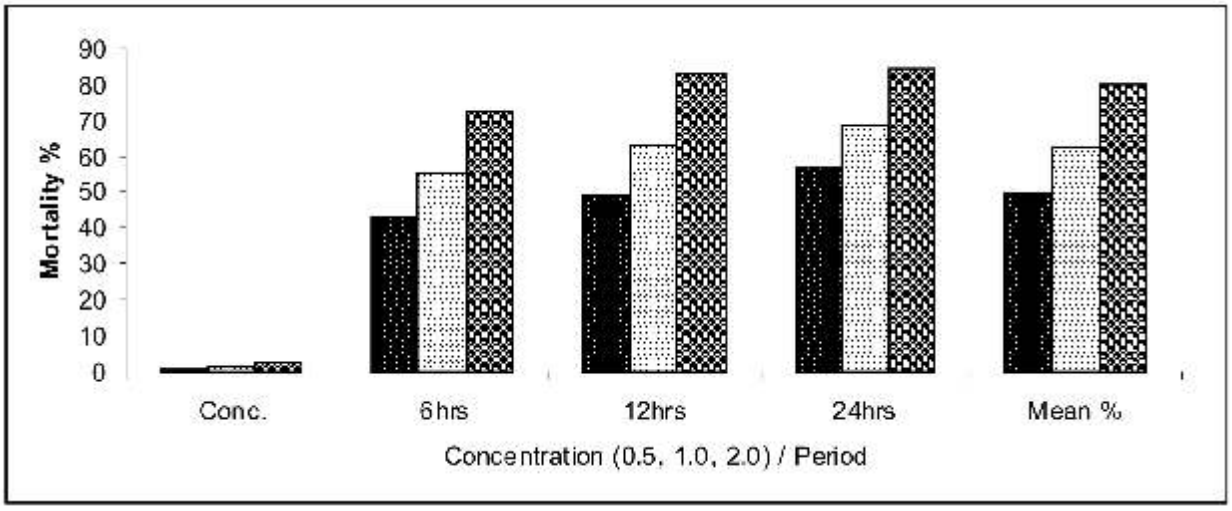

Fig.4. Mean mortality % of *H. vigintioctopunctata*. in concentrations and periods.

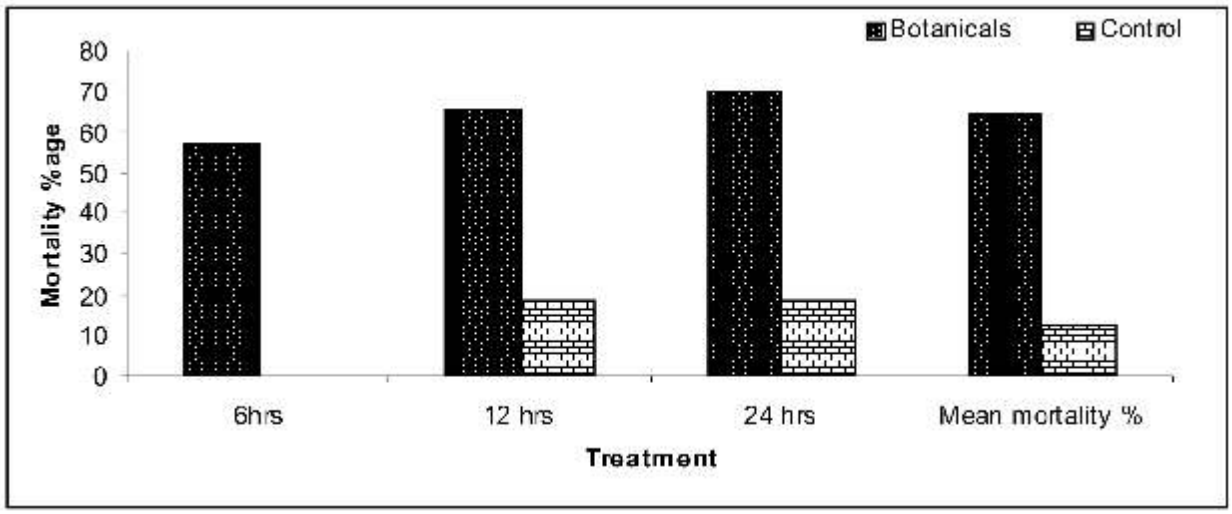

Fig. 5. Mean mortality % of *H. vigintioctopunctata*. in botanicals irrespective of control

Table 5 : Mean mortality % of *H. vigintioctopunctata*. in botanicals irrespective of control.

Treatments	6 hrs	12 hrs	24 hrs	Mean mortality %
Botanicals	57.00	65.10	69.89	63.99
Control	00.00	18.44	18.44	12.26

80.86 per cent grubs of *H.vigintioctopunctata* followed by extracts of *Lantana* unripe fruit (78.26 %), *Lantana* leaves (68.83%), *Clerodendron* leaves (67.90%), *Clerodendron* root (65.70%), *Vitex* leaves (65.50%), *Vitex* bark (64.66%), *Clerodendron* seed (64.16%), *Lippia* bark (48.93%), *Lippia* leaves (35.90%) respectively.

Similar mortality has also been reported by various workers, notably Bai and Kandaswamy, 1985; David *et al.*, 1988; Buiyah and Quiniones,1990; Raja and Albert, 2000). Kulkarni *et al.*, (1997) reported that reduction in insect-pests was due to the antifeedant properties of the extracts which caused mortality. The anti-insect responses of *Vitex negundo* was reported by Ajiwe and Okeke (1998). Rao *et al*. (2003) isolated from leaves of *V. negundo*, betulinic acid and ursolic acid and tested antifeedant activity against the larvae of castor semilooper *Achoea janata*. They concluded that ursolic acid showed more effective activity than the betulinic acid with larvae of *A. janata regarding mortility.*

Singh and Kanaujia, (2003) evaluated the insecticidal impact of NSKE (5.0 per cent) against the larvae of *Spilosoma obliqua* on castor. Saxena *et al.* (1992) found insecticidal responses of *L. camara* against *C. chinensis*. Ogendo *et al.* (2003) has evaluated the insecticidal and repellent properties of *L. camara* against stored maize grain of *Sitophilus zeamais*. They reported that after 21 days caused 85.0-93.7% insect mortality and repelled 65.0 and 62.5% of insects. These taken plants extract can be more effective as antifeedant and biopesticides for insect-pest management.

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The Histopathological Study of Brain of *Catla catla* Exposed to Dimethoate

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Abstract : The histopathological changes were evaluated in the brain tissue of Indian major carp, *Catla catla* after chronic exposure to an organophosphate pesticide, the dimethoate. The experimental group was exposed to sublethal dose of dimethoate (0.001 ppm) for four weeks. The different regions of brain were sectioned at 5-12 µ thick by paraffin embedding process. For the study of histology of nerve tissue silver impregnation technique was employed while for routine histology, the sections were stained with hematoxyline counter stained with eosin. The nerve cells showed necrosis, vacuolation, loss of cytoplasm and Nissl's bodies. The nerve bundles become loose and show dispersal of nerve fibrils in all parts of the brain. The severe damage was noticed in the optic tectum but less damage was noticed in the organelles of the Purkinje cells and also in the granular layer of cerebellum. The necrosis in axons of Mauthner cells in medullary region was prominent. The present study reveals that dimethoate even in 0.001concentraion is highly toxic to brain of the carp fingerlings and may lead to subsequent killing of fish.

Introduction

Dimethoate ($C_5H_{12}NO_3PS_2$) Tech. 95% is an organophosphate insecticide in EPA toxicity is class-II. The nerves are broadly been classified as cholinergic and adrenergic on the basis of neurotransmitters. It is unanimously accepted that AChE enzyme is present at the cholinergic synapse to deactivate the acetycholine neurotransmitter secreted at the cholinergic synapse. AChE is mainly activated by organophosphate, carbamate and some neurotoxic ligands as they pass through the P-site and phosphorelate the catalytic serine in the A-site (Johnson *et al.*, 2003). The cholinergic deficit is believed to be the main cause of cognitive decline noticed in Alzheimer's disease (AD) in human beings. According to Inestrosa and Colombres (2005), the only consistent treatment for AD symptoms has been controlled by the cholinesterase inhibitors. Inhibition of AChE results impairment of nervous tissue, respiratory and muscular functions in experimental animals as suggested by Hsssanein (2005).

Dimethoate reaches to water bodies through runoff during rainy season and damage to aquatic fauna including fishes. It is highly soluble in water. Dimethoate inhibits enzyme AChE activity and result in accumulation of ACh resulting in continuous transmission of impulse at the cholinergic synapse resulting death of the tissue in fishes (Bashamohideen & Sailbala,1989 and Kumar and Tembhre, 2010). Cardoso *et al.* (1996), Metcafe (1998), Balres (1999) and Cengiz *et al.* (2001) calculated maximal percent suppression and percent recovery of AChE activity in fishes and according to these authors the maximum activity is found in the brain and lowest in the intestine. Satydevan *et al.* (1993) and Gaur and Kumar (1993) studied AChE activity and enzyme kinetics in the brain and heart of common carp, *Cyprinus carpio* and *Channa punctatus* subjected to sublethal exposure to diamethote. According to these authors, diamethote is competitive in nature. Inhibitory kinetic study of AChE is very useful for monitoring the pesticide toxicity as suggested by Tembhre and Kumar (1994). Adams (1990), Oliveira *et al. (*2005) and Lionetto *et al.* (2005) suggested that AChE is a biomarker in environmental biomonitoring in tropical aquatic ecosystems. Parveen and Kumar (2005) described the biochemical estimation in the heart both in normal and pathological condition.

Begum and Vijayaraghhvan (1995) studied in vivo effect of dimethoate in the liver tissue of freshwater fish, *Clarias batrachus and* found that 65 mg/L for 96 h is quite toxic. Srivastava and Singh (2001) found that 17.9 mg/L for 96 h is toxic to *Channa punctatus*. Pandey *et al.* (2009) noticed low LC_{50} for 24, 48, 72 and 96 h dimethoate exposure was recorded inhibition of AChE as 3.38, 3.23, 3.08 and 2.98 µ/L respectively in *Heteropneustes fossilis*.

The behavioral changes have been reported due to dimethoate by almost all workers in this field. They found copious mucous secretion, reduced ability to maintain normal posture and balance with increasing exposure time (Kumar and Singh, 2000; Ram *et al.*, 2001; Bonita, 2004; De Mel *et al.*, 2005; Velmurugan *et al.*, 2007; Pande *et al.*, 2009; Singh *et al.*, 2010).

Some specific studies on histopathological effects of pesticides on the brain of fish were conducted by Satyadevan *et al.*(1993). Kumar *et al.* (1993) and Tembhre and Kumar (1994) studied effects of various

concentrations of organophoshate pesticides on various organs of *Cyprinus carpio* and reported the vacuolation in the cholinergic nerve cells and degeneration of Nissl's bodies and loosening of nerve bundle in various organs of fish.

Materials and Methods

A common major carp, *Catla catla* (length 3" to 4" and weight 10 $\pm$ 2 gm) were collected from Govt. Patra Fish Farm, Bhopal. After treatment with $KMNO_4$ for 2 minutes the specimens were acclimatization in glass aquaria (size 18"× 12"× 9"; capacity 25 lt.) for a week. Water was continuously aerated. The fingerlings were feed regularly with Shalimar fish food. Rogor 30% EC manufactured by Anil Products Limited, New Delhi was also used. Long term experiment was conducted on the fish for 30 days to study chronic toxicity of the pesticide. The LC_{50} (96 h) for dimethoate was determined by renewal bioassay test. The fishes were divided into two groups in glass aquaria. Ten fish were used for each group.

Group–1 was exposed to 1/5 of LC_{50} i.e. lowest sublethal concentration of pesticide.

Groups–2 was maintained in pesticide free water to serve as control.

Both the experimental and control fishes were sacrificed after 30 days. Brain was taken out from both groups, tissue was washed and cleaned in normal saline and fixed in Bouins solution and 10% formalin. The blocks were prepared according to paraffin embedding process. The sections were cut 5 to 12 µ (micron) thick. The sections were stained with haematoxylin counter stained with eosin and silver impregnation method. The microphotography was taken with the help of Olympus P.A. 6 equipment.

Results and Discussion

The behavioural changes were noticed in brain of fingerlings of *Catla catla* with the chronic exposure to sub-lethal concentration (0.001 ppm) of dimethoate. The fingerlings showed erratic swimming, increased surfacing and loss of balance and the fingerlings persistently loose balance with increasing exposure. They showed profuse secretion of mucous and decreased rate of opercular movements. Further, during 1st week, the opercular beats increased but later the opercular beats gradually decreased during 2nd, 3rd and 4th week of exposure. It is argued that decreased rate of opercular movements helps the fish in reducing absorption of poison and increased the period of their survival in such toxic environment. Regarding toxic behavior of organophosphate pesticide is in confirmation to the observations of earlier investigators (Kulshrestha and Jauher, 1986; Richmonds and Dutta, 1989; Cardoso *et al.*, 1996; Kumar *et al.*,2000; Rao *et al.*, 2005; Mahira and Kumar 2005; Singh *et al*., 2010; Kumar and Tembhre, 2010; Ricardo *et al*., 2011; and Moitra *et al.*, 2012).

As regards the fish brain, the majority of workers did not find any damaging effect of pesticide on the morphology of fish brain and they also did not find any impairment in the choroid plexi (Lal, 1969; Srivastava and Dubey, 1973; Srivstava and Kumar 1975; Bhattacharya and Mukherjee, 1978; Sastry and Sharma 1981; Kulshreshtha and Arora, 1984; Sahai and Thakur, 1989). In contrast to the observations of the above referred authors, the present investigation shows that color of brain became pink to yellow. This is due to hypoxic condition resulting effecting hemoglobin deformation on account of pesticide. The network of blood vessels was also not visible. This indicates that the pesticide penetrated into blood vessels and disrupted the normal structure of blood vessels.

In telencephalon, necrosis of nerve cells has been noticed. The gap of ypsiliformes has become large in size and the outer lining of the sulcus also showed disorganization (Fig. 1). Increased numbers of vacuoles are seen, less cytoplasm has been noticed and in some of these cells there is a gap between the cell membrane and cytoplasm. The nerve fibrils in anterior commissure become very less in number and are found in dispersed manner (Fig. 2). Neuropathological findings based upon silver impregnation noticed high degree of vacuolization in nerve cell and perikoryon became impaired at several places. The nuclei became acentric in position and there is loss of cytoplasm in nerve cells (Fig. 3). The axons and dendrites too show degeneration. The nerve bundles in both telencephalon and diencephalon showed degeneration. The optic tectum and cerebellum also showed severe necrosis. Metheissen and Robert (1982) and Singh (1985) reported encephalitis in optic tectum of *Tilapia rendalli* after exposure to endosulfan . Encephalitis, vacuolation and dispersed nerve fibres are seen in rest of layers of optic tectum (Fig. 4). Nerve fibres joining optic tectum to torus longitudinalis (Singh and Khanna 1970) are found less in number and show clear signs of degeneration (Fig. 4). Partial necrosis has been seen in axons of Mauthner's cells in medullary region, degenerated and dispersed nerve fibres are distinguished feature of the present study.

The III ventricle in the diencephalon losses its shape and became shrunken. Vacuolation were noticed in the neurosecretary cells. A great degree of vacuolation and necrosis are noticed in granular and molecular layers of cerebelli (Fig. 5). Cerebellar cortex is badly affected in molecular layer, large vacuoles,

clumped nerve fibres are very clear (Fig. 6). Purkinje's cell layer and granular layer are not much affected. In the medullary region, columns of nerve fibres are found to be degenerated. Nerve fibres are lying dispersed thus large gaps are seen among nerve fibres (Fig. 7). Cytoplasmic congestion has been noticed in the cells of medullary region (Fig. 8). The present findings are in conformity to the observations to Ricardo *et al*. (2011) and Xing *et al*. (2012).

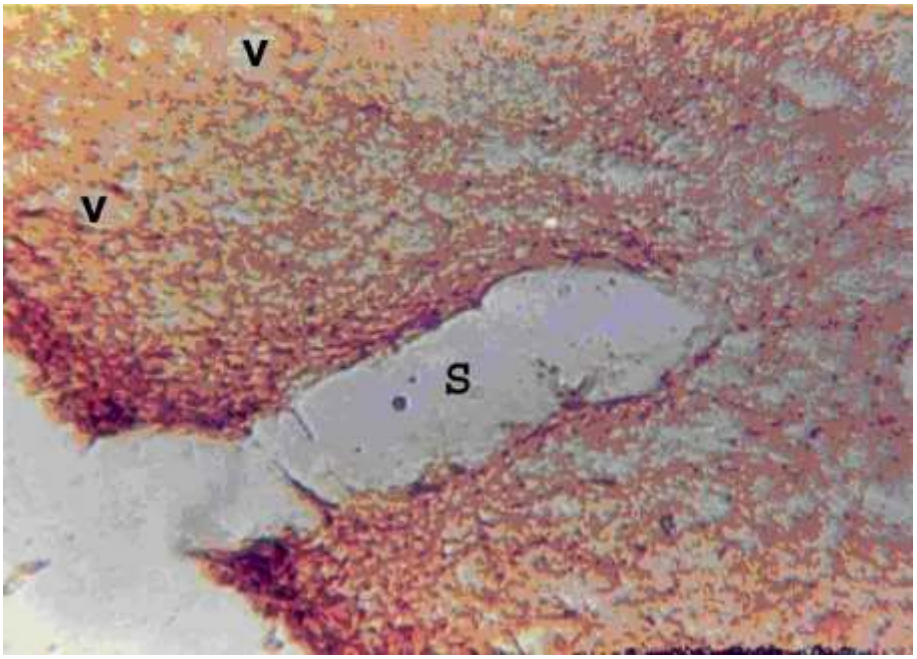

Fig. 1. Photomicrograph of T. S. of telencephalon of *Catla catla* showing large gap of suculus (S) and vacuolization (V) after chronic exposure to dimethoate (0.001 ppm). X 400

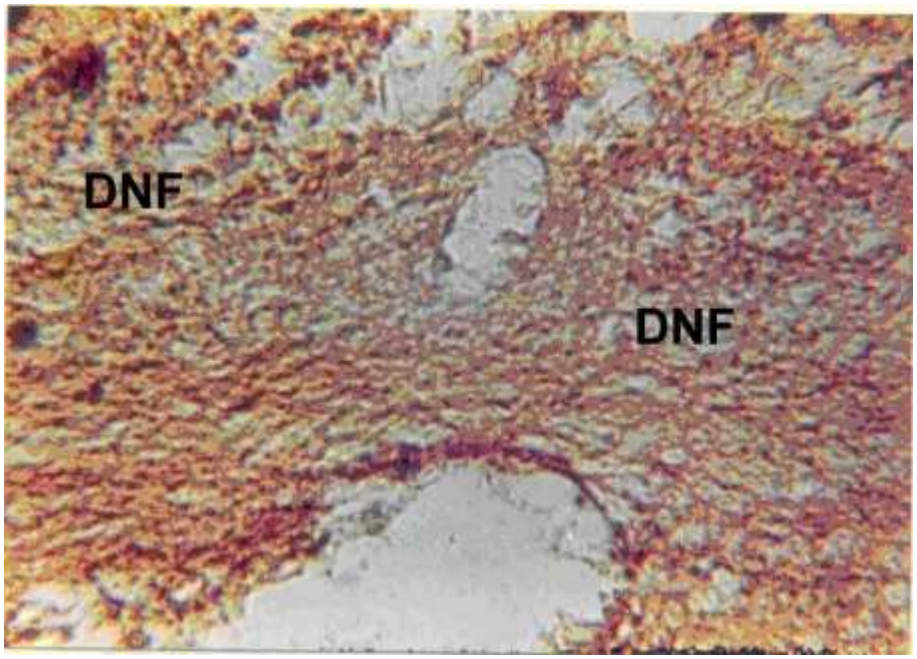

Fig. 2. Photomicrograph of T. S. of telencephalon of *Catla catla* showing meager and dispersed nerve fibrils(DNF) in anterior commissure after chronic exposure to dimethoate (0.001 ppm). X 400

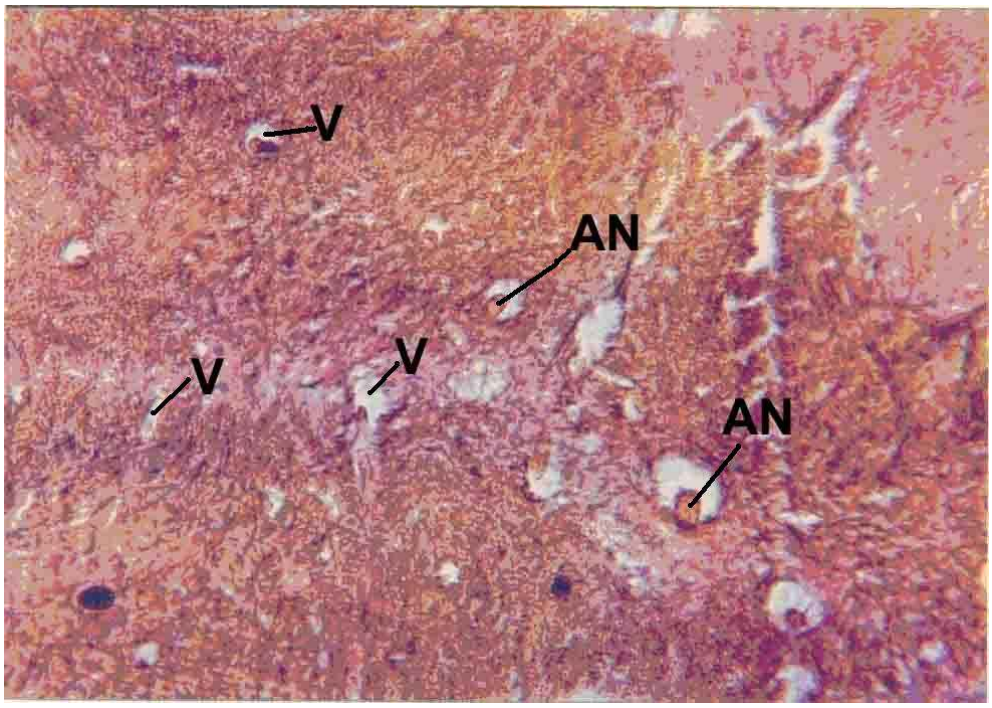

Fig. 3. Photomicrograph of T. S. of diencephalon of *Catla catla* showing nerve cells with accentric nuclei (AN) and vacuolization (V) after chronic exposure to dimethoate (0.001 ppm). X 100

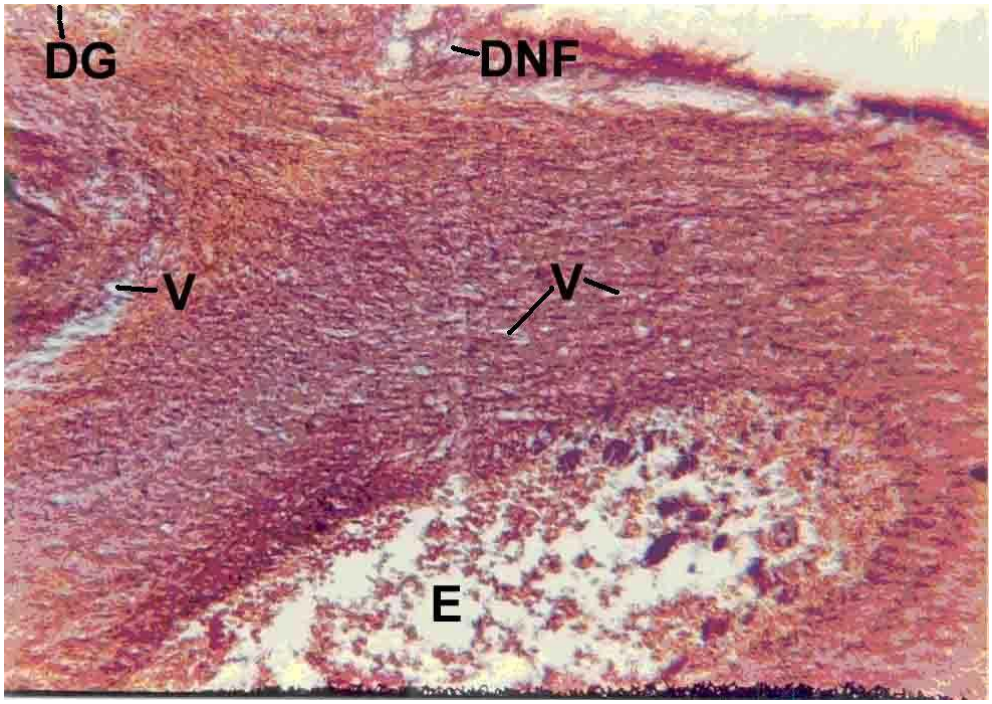

Fig. 4. Photomicrograph of T. S. of mid brain of *Catla catla* showing degeneration (DG), encephalitis (E), vacuolization (V), dispersed nerve fibres (DNF) in various layers of optic tectum after chronic exposure to dimethoate (0.001 ppm). X 100

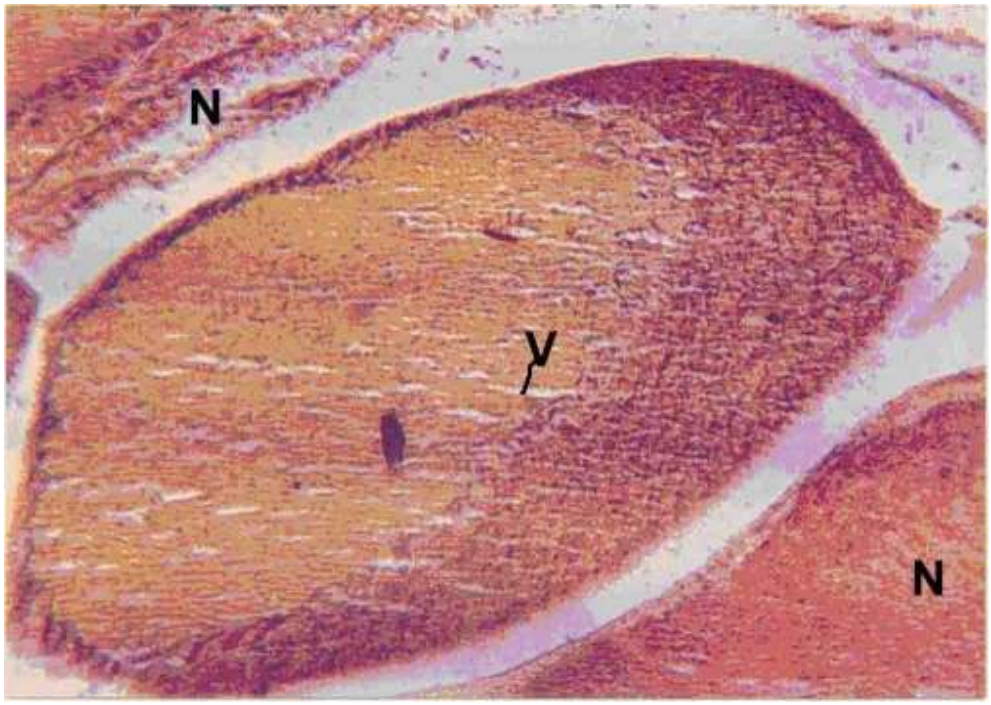

Fig 5. Photomicrograph of T. S. of mid brain of *Catla catla* showing vacuolization (V) and necrosis (N) in granular and molecular layer of valvula cerebella after chronic exposure to dimethoate (0.001 ppm). X 100

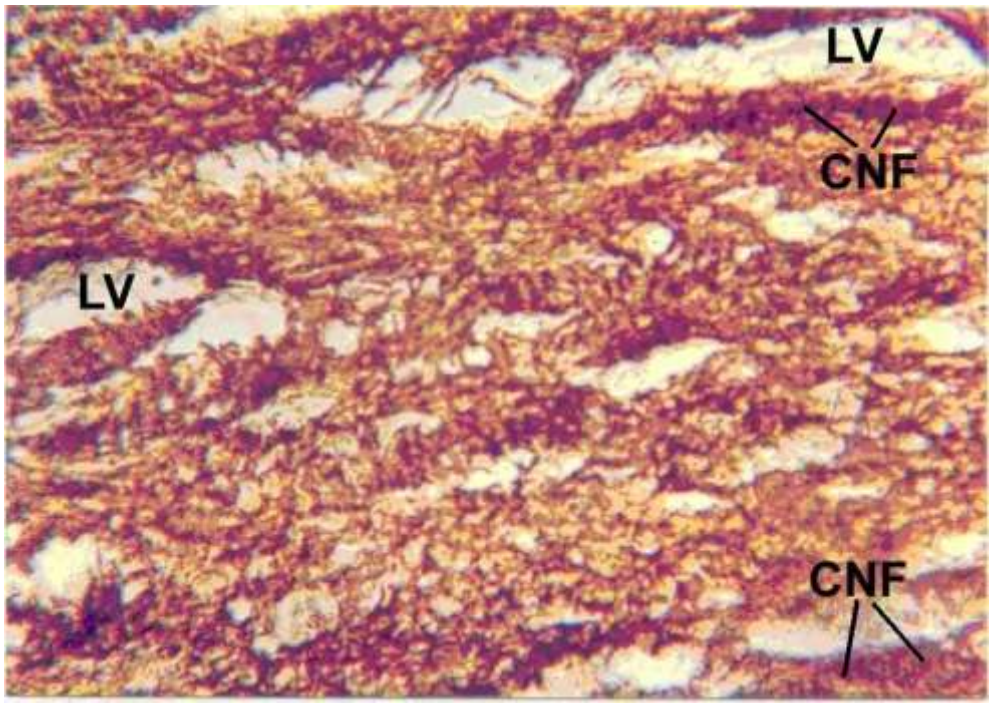

Fig. 6. Photomicrograph of T. S. of through cerebellar cortex of *Catla catla* showing large vacuoles (LV), clumped nerve fibres (CNF) in molecular layer after chronic exposure to dimethoate (0.001 ppm). X 400

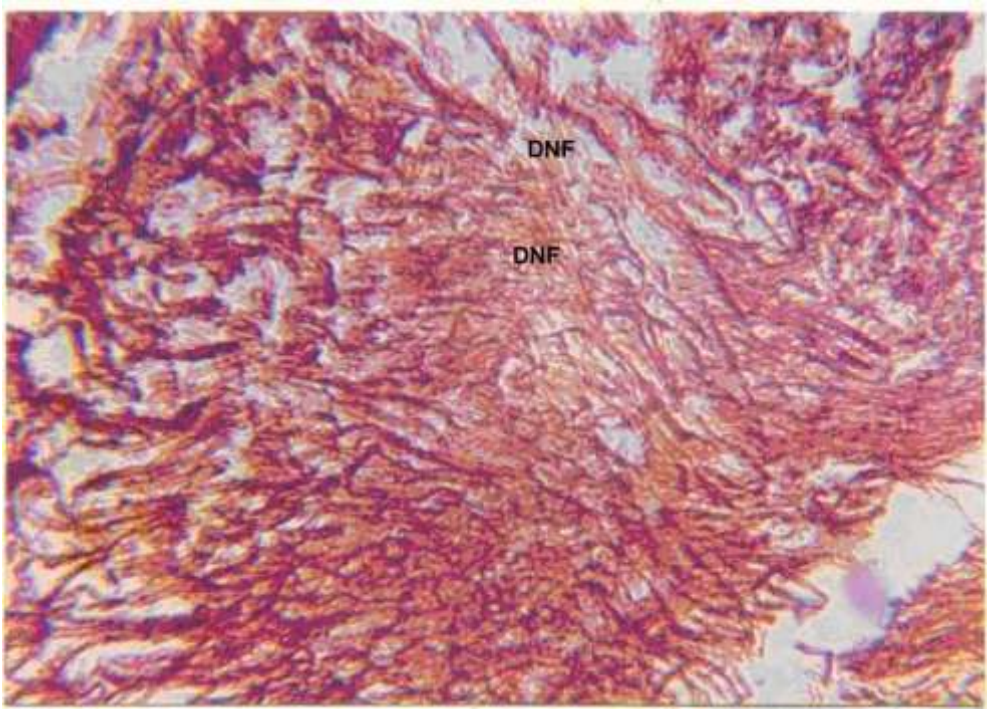

Fig. 7. Photomicrograph of T. S. of medullary region of *Catla catla* showing degenerated nerve fibres (DNF) after chronic exposure to dimethoate (0.001 ppm). X 400

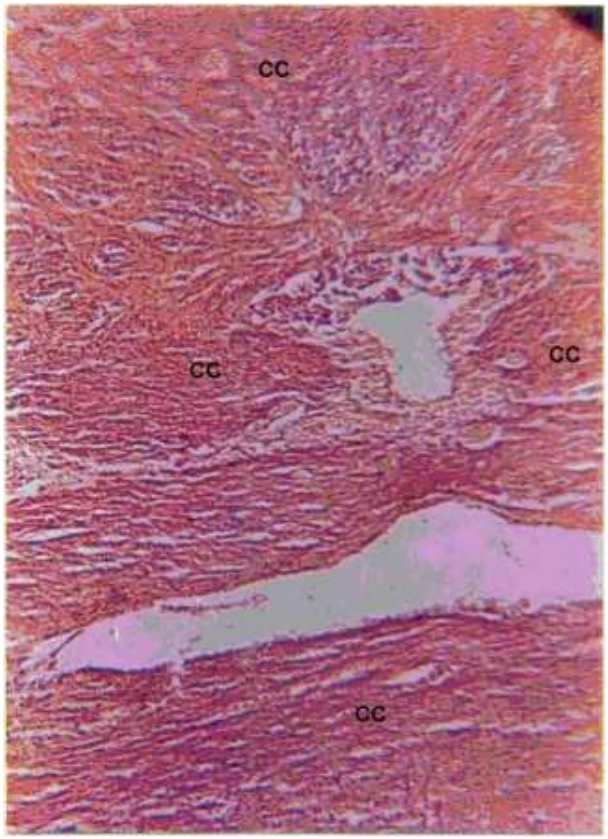

Fig. 8. Photomicrograph of T. S. of medullary region of *Catla catla* showing cytoplasmic conjestion (CC) after chronic exposure to dimethoate (0.001 ppm). X 400

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Use of FTA in nucleic acid research: An optimization study for G6PD gene with FTA

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Abstract : In the present study the standardization and then modification has been done in the protocol of Whatman FTA cards for G6PD gene which plays an important role in RBCs metabolism. The present modification showed better result in PCR program for G6PD gene. The results suggest that the optimization was best observed with 2 discs instead of 1 disc as per standard protocol given in the literature. It is noticed that best results were observed with 10 pmol of forward and reverse primers and 12 µl of master mix instead of 15 µl for total 20 µl reaction mixtures. The annealing temperature should be 61°C which is more sensitive instead of 60°C as reported in the protocol. It is also suggested that the present alteration in the protocol is best suited for Indian conditions as the study showing clean and sharp peaks in the electropherogram of 5 G6PD samples.

Key Words : G6PD, PCR, Whatman FTA Classic cards

Introduction

G-6-PD (Glucose-6-phosphate dehydrogenase) deficiency is an inherited condition in which the body does not have enough G-6-PD enzymes to help RBCs function normally because it has been identified as the only NADPH (Nicotinamide Adenine Dinucleotide Hydrogen Phosphate) producing enzyme in RBCs that is activated during oxidative stresses (Filosa *et al.*, 2006). G-6-PD deficiency is relatively common in the population exposed to malaria and the incidence of G-6-PD deficiency seems to be relatively high in places where *falciparum* malaria has been a life-threatening factor for centuries (Motulsky and Allison, 1960). Researchers have found evidences that the parasite that causes this disease does not survive well in G-6-PD deficient cells. This is a selective advantage against malaria among the population where malaria was once endemic. An incidence of 25-28 % G-6-PD deficiency among tribes of Bastar district (High risk malarial region) has been reported (Tomar *et al*, 1983).

This clinical characterization of the enzyme makes it significant for population genetic studies. Genetically, it is an X-linked disorder located on the telomeric region of the long arm of the X-chromosome (band Xq28). The G-6-PD gene is 18.5 kb in size and consists of 13 exons, which are the regions of the DNA that code for the enzyme and 12 introns, which are intervening sequences (Scriver *et al.*, 1995). A mutant G-6-PD enzyme may differ person to person as it can be in the form of point mutations or can range from one to several base pair deletions or substitution in the DNA.

The present paper is thus a part of large study plan of finding such type of mutation and variants in the tribes of M.P. And therefore it deals with the very basic and important process of standardization of the working protocol of FTA (Fast Technology of Analysis) that best suited to Indian laboratory conditions. It includes the preparation of DNA template and optimization of its PCR (Polymerase Chain Reaction) program to screen G6PD gene mutation, by sequencing of the amplicons. For the purpose, the use of Whatman FTA classic card is remarkably an advanced and convenient way to collect and isolate the nucleic acid samples for analysis. In the Present paper we have optimized the FTA protocol for G-6-PD gene and its PCR program for DNA amplification and we have confirmed the optimized conditions via sequencing.

FTA — Fast Technology of Analysis

Leigh Burgoyne was an inventor of FTA technology. He wanted to collect nucleic acid samples (especially from blood), safely transport and store these prior to analysis by PCR or RFLP (Restriction Fragment Length Polymorphism). It was essentially required a convenient medium that would preserve the nucleic acid material and could facilitate fast analysis of multiple samples for a population based study. The idea for the trade mark 'FTA' was chosen to mean Fast Technology of Analysis of nucleic acids. USA patented Whatman FTA cards are impregnated with a chemical reaction that lyses cell membranes and denatures proteins upon contact. (Fig. I)

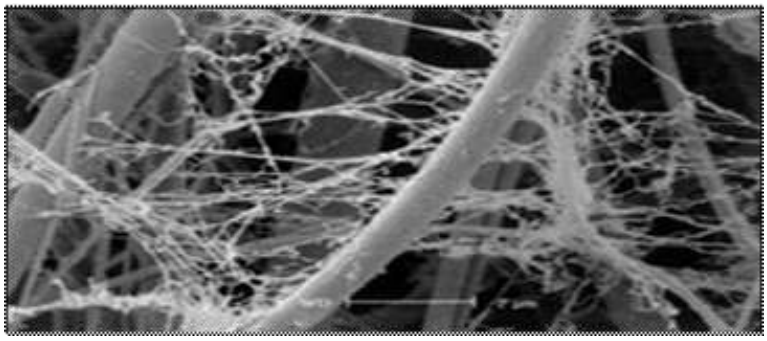

Fig. 1. Electron micrograph of entrapped DNA into FTA matrix

Materials and Methods

Only after consent of the subjects, random sampling has been done in malarial prone areas of M.P. About 1 ml blood is temporarily collected in EDTA (Ethylene Di-amine Tetra Acetic Acid) vaccutainers, kept in thermocol icebox and carried to laboratory to store it at 4°C till further processing on to FTA Classic cards.

A) DNA preparation using FTA cards

The preparation of DNA was performed using the Whatman FTA classic cards. The methodology is based on step wise optimization with the efficient and cost effective alteration in the protocol that best suited to Indian laboratory conditions. The use of Whatman FTA classic cards is as follows:

1. Label the Whatman FTA classic cards properly and pour the blood sample about 200-250µl on the circle.
2. Allow the sample to dry for 1 hr at room temperature and store it at the same.
3. Remove (punch out) a small disc (1.2 mm) from a dried sample spot and place it into a PCR tube.
4. 200µl of washing reagent was added to PCR tube and incubated for 15-20 minutes at room temperature, discard all spent FTA reagent by pipetting.
5. The process was repeated for two washes with FTA purification reagent.
6. The same process was repeated twice with 100 µl TE (Tris EDTA) buffer and the entire spent TE buffer was removed and discarded by pipetting.
7. The disc was allowed to dry at room temp for overnight and then it is ready for further PCR amplification.

B) Genetic analysis

i) PCR amplification (2720 AB Thermal Cycler)

The optimization of PCR condition includes the preparation of DNA template (number of FTA discs), primer concentration, volume of PCR master mix and cycling conditions to get better results in domestic conditions. PCR program commonly used for G6PD is 94°C for 2 min, 94°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec, for 45 cycles72°C for 4 min and finally 4°C for infinity (Beutler and Yoshida, 1993) and 94°C for 2 min, 94°C for 30 sec, 60°C for 60 sec, 72°C for 30 sec, for 34 cycles 72°C for 4 min and finally 4°C for infinity (Guindo *et al*, 2007) but the condition for PCR were modified with the use of FTA cards and optimized as: 94°C for 2 min, 94°C for 30 sec, 61°C for 30 sec, 72°C for 1 min, for 40 cycles 72°C for 4 min and finally 4°C for infinity, after working on different annealing temperatures (54.9°C. 55.1°C, 55.8°C, 56.8°C, 58.1°C, 59.5°C, 61.0°C, 63.7°C, 64.8°C, 65.6°C, 66°C).

The PCR condition was standardized with different number of FTA discs (represents the amount of DNA) starting from 2 to 4. The details are shown in Table I. The primer sequences chosen were of exon 3 and taken from NCBI (National Centre for Biological Information) (G6PD forward: ATACTTCTGTGGAGTGGCAGTGTT and Reverse: CTTGTCCCACTCCCCA AGTC). Optimization of primer concentration was done with 15pmol and 10pmol. The master mix was used in the volume of 10µl and 15 µl to make up the total reaction volume of 15 µl and 20 µl respectively. By observing the better results with 10 pmol of primer concentration, the condition were further modified in order to minimise the use of PCR master mix to make it more cost effective for population genetic studies. And it was done by taking 12 µl of master mix and 6 µl of water (Table II). Following the PCR, the products of 260bp were electrophoresed at 100V in 1% agarose gel. The PCR products were then visualized under UV light in transilluminator. On successful obtaining of a single band devoid of any primer-dimer, the PCR products were then sequenced.

Table I: Details of number of FTA discs, primers, master mix conditions

No. of discs	**2**	**2**	**3**	**3**	**4**	**4**
Primers (10 pmol)	2	2	2	2	2	2
Master mix	10	15	10	15	10	15
Water	3	3	3	3	3	3
Total	**15**	**20**	**15**	**20**	**15**	**20**

Table II : Modification in PCR master mix

No. of discs	**2**	**2**
Primers (10 pmol)	2	2
Master mix	12	15
Water	6	3
Total	**20**	**20**

ii) Sequencing of the PCR product

The sequencing of the PCR products was outsourced from Sequencher Tech Pvt. Ltd., Ahmedabad (Gujrat), India.

C) Data analysis

Following the practical work the obtained sequences were analyzed using appropriate software tools such as ChromasPro, Codon Code Aligner, clustal-X and online available BLAST programs.

Results and Discussion

The results of the standardization of the annealing temperature are shown in (Fig. II). The results shows that optimization of working condition for G6PD gene was best observed with 2 discs of Whatman FTA classic cards and best results were obtained with 10 pmol of both forward and reverse primers (Fig. III). 15 pmol primer concentrations also show bands but with primer dimer in all the discs (Fig. IV). In case of PCR mater mix, the best results were obtained in 20 µl reaction mixture volume with 2 discs (Fig. III). Although DNA bands were also present with 15 µl volume with 3 and 4 discs, but the band intensity was very light that cannot be suggested for further sequencing.

Sequencing results of 5 samples with forward primer were obtained which was essential, in order to confirm the optimized conditions. As shown in (Fig. V), the peaks are clean and sharp without any noise indicate, proportionate primer concentration and it confirms the accurate use of 10 pmol of primer (Fig. VI). The sequence of 240bp was obtained, when the expected gene size was 252bp. This supports the fact that the primer concentration and PCR cyclic condition is accurately optimized and it can varies only as per gene

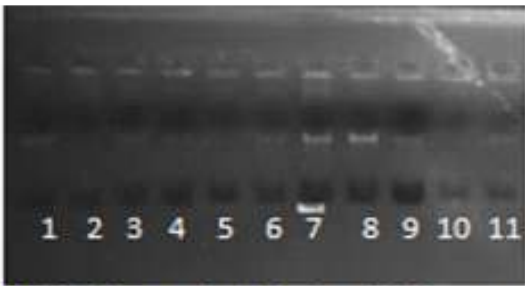

Fig. II. Gel picture of optimization of different annealing temperatures (54.9°C, 55.1°C, 55.8°C, 56.8°C, 58.1°C, 59.5°C, 61.0°C, 63.7°C, 64.8°C, 65.6°C, 66°C). Lane 7 with annealing temperature 61.0°C shows sharp DNA band.

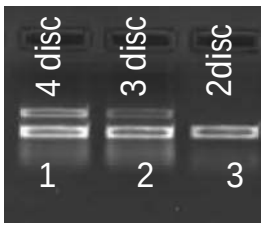

Fig. III. Gel picture showing DNA bands with 10 pmol of primer in different number of FTA disc.

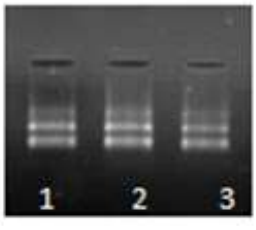

Fig. IV. Gel picture showing DNA bands with 10 pmol of primer in different number of FTA disc.

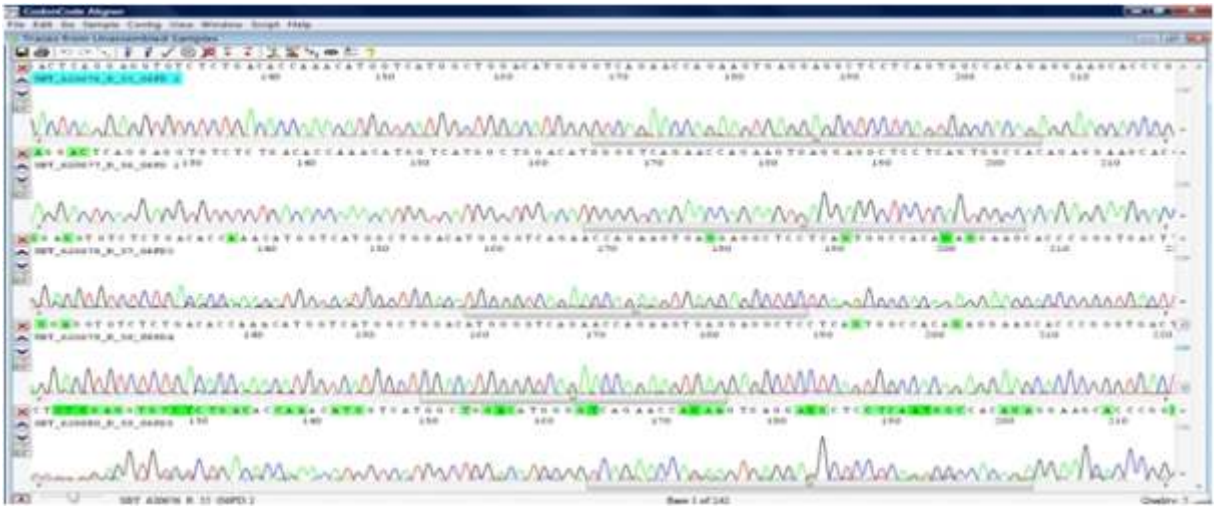

Fig. V. Electropherogram of 5 G6PD samples showing clean and sharp peaks

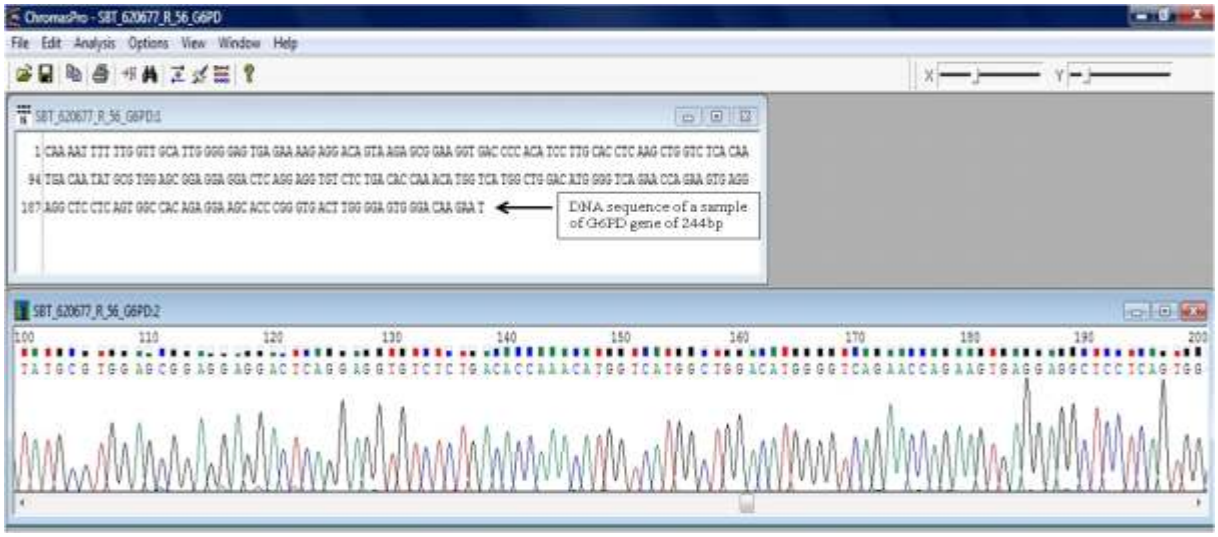

Fig. VI. Electropherogram of 5 G6PD samples showing DNA sequence of more than 240bp

and the primers that researchers are using.

Conclusion

This work concludes that in the field of molecular biology, among different methods of DNA isolation, the use of Whatman FTA classic cards is a very efficient and convenient technique of DNA collection, isolation and even preservation. The present paper has thus optimized the FTA protocol for DNA isolation and amplification for G-6-PD gene in order to make the population genetic studies more efficient and practically more convenient.

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Potential use of Glucanase from *Ampelomyces quisqualis* DSM 2222 in Food and Textile Processing

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Abstract : Agro substrates like barley and oats were used for the production of glucanase from *Ampelomyces quisqualis* DSM 2222. Solid barley medium adjusted to pH 5.0 and supplemented with 0.1% KNO_3 was used for harvesting the enzyme.

The enzyme was purified at 60% saturation with ammonium sulphate followed by ultrafiltration using a 30kD cellulose acetate filter aid. Maximum glucanase activity of 533.3 units/ min was associated with the retentate and having a protein content of 3.3 mg/ ml. The enzyme yield was 0.22 mg of protein per gram of solid barley. This enzyme exhibited maximum activity in the presence of 0.05% Cu^{2+}, insensitivity towards Na^{+}, K^{+}, Mg^{2+}, Ca^{2+} and optimum activity in the pH range 5-6. However, the enzyme was completely inactivated in presence of Hg^{2+}.

Enzyme produced by *A. quisqualis* was able to show a 46.16% reduction in viscosity as compared to a 4.7% exhibited by *Rhizopus microsporus var. microsporus*, thus, indicating its potential use in the brewing industry. Bioscouring of cotton fabric was also carried out using a standard glucanase and that produced by *A. quisqualis*. The results obtained were comparable to the standard enzyme but holds a promising potential for use in the textile industry.

Key words : *Ampelomyces quisqualis* DSM 2222, Glucanase, Barley, Ultrafiltration, Viscosity, Bioscouring.

Introduction

Ampelomyces quisqualis is a mycoparasite of many fungal species that cause powdery mildews (Rotem *et al*., 1999). The interactions between powdery mildew fungi (biotrophic parasites of many plants) and pycnidial fungi belonging to the genus *Ampelomyces* are probably one of the most evident cases of interfungal parasitic relationships in nature, possibly because this relationship is common and takes place exclusively on aerial plant surfaces that facilitate its direct observation.

Glucans are polysaccharides that only contain glucose as structural components and are important structural compounds in the cell walls of plants and fungi. Endoglucanases and exoglucanases are secreted into the cell wall by many organisms and the putative roles for these include localized breakdown of â -glucan for wall expansion, mobilization of glucan for use as a fuel and the hydrolysis of exogenous material for uptake as a nutrient (Stubbs *et al.,* 1999).

Degradation of the β-D-glucans is one of the main processes of malting. The enzyme endo-(1,3), (1,4)- β -glucanase (EC 3.2.1.73), which is synthesized during germination, is a principal enzyme which hydrolyzes the β -glucan of the whole grain from an initial level of 3.0–4.5% to about 0.2–1.0% in the malting process. The hydrolysis is controlled primarily by the germination temperature and grain moisture content. Efficient degradation of the β-glucans is important for quality of malt. Dissolved β -glucans create viscous aqueous solutions, which may cause lautering and filtering problems in the brewery. Germination is thus, crucial stage of malting. Most enzymes of the grain including â-glucanases are born and are active during germination. Enzymatic hydrolysis results in major changes of the grain structure during germination. Thus, in light of malt quality, degradation of β -glucans is an important process (Kussela *et al.,* 2004).

Cotton composed almost entirely of cellulose, is the most abundant polymer used today in the textile industry. Worldwide about 50% of the fibres consumed is cotton. Cellulases catalyze the degradation of cellulose. All cellulases have an identical chemical specificity towards the β (1,4) glycosidic bonds, but they differ in terms of the site of attack and are differentiated into exoglucanase and endoglucanase activities. The exoglucanases or cellobiohydrolases (CBH) cleave cellobiose units from the end of the polysaccharide chains and typically exhibit relatively high activities on crystalline cellulose. The Endoglucanases (EG) make more random cuts in the middle of the long chains, thereby producing new chain ends for the cellobiohydrolases to act upon.

The current study involves designing a medium that supports maximum glucanase production from *A. quisqualis*, partially purify it study its effect on reducing viscosity of mash used for beer making, and bioscouring

in textiles.

Materials and Methods

Procurement, Maintenance & Identification of culture:

Ampelomyces quisqualis DSM 2222 was obtained from IMTECH, Chandigarh. The culture was routinely maintained and sub-cultured in sterile Potato Dextrose Agar slants. The culture was confirmed by Light microscopy and a Scanning Electron Microscopy at 25, 000 kV using a Large Field Detector.

Selection of crude agro substrates for enzyme production:

A standardized inoculum of 10^6 spores/gm of *Ampelomyces quisqualis* DSM 2222 was inoculated in 10gms of autoclaved barley & oats (initially moistened by steeping under distilled water for 15 minutes). Each of the flasks was incubated under dark conditions at room temperature for 1 week. The substrate was mashed after addition of distilled water and the clear supernatant was assayed for glucanase activity by the method of DNSA estimation for reducing sugars (Plummer, 1999). A solid state fermentation by using 10gms of barley as well as a submerged fermentation using 10gms of barley in 100 ml distilled water was carried out. Optimization of medium parameters for enzyme production was done as suggested by Rajoka (2004).

A. quisqualis at 10^6 spores/ gm of barley was inoculated into barley medium adjusted to pH 2.6, 3, 4, 5, 6 & 7 respectively. The flasks were incubated and activity determined as mentioned previously. Further, different nitrogen sources like yeast extract, ammonium salt, inorganic nitrogen (KNO_3) & urea were used at a concentration of 0.1%, 0.5%, 2% & 4%.

Glucanase assay (Celestino *et al*., 2006) β-1, 3 glucanase activity was assayed using 1% barley as substrate. It was prepared by pulverizing barley then sieving it to obtain a fine powder. 1 gm of this powder was then added to 100 ml of distilled water and digested in a boiling water bath for 10 minutes. The resultant was used in all enzyme assays to determine activity of the developed glucanases. 1 ml of enzyme solution was added to 1 ml of substrate and the mixture was incubated at room temperature for 10 minutes. After incubation 1 ml of DNSA reagent was added and treated for 5 minutes in a boiling water bath. Absorbance was measured at 530 nm using a colorimeter.

Bulk production of enzyme

Solid state fermentation was carried out using three 1000 ml flasks containing 100gms of barley adjusted to pH 5.0 and supplemented with 0.1 % KNO_3. Each was inoculated with 10^6 spores/ gm. Later, the substrate was mashed by addition of distilled water and then centrifuged at 7500 rpm for 15 minutes. Around 600 ml of metabolic filtrate was obtained which was then subjected to further purification strategies.

Purification and Characterization of enzyme (Colowick & Kaplan, 1957; Cruz and Llobell, 1999)

Protein in the metabolic filtrate was precipitated by solid $(NH_4)_2SO_4$ (Martin *et al.*, 2005). The salted out protein was subjected to ultra filtration in stirred cell (Amicon 8050) using a 30kD cellulose acetate membrane. Optimal pH for enzymatic activity was investigated by using 0.1M citrate phosphate buffer within the pH range 3.0–9.0, while temperatures studied for activity were 4°C, 37°C, 30 °C and 45°C. Effect of metal ions like Na^+, K^+, Mg^{2+}, Ca^{2+}, Cu^{2+} & Hg^{2+} on the enzyme activity was determined at a concentration of 0.01%, 0.05% & 0.1%.

Viscosity Study of Glucanase (Celestino *et al*., 2006)

12.5 g of malt was pulverized in a grinder, drizzled into a sieve of 0.2 mm spacing, and dissolved in citrate-phosphate buffer (0.1M citrate-phosphate buffer, pH 5.0), pre-heated to 45°C. Reaction was initiated with 1.0 ml of enzyme sample and allowed to proceed for 30min at 45°C, followed by other periods of 10 min at 50°C, 15 min at 60°C, 60 min at 70°C, and 5 min of boiling. The reaction was then stopped by the addition of 100 ml of cold water and immediate cooling in an ice-water bath.

Viscosity studies were carried out subjecting 200 ml of filtered mash to Brookfield viscometer DVII+ Pro at 25°C. Mash viscosity was also measured in absence of enzyme. The specific viscosity rate was determined in centipoise by using a Universal LV spindle. The software Rheocalc V 2.5 Rheometer was used to operate the Viscometer with the computer.

Bioscouring of cotton using glucanase (Karapinar and Sariisik, 2004, Dhamija and Chopra, 2007)

Desizing of cotton fabric was done using 44 grams of grey cotton. The extent of desizing was measured in terms of coloration of the Tegewa scale. Bioscouring of desized fabric was carried out using 2%, 3% & 4% of partially purified enzyme on 4 gms of fabric. 4 ml of 1% wetting agent was added and the apparent volume was made upto 80 ml. This system was loaded onto a Rota dyeing machine. The process was carried out at 37°C for 1.5 hr and 3 hr. The efficiency of Bioscouring was checked using following four tests.

- **Test for water absorbency**

It was evaluated using an AATCC (American Association of Textile Chemists & Colorists) Test

method 79-1995 after scouring in comparison with the grey fabric.

- **Test for loss in weight of fabric:**

Grey fabric and bioscoured fabric was kept in oven (100◦C) for 3 hours. It was then weighed till constant weight was obtained. Weight loss was expressed as a percentage with respect to the initial dry weight.

- **Test for Tensile Strength of bioscoured cotton fabric**

The tensile strength of cotton fabric was measured using H5KS, Tinius Olsen, Qmat 5.17 S series. The fabric was cut in strips of 2.5 X 14 cm in size. The tensile strength of fabric was measured in Kilogram force (Kgf).

- **Wicking Height**

This is a test for quantitative measurement of water absorbency. The fabric was cut in strips of 2 cm x 10 cm., fixed on one side and dipped for 60 seconds in a dye solution till 5 mm length in a trough. The rise of dye was measured in cm from above the 5 mm line, and recorded as the wicking height.

Results and Discussions

Lyophilized culture of *Ampelomyces quisqualis* DSM 2222 was successfully revived and confirmed by Light microscopy as well as a Scanning Electron Microscopy. Stipitate pycnidia developing into slow-growing colonies characterize *A. quisqualis* (Kiss, 1998, Sullivan and White, 2000 and Adams, 2004).

Agro substrates viz. oats and barley without any supplementation were selected to produce glucanase due to their high glucan content of 13-17 % (Lazaridou and Biliaderis, 2007). Oats medium did not support the growth of *A. quisqualis*. Hence, only barley was used for glucanase production. Solid substrate fermentation and submerged fermentation were studied for glucanase production. Solid substrate fermentation gave a 1.83 times more enzyme activity i.e. 320 units/ min to 174 units/ min than the submerged fermentation. One unit of â-glucanase activity (U) was defined as the amount of enzyme required to form 1 ìmol reducing sugar (glucose) per minute (Hrmova *et al.*, 1996).

Glucanases being extracellular the effect of pH on glucanase production was determined. A wide range of acidic pH were studied and results indicated that pH of the medium did not affect the enzyme production as the pH of all the media after growth was found to be ranging between 4.5- 5.5 (**Table 1**). This indicates that the fungus probably produces some metabolites which alter the pH of the medium and makes it conducive to grow. Results obtained showed that pH of the medium after growth of the fungus were between 4.5- 5.5, the pH of the medium for bulk production of enzyme was adjusted to pH 5.

The effect of nitrogen source on glucanase production was determined using varying concentrations of both organic and inorganic nitrogenous compounds like yeast extract, ammonium salt, inorganic nitrogen (KNO_3) & urea. With respect to the results obtained, 0.1% KNO_3 was used during the bulk production of glucanase, since the organism was able to exhibit maximum enzyme activity (**Table 2**). Excess of nitrogen source i.e. 4% KNO_3 affected the C: N ratio and hence, even though it could utilize the carbon source the enzyme production was affected. In *Cellulomonas flavigena*, the effect of nitrogen sources was tested by replacing $NaNO_3$ in the medium with other compounds, maintaining equimolar amount of nitrogen at 0.164 g/liter. $NaNO_3$, KNO_3 and NH_4NO_3 were the best sources since *C. flavige*na possessed strong nitrate reductase activity which was induced by NO_3 ions to an optimal level and repressed by free NH_4 ions in the growth medium (Rajoka *et al*. 2004).

Enzyme was bulk produced, and the metabolic filtrate was surrendered to a two step purification procedure. Maximum protein was precipitated with 60% ammonium sulphate. Glucanase produced by *A. quisqualis* has a molecular weight of 84kDa (Rotem *et al.*, 1999), thus the filter aid used for ultrafiltration had a MWCO of 30 kDa. Glucanase activity was exhibited only by the retentate fraction. The protein content of

Table 1 : Effect of pH on glucanase production

pH	Enzyme Activity in Units/ min
2.6	300
3	270
4	250
5	270
6	270
7	300

both retentate and ultrafiltrate were determined by the Folin- Ciocalteau method using BSA as standard. Maximum glucanase activity of 533.3 units/ min was associated with the retentate and had a protein content of 3.3 mg/ ml (**Table 3**). The culture supernatant of *Rhizopus microsporus var. microsporus* grown in liquid medium containing chitin was concentrated 10-fold by ultrafiltration, using a 10 kDa cut-off membrane. No â-glucanase activity was found in ultrafiltrate whereas the retentate exhibited a protein content of 1.574 mg and an enzyme activity of 0.228 units (Celestino *et al.*, 2006).

The purified enzyme exhibited optimum activity at pH 5. â-1,6 glucanase from *Acremonium persicinum* also exhibited maximum activity at pH 5 (Pitson *et al.*, 1996). Aono *et al.* (1991) reported that â-1, 3 glucanase produced by *Bacillus circulans* IAM 1165 had activity over a broad pH range & was active at pH 4.5 to 9.0.

The purified enzyme showed maximum activity at 37°C whereas the activity reduced at 45°C indicating that the enzyme is not thermostable and would find application only at ambient temperature. â-(1, 6) glucanase from *Acremonium persicinum* also displayed short-term stability up to 50°C (Pitson *et al.*, 1996). *Rhizopus microsporus var. microsporus* exhibited maximum glucanase activity at 50°C and 60°C,the optimum temperature for glucan hydrolysis being 55°C (Celestino *et al.*, 2006).

The effect of metal ions like Na^+, K^+, Mg^{2+}, Ca^{2+}, Cu^{2+} & Hg^{2+} were studied. The results obtained indicated that Hg^{2+} completely inhibited enzyme activity indicating that it can be used as an enzyme inhibitor. The enzyme was insensitive to Na^+, K^+, Mg^{2+}, Ca^{2+} & Cu^{2+}. Cu^{2+} at a concentration of 0.05% gave maximum enzyme activity indicating that it enhances the enzyme activity and may be acting as a cofactor. The glucanase produced by *Rhizopus microsporus var. microsporus* was sensitive to copper unlike the one produced by *A. quisqualis* and fairly sensitive to zinc and manganese, but insensitive to magnesium, calcium and aluminum. Glucanases produced by *Rhizopus oryzae*, *Bacillus clausii*, *Bacillus halodurans* and *Trichoderma harzianum* also exhibit sensitivity to the divalent metal ion copper (Celestino *et al.*, 2006).

A 46.16% reduction in viscosity was obtained, indicating its potential use in the brewing industry. Viscosity reduction with the help of glucanase produced by *Rhizopus microsporus var. microsporus* was reported to be 4.7% (Celestino *et al.*, 2006). Hence, the glucanase produced by *A. quisqualis* was more efficient. Glucanase 5XL and Glucanase 1XL are commercial

Table 2 : Effect of nitrogen source on glucanase production

Nitrogen Source & its concentration	Enzyme Activity in Units/ min	Nitrogen Source & its concentration	Enzyme Activity in Units/ min
0.1%		**2%**	
Urea	303.3	Urea	65.3
$(NH_4)_2SO_4$	326.6	KNO_3	233.3
KNO_3	349.9	**4%**	
Yeast Extract	233.3	Urea	No Activity
0.5%		KNO_3	314.9
Urea	256		
$(NH_4)_2SO_4$	225		
KNO_3	314.9		
Yeast Extract	177		

Table 3 : Ultrafiltration study of spent medium

Sample	Enzyme Activity	Protein Content
Retentate	533.3 units/ min	3.3 mg/ ml
Ultrafiltrate	231.0 units/ min	1.86 mg/ ml

Table 4 : Studies of Protein Purification

Parameter	Volume	Enzyme Units	Protein Content	Specific Activity	Percentage Yield	Folds of Purification
Spent Medium	600 ml	170 units/ min	3 mg/ ml	0.566	100%	1
Purified Enzyme	20 ml	440 units/ min	3.3 mg/ ml	1.33	258.82%	2.34

enzymes produced by *Trichoderma spps* used in reduction of viscosity of barley mash.

The results obtained were statistically analyzed by statistical package SPSS version 13.The data was subjected to paired t test and significant differences between test and control were recorded. Comparisons were made at 95% confidence interval. The entire experiment was divided into two groups:

Group A: Enzymatically treated barley mash with a mean of 4.96 and 95% confidence interval for mean was 4.207 through 5.713. The median value was found to be 5.00 and the average absolute deviation from median was found to be 0.233.

Group B: Untreated barley mash with a mean of 9.29 and 95% confidence interval for mean was 8.541 through 10.05. The median value was found to be 8.75 and the average absolute deviation from median was found to be 0.776.

The final result obtained shows the standard deviation value obtained as 0.828. t= -9.07 i.e. the probability of test and control being same according to null hypothesis is 0.0001. This proves enzymatic treatment to reduce the viscosity of barley mash using glucanase was useful.

Efficiency of bioscouring was determined using four tests. Water absorbency of fabric increases with decrease in the layer coating the grey fabric. The results obtained indicate that the enzymatic treatment has successfully removed this coating of the fabric. The measurement of weight loss is a quantitative assessment of the size. Also, the weight loss expected after scouring is approximately 3-4% due to removal of impurities from the fabric. The results obtained for loss in weight of fabric after scouring is depicted in **Fig. 1**. Tensile strength is an intensive property and, consequently, does not depend on the size of the test specimen. However, it is dependent on the preparation of the specimen and the temperature of the test environment and material. The results obtained for bioscoured fabric is depicted in **Fig. 2.** Thus standard enzyme at 3% enzyme with 1.5 hours of treatment gave best results, while the enzyme from *A. quisqualis* at 4% enzyme concentration with 1.5 hours of incubation gave best results. Thus, unlike amylases, glucanase are not prospective agents for desizing.

Conclusion

The glucanase produced by *A. quisqualis* though a potential candidate needs further research work to clarify its possibility to be used as a Bioscouring agent. Furthermore, it can be combined with pectinases in order to increase its efficiency in Bioscouring. Hence, development of a consortium of enzymes for better Bioscouring needs deeper research.

Fig. 1. Test for loss in weight of fabric

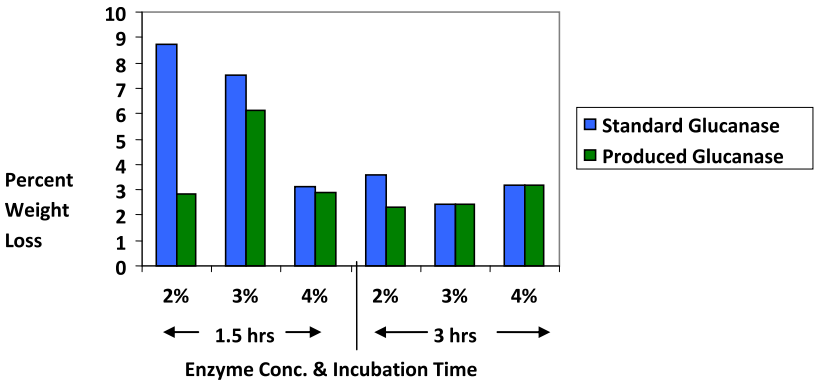


Fig. 2. Testing of Tensile Strength

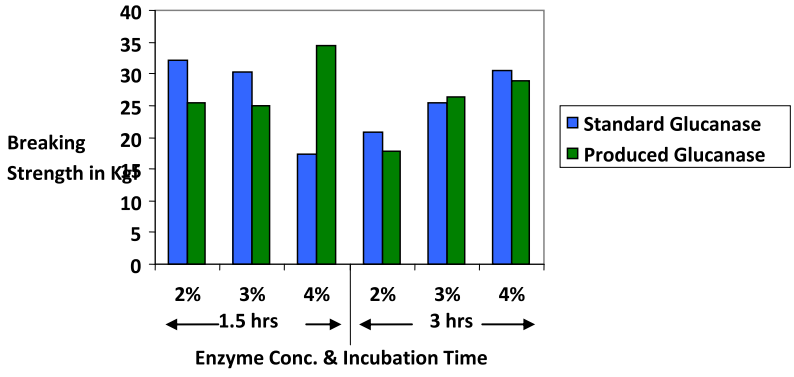
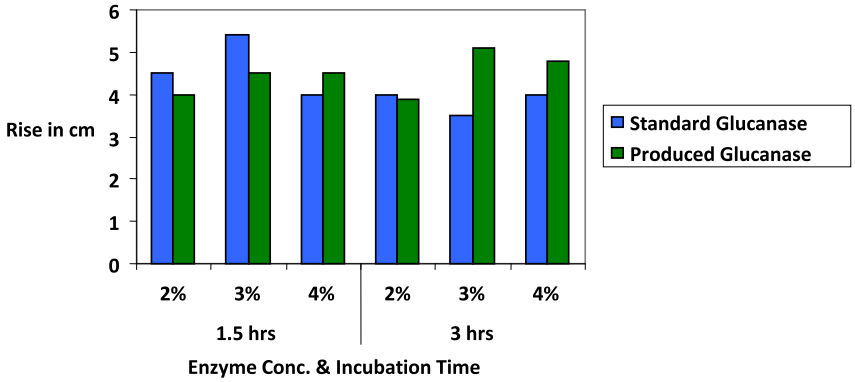


Fig. 3. Determination of Wicking Height



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Carotid Intima-media Thickness as a Surrogate Marker of Atherosclerosis and its Correlation with Coronary Risk Factors and Angiographic Severity of Coronary Artery Disease.

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Abstract : Carotid arterial intima-media thickness (CIMT) has been a good indicator of the presence and extent of coronary artery disease(CAD) in observational studies. Since treadmill testing and stress echocardiography can have limited specificity in diagnosing CAD, other surrogate markers of CAD and Atherosclerosis are required. Biochemical investigations for risk factor's and B-mode ultrasound examinations were performed to calculate CIMT. All the patients were catheterized percutaneously via the femoral vessels with standard Judkins technique and angiographic scoring of CAD was performed. CIMT was higher in subjects with hypertension, diabetes mellitus, smoking, dyslipidaemia, and males. The extent and severity scores for CAD were all positively correlated with CIMT ($p<0.0001$). A significant, nearly linear correlation between CIMT and advancing CAD ($p<0.0001$) was found. Patients with one, two and three vessel CAD had significantly higher CIMT than the patients without CAD. The univariate logistic regression analysis showed that increased CIMT levels were associated with severity of atherosclerosis. There is a strong correlation between carotid atherosclerosis and coronary atherosclerosis, and CIMT is a good predictor of presence and extent of CAD, hence it can be used as a surrogate marker in the prediction of pre-clinical atherosclerosis and CAD.

Key words : Myocardial infarction, Coronary artery Byepass graft, Cerebrovascular accident, Triglycerides, Highdensity lipoprotein, Lowdensity lipoprotein.

Imtroduction

The quantitative assessment of atherosclerosis in populations is essential for a better understanding of the pathophysiology of this disease and for the consequent development of optimal disease prevention stratergies. Thickening of the intima-media at any local site is generally considered to be an early marker of generalized atherosclerosis. (Mohan *et al.*, 2000). Most epidemiological and clinical studies in progress are based on measurement of the carotid arteries intima-media studies have shown that the extent of extra cranial carotid and coronary atherosclerosis is correlated with Pearson's correlation coefficient values of r = 0.4-0.6 (Holme I *et al.*, 1981). For these reasons, CIMT has been suggested as a surrogate marker for coronary atherosclerosis (O'Leary, 1999; Hodis *et al.*, 1998) In adults, CIMT ranges from 0.25 to 1.5 mm and value above 0.9 mm is often regarded as abnormal as suggested by Zwiebel I 2000. CIMT has been proposed as a quantitative index of atherosclerosis and is of value in guiding disease progression and the effects of treatment. Age is one of the most powerful determinants of CIMT with increases of 0.01-0.02 mm per year (Howard *et al.*,1993). CIMT is used as a non-invasive surrogate end point to measure progression of atherosclerosis; furthermore, carotid arterial CIMT has been a good indicator of the presence and extent of coronary artery disease (CAD) in observational studies(Geroulakos *et al.*, 1994; Crouse III JR, *et a*l., 1995). Despite methodological differences with previous studies, the association found between CIMT and known risk factors for atherosclerosis such as age, hypertension, lipid abnormalities, smoking and diabetes are in good agreement. Age and hypertension appeared to be the strongest determinants of the presence of plaque and increased CIMT(O'Leary *et al.*, 1992)

Materials and Methods

We retrospectively reviewed the association between carotid artery atherosclerosis, valued from the CIMT and the presence of atherogenic plaques, and CAD in 100 patients who were subjected for coronary angiography. Subjects were excluded if they had a past history of coronary artery bypass graft surgery (CABG), coronary angioplasty, carotid surgery and cerebrovascular accident (CVA).

Ultrasound

B-mode ultrasound examinations were performed using a 7.0 MHz linear array transducer. Three scanning angles were used in each case: anterior oblique, lateral and posterior oblique (Wofford *et al.*, 1991).

Three segments were identified on each side: (1)

the distal 1.0 cm of the common carotid proximal to the bifurcation; (2) the bifurcation itself and (3) the proximal 1.0 cm of the internal carotid artery[Howard G et al ;1993]At each of the three segments for both near and far wall in the left and right carotid arteries, 2 interfaces were identified: (1) **on the near wall** - the first interface is the adventitial-medial boundary and the second is the intimal lumen boundary, (2) **on the far wall** - the first interface is the lumen-intima and the second is medial-adventitial. These define the CIMT on the near and far walls, respectively. After measuring all twelve areas the maximum value was taken. The maximum CIMT and not the mean value was taken into consideration for calculating the results.

Laboratory methods

Blood samples were taken after a fasting period of 12 hours in all subjects. Baseline biochemistry included lipid profile and liver function tests to rule out any other systemic illness or a secondary cause of dyslipidemia. Total cholesterol (TC), triglycerides (TG) and high density lipoprotein (HDL) cholesterol were analyzed using enzymatic methods. Low density lipoprotein (LDL) cholesterol was computed (Friedewald *et al.*,1972). The reports were retrospectively analysed and correlated with CIMT and angiographic severity of CAD.

Coronary angiography and scoring

All the patients were catheterized percutaneously via the femoral vessels with standard Judkins technique (Judkin, 1976). Angiographic scoring was performed. Coronary angiograms were interpreted visually and always analyzed in two orthogonal views and scored.

With the severity score, the number of coronary vessels with luminal stenosis more than 70% was scored from 0 to 3 (for right, left anterior descending and circumflex arteries). Left main stenosis more than 70% was scored as one-vessel disease (Rohani *et al;* 2005).

Statistical analysis

Continuous variables were expressed in mean ± standard deviation and categorical variables in numbers with percentage. Continuous variables were compared using Student t-test and categorical variables using chi-square test. Association of CIMT with other variables was ascertained using regression analysis.

Results

We retrospectively analyzed and correlated CIMT and angiographic extent and severity of CAD in 100 patients who underwent elective coronary angiography to assess the strength of any relation between CIMT and CAD. There were 89 (89%) men and 11 (11%) women in the study with mean age of 37 ±17 years. The baseline characteristics of the study cohort are summarized in Table 1.

Overall mean CIMT for 100 subjects was 1.05 ± 0.32 mm. The mean CIMT was more in men (1.40 mm),

Table 1 : Risk factor profiles and variable of the patients.

PATIENTS VARIABLE	N%
Mean age(years)	37±17
Males	89(89%)
Females	11(11%)
Smoking	56(56%)
Hypertension	68(68%)
Diabetes mellitus	34(34%)
Total Cholestrol(mg/dl)	196±34
LDL-cholestrol(mg/dl)	134±36
HDL-cholestrol(mg/dl)	32.6±5
Triglycerides(mg/dl)	169±89
Mean CIMT(mm)	1.05±0.32
LDL = low density lipoprotein; HDL= high density lipoprotein; CIMT = carotid intima-media thickness; MI= myocardial infarction; UA = unstable angina. Data is expressed as mean and standard deviation for continuous variables and as percentage for categorical variables.	

subjects with hypertension (1.37 mm), smoking (1.25 mm), diabetes (1.39 mm), total cholesterol >200 mg/dl (1.37 mm), LDL-cholesterol >100 mg/dl (1.48 mm), triglycerides > 150 mg/dl (1.12 mm), HDL-cholesterol <40 mg/dl (1.28 mm) and patients with lower left ventricular ejection fraction (1.47mm) when compared with women (1.31mm), non-hypertensive subjects (0.9mm), non-smokers (0.93mm), nondiabetics (1.05mm), total cholesterol <200 mg/dl (0.98 mm). LDL-cholesterol <100 mg/dl (0.85 mm), triglycerides <150 mg/dl (0.85mm). HDL-cholesterol >40 mg/dl (0.89mm) and patients with normal left ventricular ejection fraction (0.89 mm) (Table 2).

The p-value was significant in correlation of mean CIMT and hypertension ($p < 0.05$), smoking ($p < 0.05$), diabetes mellitus ($p < 0.05$), total cholesterol ($p< 0.05$), LDL-cholesterol ($p < 0.05$), HDL-cholesterol ($p < 0.05$), left ventricular ejection fraction ($p < 0.05$). Even though there was a significant difference in correlation of the mean CIMT with triglyceride levels the p-value was statistically not significant ($p > 0.05$).

Total cholesterol (TC), LDL-cholesterol (LDLC), HDL cholesterol (HDLC) levels were

Table 2 : Correlation of variables of Risk factor with mean CIMT

VARIABLE	MEAN CIMT (mm)	P-VALUE
HYPERTENSION		
Hypertensives	1.37	$p < 0.05$
Non-hypertensives	0.9	
SMOKING		
Smokers	1.25	$p < 0.05$
Non-smokers	0.93	
DIABETES MELLITUS		
Diabetics	1.39	$p < 0.05$
Non-diabetics	1.05	
TOTAL CHOLESTEROL		
TC > 200 mg/dl	1.37	$p < 0.05$
TC < 200 mg/dl	0.98	
LDL-CHOLESTEROL		
LDLC > 100 mg/dl	1.48	$p < 0.05$
LDLC < 100 mg/dl	0.85	
TRIGLYCERIDES		
TG > 150 mg/dl	1.12	$p > 0.05$
TG < 150 mg/dl	0.85	
HDL-CHOLESTEROL		
HDLC < 40 mg/dl	1.28	$p < 0.05$
HDLC> 4 mg/dl	0.89	
LVEF		
Lower LVEF	1.47	$P < 0.05$
Normal LVEF	0.89	
SEX		
Male	1.40	P>0.005
Female	1.31	

CIMT= carotid intima-media thickness; TC = total cholesterol; LDLC = low density lipoprotein cholesterol; HDLC = high density lipoprotein cholesterol; TG - triglycerides; LVEF = left ventricular ejection fraction. (a Indicates significant p-value.)

compared in patients with normal and elevated levels of CIMT. In subjects having high CIMT (>0.9 mm) the TC, LDLC and HDLC levels were 210±14 mg/dl, 146.13 mg/dl and 30.40 mg/dl respectively, whereas in subjects having normal CIMT (<0.9 mm) the TC, LDLC and HDLC levels were 170.30± 10mg/dl, 104.4± 12 mg/dl and 40.04± 6 mg/dl respectively. In all the 3 cases p-value was significant ($p < 0.05$). Univariate analysis showed that increased CIMT levels were associated with hypertension, smoking, and triglycerides (Table 3). Multivariate logistic regression analysis showed that an increased level of CIMT was associated with hypertension and triglycerides (Table 4).

A comparison between CIMT and risk factors was done and from this statistically significant elevated CIMT was found in multiple risk factors when compared with single and double risk factors respectively. Patients with three or more risk factors had higher CIMT (1.26 mm) then patients with two or less risk factors (0.86 mm) and p-value was found significant ($p < 0.05$) (Fig. 1).

Table 3 : Univariate logistic regression for association of CIMT with risk factors

Independent variable	Coefficient	S.E	Odd ratio	95% CI	p-Value
Hypertension	0.824	0.335	2.281	(1.18-4.40)	0.01[a]
Smoking	0.999	0.339	2.715	(1.39-5.28)	0.003[a]
Total cholesterol	0.988	0.397	2.687	(1.23-5.85)	0.01[a]
LDL-cholesterol	0.538	0.275	1.712	(0.99 -2.93)	0.05[a]
Triglycerides	0.505	0.185	1.65	(1.15-2.38)	0.01[a]

CIMT as dependent variable and other variables as independent variable.
S.E = standard error; LDL = low density lipoprotein.
(a Indicates significant p-value (p < 0.05).)

Table 4 : Associations of CIMT with risk factors in multivariate logistics regression

Independent variable	Coefficient	S.E	Odd ratio	95%CI	p-value
Hypertension	0.643	0.581	1.93	(0.62-2.60)	0.005[a]
Triglycerides	0.803	0.387	2.233	(1.046-4.40)	0.05[a]

CIMT as dependent variable and other variables as independent variable
SE-Standard error
(a indicates significant p value (P<0.05))

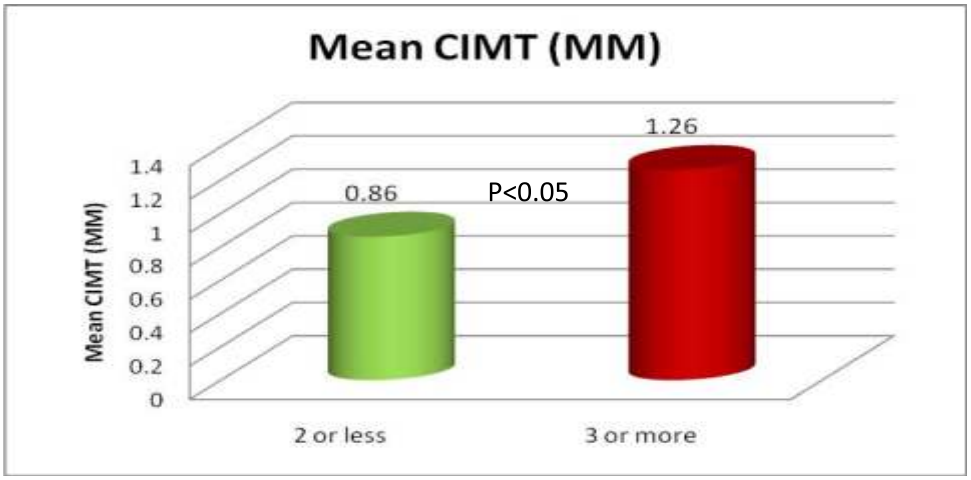

Fig. 1. CIMT correlated with number of risk factor for CAD

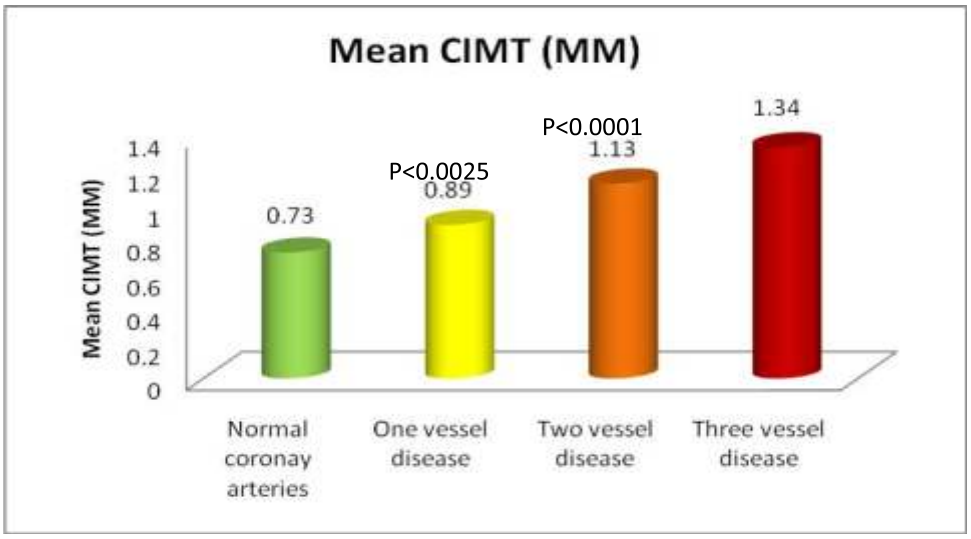

Fig.2. Correlation of CIMT with extent of CAD.

The severity scores for CAD were all positively correlated ($p < 0.0001$ in every case). The mean measured CIMT calculated for patients with normal coronary arteries was 0.73 mm and in patients with angiographic proven CAD was 1.32 mm. The mean CIMT was found to be 0.89 mm. 1.13 mm and 1.34 mm in patients with one-vessel CAD, two vessel CAD and three-vessel CAD respectively. A significant, nearly linear correlation between CIMT and advancing CAD ($p < 0.0001$) was found. Patients with one, two and three vessel CAD had significantly higher CIMT than the patients without CAD (Fig. 2).

We observed significant differences in CIMT between patients with one and two-vessel CAD ($P < 0.0025$) as well as between two and three-vessel CAD ($p < 0.0001$) (Fig. 2).

The correlation between CIMT and vessel score showed that elevated CIMT was seen in three vessel disease or two vessel disease group, when compared with normal CIMT seen in normal coronaries or one vessel disease group. The univariate analysis showed that increased CIMT levels were associated with severity and extent of CAD.

Discussion

In this study we evaluated the relationship between carotid disease and the presence and severity of CAD by coronary angiography in patients with risk factors subjected to coronary angiography. The present study showed strong correlation between CIMT and conventional atherosclerotic risk factors. Patients particularly with multiple risk factors were having elevated CIMT. In this study our main finding was that CIMT and carotid disease was significantly related to the presence of severe CAD. Furthermore, in patients with impaired left ventricular systolic performance, the presence of higher CIMT and carotid disease reflects the presence of severe CAD.

Autopsy studies have demonstrated a strong correlation between the extent of extra cranial carotid and coronary atherosclerosis (Mitchell *et al*., 1962). Non-invasive measurements that relate to the severity of coronary atherosclerosis have been sought for clinical screening of patients with chest pain syndromes (Admas *et al.*, 1995). Thus CIMT has been suggested as a surrogate marker for coronary atherosclerosis for use in clinical trials. Craven *et al.* (1990) have suggested that B-mode score is strongly and independently associated with CAD in patients aged >50 years and is at least as useful as well-known risk factors for identifying patients with CAD; Salonen *et al.* (1991) reported that greater common carotid CIMT values in middle-aged men may be independently associated with higher subsequent risk of acute coronary events.

However, possible additional associations between carotid disease and the severity of CAD have not been well addressed. To investigate this issue further, we extended our attention to the exact relationship between carotid disease and CAD. We found that increased CIMT and carotid disease could indicate the presence of severe CAD in patients undergoing coronary angiography for chest pain. Moreover, the combination of carotid disease with impaired left ventricular systolic performance could predict the presence of severe CAD. Also, the absence of carotid disease in a patient with normal left ventricular systolic performance may reflect the absence of severe CAD.

In concordance with the above, Hertzer *et al.* (1985) studied patients with asymptomatic carotid

bruits or transient ischemic attacks and revealed severe CAD in 37% of patients without suspected CAD.

Our study correlated well with the study conducted by Kablak *et al* (2004) which showed that CIMT increases with advancing CAD, significant increase in CIMT was observed among patients with one, two and three-vessel disease and patients with mean CIMT over 1.15 mm have a 94% likelihood of having CAD. CIMT also showed a positive association with traditional risk factors like male sex, age, obesity, hypertension, serum cholesterol, smoking and diabetes (Howard *et al.*,1993; Kitamura *et al.*,2004)]

Hence it is concluded that CIMT is a simple, non invasive and reproducible clinical tool to evaluate atherosclerosis. There was a strong correlation between carotid atherosclerosis and coronary atherosclerosis, and CIMT is a good predictor of presence and extent of CAD, hence it can be used as a marker for prediction of pre-clinical artherosclerosis and CAD.

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Impact of Nitrogenous Fertilizers on the Quality of Shallow Groundwater in the Upper Alluvial Plains of Narmada Valley Between Hoshandabad and Bhilaria, M.P., India

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Abstract : The present study has been undertaken to motivate the farmers to use the high nitrate water for irrigation as a substitute of nitrogenous fertilizers available in over a stretch of about 45 kms from Hoshangabad to Bhilaria. The study area falls in the toposheets Nos. 55F/9, 55F/10, 55F/6 which is mostly covered by alluvial plains comprising the mist fringing rock, the Deccan trap lava flows of Basaltic composition of Cretaceous-Eocene age. In order to evaluate the quality, 25 representative groundwater samples from shallow aquifers were collected from the study area. The collected water samples were analised by using the standard methods as proposed by APHA for drinking and agricultural purpose.

The result demonstrated that majority of the groundwater samples have nitrate concentration less than 50mg/l and 45 mg/l which is the upper mandatory limit as per water quality guidelines proposed by WHO and Indian Standards Specifications respectively However, few of the samples have nitrate concentration more than the permissible limit which indicates that they are fit for agriculture but not for the drinking purpose. Higher concentration of nitrate in shallow groundwater causes several disease such as methaemoglobinemia (blue baby syndrome), Alzheimer's disease, vascular dementia, netural tube defects and gastro-intestinal cancers.

The investigation also suggests that farmers of the study area should be motivated to adopt sprinkler, drip or trickle techniques for irrigation in place of flood irrigation. The farmers of the study area should use proper quality and quantity of fertilizers as per the requirement of the crops and as per manufacturing instructions.

Introduction

In recent years, the groundwater has become the major source of water supply for the drinking and irrigation sectors of many countries.Disposal of sewage wastes without treatment from urban areas, effluents from, increasing use of fertilizers, pesticides, insecticides etc. for better agricultural industries production are the principal cause of deterioration in the quality of natural waters. In order to achieve green revolution, the farmers are using high doses of nitrogenous fertilizers. At the same time, they are also applying flood irrigation. Such unscientific steps of the farmers have resulted in the ground water pollution by Nitrate, through return irrigational flows.

In the present study, an attempt has been made to evaluate the shallow ground waters of the study area, due to excessive use of fertilizers through return irrigational flows. The suitability of shallow ground water for drinking and irrigational purposes have also been worked out on the basis of water quality guide lines as proposed by WHO (2006), ISI (2004) and Ayers and Westcot (1994). The main purpose of the present study is to throw light on distribution of nitrate and possible cause of nitrate pollution in the area.

The present study covers an area of about 45 sq. kms. The area of present investigation falls on the Survey of India Toposheet Nos. 55F/9, 55F/10 and 55F/41. The area of present study is mostly covered with alluvium. Most of the study area is occupied by clay and clayey loam soils, commonly known as “Black cotton soils”.

Material and Methods

In order to know the chemistry of ground water quality, ground water samples from shallow aquifers were collected from the study area. In the present study, the standard procedures as suggested by Arnold *et al* (1998, APHA) have been followed in the chemical analysis. The nitrate concentration was determined by chromotrophic acid method using spectrophotometer at 410 nm, providing a light path of 1 cm.

Results and Discussion

In the area of present investigation, 25 ground water samples were collected from shallow aquifers. The ground water of the study area was found to be clear, colourless having no objectionable taste. The ranges of major cations and anions of ground water are given in **Table 1.** The nitrate concentration of ground water are presented in **Table 2.**

Frequency and percent frequency have been worked out separately as per drinking and irrigational specification and presented in **Table 3.**

Table 1 : Concentration of Major Cations and Anions in Groundwater of the Study area

Well No.	Name of Village	Temp. °C	pH	EC	TDS	CATION'S				T.H. as $CaCO_3$	ANION'S					
						Na^+	K^+	Ca^{++}	Mg^{++}		HCO_3	CO_3^-	Cl^-	NO_3^-	SO_4^-	PO_4^{---}
1	Phephartal	35.3	7.7	686	352	16	1.0	56	12	175	240	-	42	56	22	0.250
2	Hasulpur	35.0	7.5	659	335	16	1.4	40	13	200	134	-	41	30	27	0.578
3	Randhal	33.9	7.1	690	350	18	1.5	35	20	160	218	-	37	29	23	0.429
4	Khoksar	33.8	6.5	673	495	16	1.7	45	17	190	236	-	35	42	21	0.229
5	Kharkhedi	34.2	7.6	590	324	12	0.9	38	15	205	175	-	30	38	18	0.208
6	Mampa	33.7	7.6	747	339	18	1.8	41	14	125	218	-	34	56	19	0.206
7	Makrai	34.8	7.6	306	540	18	0.7	48	13	175	212	-	49	30	24	0.180
8	Pipalbaher	35.2	7.4	490	340	13	0.9	37	14	180	195	-	38	36	16	0.207
9	Dimawar	35.4	7.3	633	510	10	0.5	39	20	205	230	-	30	56	20	0.203
10	Bhainsadeh	34.9	7.5	748	499	20	0.6	44	25	185	264	-	49	50	12	0.172
11	Shivpur	33.8	7.8	427	650	16	0.4	40	8	150	218	-	28	58	29	0.570
12	Budhni	33.4	7.6	406	600	10	1.5	42	13	175	191	-	27	30	17	0.250
13	Pilikarar	35.4	7.5	795	403	12	1.7	35	16	205	203	-	38	42	14	0.283
14	Devgaon	35.3	7.2	636	800	10	1.1	38	10	185	180	-	30	45	20	0.254
15	Holipura	34.8	6.9	255	129	11	1.2	40	9	170	175	-	30	16	12	0.165
16	Mimor	32.7	7.4	905	457	22	2.3	30	12	165	161	-	39	55	28	0.183
17	Pathora	33.0	7.4	337	700	10	0.5	30	15	220	159	-	31	30	22	0.307
18	Ganjit	33.3	7.4	605	510	20	1.7	64	12	160	250	-	26	18	29	0.268
19	Aawali	33.4	7.5	740	450	15	1.3	60	15	180	210	-	20	35	28	0.302
20	Pangara	34.1	7.8	380	430	18	0.7	38	18	230	185	-	28	40	18	0.264
21	Dhankot	32.9	6.9	435	630	12	0.8	44	10	195	170	-	31	48	20	0.280
22	Chhidgaon	31.0	7.6	563	510	16	1.3	26	15	225	168	-	21	25	26	0.165
23	Mardanpur	35.8	7.5	747	378	16	1.8	32	11	165	162	-	28	32	26	0.237
24	Nehlai	34.4	7.4	474	540	15	1.6	28	20	195	200	-	33	50	12	0.180
25	Mathmi	34.8	7.5	396	480	13	1.2	35	18	176	209	-	33	54	16	0.209

Nitrogen is a vital plant nutrient and most of the crops have very large requirements of nitrogen. In order to meet the deficiency of nitrogen in soil, farmers are using nitrogenous fertilizers. A number of workers like Handa, 1977, 1983, 1986, 1987; Kaka, 1981; Handa et al., 1982; Kumar, 1983; Sehgal et al., 1989; Lunkad, 1994; Rao, 1998; Rao et al., 2006; Tamta et al., 1991; Parashar, 1994, 2001; Jhariya et al., 2012 from India and abroad have worked on the presence of high concentration of nitrate in ground water which generally varies from 3 mg/l to 1800 mg/l. They have reported and identified the possible source of nitrate.

Nitrate is considered as a second most common pollutant of ground water next to pesticides (Bachmat, 1994). These fertilizers ultimately tend to get oxidized to nitrates. A part of the added fertilizer leaches down to the saturated zone along with the downward percolating return irrigational flows and increases the nitrate concentration in the ground water and affects its quality. The use of sewage water for irrigation may also contribute small amount of nitrate to ground water. Handa (1983) mentions that exact sources of nitrate to ground water cannot be specified but the main source appears to be the input of nitrogenous fertilizers. As per the guidelines of WHO (2006), the nitrate concentration in drinking water should not exceed 50.0 mg/l and according to ISI (2004), the nitrate concentration should not exceed 45 mg/l, which is the maximum permissible limit. Regular intake of higher concentration of nitrate in food causes blue baby disease (Methaemoglobinemia) in babies. High doses of nitrates may directly affect the central nervous system, cardio vascular system and occurrence of goiter. Other health problems associated with nitrate toxicity includes oral, colon and rectum cancer or other gastrointestinal cancers.

In order to assess the ground water for drinking purpose, the physical and chemical characters of shallow ground water have been evaluated on the basis of water quality standards proposed by World Health Organization (2006) and Indian Standard Specifications for drinking water IS : 10500 (2004).

In physical characters, the ground water of the present area is colourless, odourless and has no objectionable taste. Thus, the ground water of the area at large is suitable for drinking purposes.

When the chemical characters of ground water

Table 2 : Nitrate Concentration in Ground Water of the study area

Well No.	Toposheet No.	Name of Nearest Village	Nitrate concentration in mg/l
1	55F/10	Phephartal	56
2	55F/10	Hasulpur	30
3	55F/10	Randhal	29
4	55F/10	Khoksar	42
5	55F/10	Kharkhedi	38
6	55F/10	Nanpa	56
7	55F/6	Makrai	30
8	55F/6	Pipalbaher	36
9	55F/6	Dimawar	56
10	55F/6	Bhainsadeh	50
11	55F/6	Shivpur	58
12	55F/9	Budhni	30
13	55F/9	Pilikarar	42
14	55F/9	Devgaon	45
15	55F/10	Holipura	16
16	55F/10	Ninor	55
17	55F/10	Pathora	30
18	55F/10	Ganjit	18
19	55F/10	Murjaha	35
20	55F/6	Pangara	40
21	55F/6	Dhankot	48
22	55F/6	Chhidgaon	25
23	55F/6	Mardanpur	32
24	55F/6	Nehlai	50
25	55F/6	Manjhil	54

(Table 2) are compared with the water quality guidelines of WHO (2006) and ISI (2004) as given in **Table 4**, it reveals that nitrate content from majority of ground water of the study area is well below the mandatory limit of 50 and 45 mg/l respectively, thus it is unpolluted water. All other cations and anions of ground waters are also well within the permissible limits of drinking water quality guidelines and thus they are suitable for drinking purposes.

However, 08 of the ground water samples from shallow aquifers have nitrate concentration more than 45 mg/l which shows the possible nitrate pollution in the area.

Majority of the area is occupied by clayey soil, which confirms that the fixation of NO_3 ions in clayey soil is possible. Continuous presence of high dose of nitrogenous fertilizers application along flood irrigation causing the leaching of NO_3 ions from soil profile to ground water regime is possible. If such a situation prevails for a longer period in the study area it is likely to cause the nitrate pollution. On the basis of the nature of nitrate distribution in the area it clearly demonstrates that it represents the point pollution, which are in the budding stage but it is likely to give high dimension with the passage of time and continuous use of nitrogenous fertilizers.

Ayers and Westcot (1994), proposed a modified water quality guidelines to assess the agricultural water quality. In the purposed guidelines presented in **Table 5,** they have classified and grouped the water quality into four categories namely salinity, water infiltration, Specific ion toxicity and miscellaneous effects. Each of the water quality problems has been further classified into the following three categories based upon the degree of restriction on their use.

1. None restriction category.
2. Slight to moderate restriction category.
3. Severe restriction category.

As per the guidelines of Ayer's and Westcot (1994), nitrate content up to 22.5 mg/l, cause no toxic effect, while concentration ranging from 22.5-135 mg/l causes slight to moderate toxicity and above 135 mg/l, nitrate concentration have severe restriction on irrigational use.

In the present study, the nitrate concentration varies from 16 to 58 mg/l. The frequency distribution of Nitrates in shallow ground water as shown in **Table 3**, which reveals that 5% ground water samples fall in the nitrate concentration range of up to 22.5 which reveals that this water may not be used for sensitive crops. **Table 3** further reveals that majority of ground water samples (95%) fall in the nitrate concentration ranges from 22.5 to 135 mg/l. As per the water quality guidelines proposed by Ayer and Westcot (1994), it clearly indicates that these waters have slight to moderate restriction with respect to sensitive crops. However, these water can be used for tolerant and semitolerant crops. As per the guidelines of Ayers and Westcot "Restrictions on use" does not indicate that the water is unsuitable for agricultural purposes but indicate that there may be a limitation of choice of crops or special management may be needed to maintain full production capability.

Table 3 : Frequency Distribution of Nitrate Concentration in Ground Water of the Study Area

S. No.	Nitrate concentration range in mg/l	Water classes	Frequency distribution	
			No. of Samples (F)	% (%f)
1	**As per drinking specification of WHO Recommendations Vol. I & II (2006)**			
	<50	Maximum Desirable	21	84
	>50	Polluted Water	04	16
		Total	**25**	**100%**
2	**As per drinking specification of Indian Standards of Institution (ISI, 2004)**			
	<45	Maximum Desirable	17	80
	>45	Polluted Water	08	20
		Total	**25**	**100%**
3	**As per Ayer's and Westcot Agricultural Water Quality Guidelines (1994)**			
	<22.5	None Restrictions	02	05
	22.5 to 135	Slight to moderate Restrictions	23	95
	>135	Severe Restrictions	Nil	Nil
		Total	**25**	**100%**

Table 4 : Water Quality Standards for Drinking Water Purposes

S.No.	Parameters	W.H.O. (2006)		I.S.I. (2004)	
		Highest Desirable	Maximum Permissible	Highest Desirable	Maximum Permissible
	Physical				
1	Turbidity (JTU units)	5	25	10	25
2	Colour, Hazen Units (on platinum cobalt scale)	5	25	5	50
3	Taste and Odour	-Unobjectionable-		-Unobjectionable-	
	Chemical				
1	pH	7.0-8.5	6.5-9.2	6.5-8.5	6.5-9.2
2	Total Dissolved Solids (mg/l)	500	1500	500	1500
3	Total Hardness as $CaCO_3$ (mg/l)	100	500	300	600
4	Calcium (mg/l)	75	200	75	200
5	Magnesium (mg/l)	<30*	150	30	100
6	Chloride (mg/l)	200	600	250	1000
7	Sulphate (mg/l)	200	400	150	upto 400**
8	Flouride (mg/l)	0.6-0.9	1.5	0.6-1.2	1.5
9	Nitrate (mg/l)	--	50	45	No relaxation

Table 5 : Agricultural Water Quality Guidelines (Ayer's and Westcot, 1994)

Potential Irrigation Problem	Units	Degree of Restriction on use		
		None	Slight to Moderate	Severe
Salinity (affects crop water availability)				
* Ec_w	mmhos/cm	< 700	700-3000	> 3000
* TDS	mg/l	< 450	450-2000	> 2000
Infiltration (affects infiltration rate of water into the soil. Evaluate using Ec_w and SAR together)				
SAR = 0-3 and Ec_w =		> 0.7	0.7-0.2	< 0.2
SAR = 3-6 and Ec_w =		> 1.2	1.2-0.3	< 0.3
SAR = 6-12 and Ec_w =	SAR	> 1.9	1.9-0.5	< 0.5
SAR = 12-20 and Ec_w =		> 2.9	2.9-1.3	< 1.3
SAR = 20-40 and Ec_w =		> 5.0	5.0-2.9	< 2.9
Specific Ion Toxicity (affects sensitive crops)				
Sodium (Na)				
* Surface Irrigation	me/l	< 3	3-9	> 9
* Sprinkler Irrigation	me/l	< 3	> 3	--
Chloride (Cl)				
* Surface Irrigation	me/l	< 3	4-10	> 10
* Sprinkler Irrigation	me/l	< 4	> 3	--
Boron Miscellaenous Effects (affects susceptible crops)	me/l	< 0.7	0.7-3.0	> 3.0
Nitrogen (NO_3 - N)	me/l	< 5	5-30	> 30
(Nitrate)	mg/l	< 22.5	22.5-135	> 135
Bicarbonate (HCO_3) (Overhead sprinkling only)	me/l	< 1.5	1.5-8.5	> 8.5
pH	Normal range 6.5-8.4			

Conclusion and Suggestions

On the basis of the quality of ground water of the study area, it is concluded that the concentration of nitrate in majority of the ground water samples are well below the mandatory limit. However, few of the samples have more nitrate concentration beyond the permissible limit. It is feared that these concentration unlikely to be augmented in due course of time by continuous use of Nitrogenous fertilizers.

On the basis of the hydrochemical studies, the following suggestions can be made:-

1. Organic fertilizers should be preferred over inorganic fertilizers (chemical fertilizers)
2. The farmers of the study area should be motivated to adopt sprinkler, drip or trickle techniques for irrigation in place of flood irrigation.
3. The farmers of the study area should use proper quality and quantity of fertilizers as per the requirement of the crops and as per manufacturing instructions.

4. The farmers must use the high nitrate water for irrigation as a substitute of nitrogenous fertilizers.

5. The ground water having the nitrate concentration below 22.5 mg/l should not be used for irrigation with respect to sensitive crops.

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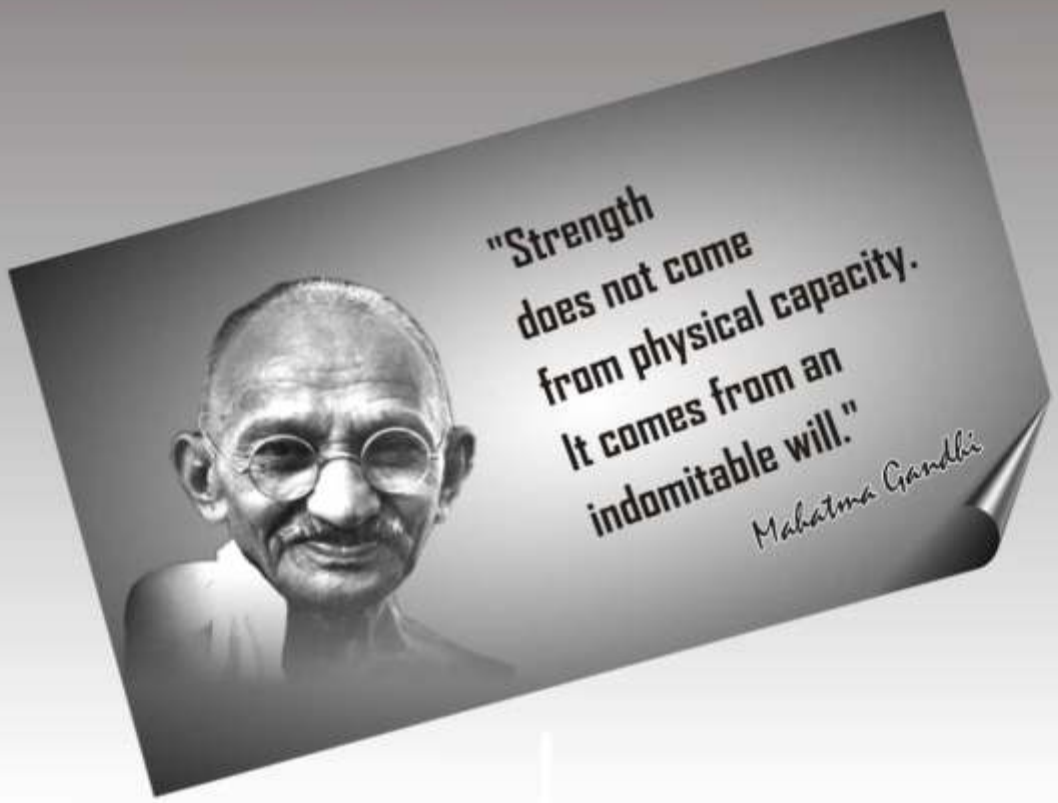

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