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Contents

Effects of Pendimethalin on freshwater fish *Channa punctata* (Bloch): Liver enzyme profile as biomarker of exposure

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ABSTRACT

The aquatic animals specially the fishes are directly exposed to the toxic chemicals including herbicides, pesticides etc. used in the agricultural fields. An investigation was carried out to observe toxicity of the herbicide pendimethalin EC 98.8% in liver of *Channa punctata*, the common fresh water fish species belonging to the *Channidae* family. 96 h LC_{50} value was calculated (2.20 mg/L) and on its basis fishes were exposed to sub lethal concentrations of 0.220 mg/L (10% of 96 h LC_{50} value), 0.440 mg/L (20% of 96 h

LC_{50} value) and 0.660 mg/L (30% of 96 h LC_{50} value) for 96 hours and studied their effects on hepato-somatic index (HSI) and liver enzyme GOT, GPT, ALP level. Fishes were divided into 5 groups, one normal control, one solvent (DMSO) exposed and 3 sub lethal concentrations of pendimethalin exposed under normal laboratory conditions. A significant decrease in hepato-somatic index (HSI) was observed in treated fishes over the controlled ones. The level of 3 major hepatic enzymes viz. GOT, GPT, ALP were found to be significantly higher in serum of treated fishes in contrast to the controlled fishes. This result shows that the increase in concentration of pendimethalin causes hyper toxicity in the liver which affects the liver enzymes profile together with the weight of liver. This study may reveal that the herbicide Pendimethalin is a potent water pollutant which causes toxicity to fish liver concerning its effect on productivity and survival of the fish species. This may spread this toxic effect into human body through food chain.

Keywords: Pendimethalin, *Channa punctata*, GOT, GPT, ALP, Hepato-somatic index.

Introduction :

Due to the urbanization, industrialization and agricultural activities; freshwater bodies are highly polluted with different kinds of chemicals that affects directly or indirectly the biotic community of the ecosystem. The continuous releases of various chemicals from different industries as well as herbicides and pesticides from agricultural fields impair water quality and become unsuitable for aquatic organisms due to their persistence, bioaccumulation, toxicity and bio magnifications in the food chain (Palaniappan *et al.*, 2009) Release of various chemicals from different industries and agricultural fields are finally suspended in water bodies become a major problem worldwide (Ghosh *et al.*, 2006). Herbicides are the most widely used chemicals in agriculture (National Academy of Sciences, 1993) for controlling unwanted grasses and broad leaf weeds. Now a days, herbicides and pesticides have been recognized as one of the serious pollutants of the aquatic ecosystems because of their toxicity, persistency and tendency to accumulate in the organisms (Joseph *et al.*, 2010).

Among the aquatic organisms, fishes mainly accumulate these toxic substances directly from contaminated water and indirectly *via* the food chain (Sasaki *et al.*, 1997) which possess a great threat to the fishes (Sharma and Singh 2006), and also to the human population consuming affected fish (Khalili *et al.*, 2012). This is because fish constitute one of the major sources of protein rich food for mankind (Sharma and Singh 2007).

Pendimethalin, the common name of N-(1-ethylpropyl)-2, 6-dinitro-3, 4-xylidine, is an herbicide that is widely used in Agricultural fields to control annual grasses and certain Broad leaf weeds (Bandyopadhyay and Choudhury 2009). This herbicide has been considered to be a moderately persistent, bio accumulative toxic compound (Roca *et al.*, 2008) that have the ability to bio magnify, and can bio concentrate up to 70,000 times their original concentrations (Ritter *et al.*, 2007). It contains dinitroanilines, which reportedly it could result in the formation of carcinogenic nitrosamines (Environmental Protection Agency Guidance, Washington D.C. 1985). It is a widely used herbicide, has been classified as a group C possible human carcinogen by

(U.S. Environmental Protection Agency; 1992) which is highly toxic to fish and aquatic invertebrates (Meister RT. 1992).

The liver is the primary organ that has a number of enzymes that plays vital role in metabolic reactions, excretion as well as detoxification and storage; alteration of which helps in diagnosis of disorders in certain tissues.

The mechanism by which the herbicides exert their toxic effects on fishes depends largely on the biochemical process in the animal body. It has been shown by many researchers that due to the pesticidal affect liver enzyme activities altered in many fishes (Prashant 2007; Malla and Bashamhideen, 1995). Many recent laboratory and field studies have suggested that the measurement of enzymatic activities might be an effective indicator of exposure to chemical pollution (Dellali *et al*., 2010). The alterations in the level of SGOT, SGPT and SALP were also observed by several workers (Ogueji okechukwu and Auta, 2007; Prashanth and Neelagund, 2008). These enzymes serve as strategic links between protein and carbohydrate metabolism and considered as sensitive indicators of stress. Because of this reason enzyme analysis are becoming increasingly important for the determination of toxic effects of chemical pollutants in the field of environmental toxicology. Hence the present piece of work includes the acute effects of pendimethalin for 96 hrs on liver enzyme levels like GOT, GPT and ALP in a commercially important fresh water air-breathing fishes, *Channa punctata*.

Materials and methods:

Adult and healthy experimental fish, *Channa punctata* locally known as goroi fish (weighting approximately 80±5 g and length 15-18 cm) were collected from local fish markets of Guwahati, Assam. The fish belongs to the class Actinopterygii, order Perciformes and family Channidae. The fishes were collected during the resting phase i.e. in the month of March. Before acclimatization, fishes were treated with 0.2% $KMnO_4$ solution for 30 seconds (Herwig, 1978; Gupta *et al.*, 2002; Zahra and Shreshth, 2006; Pandey *et al.*, 2009) to check any fungal infection. The fishes were then acclimatized in the laboratory conditions at 28 ± 2^0c for 15 days in a glass aquarium (100 liters) filled with dechlorinated tape water prior to experimentation. During this experiment, physiological properties of water were also tested in the laboratory condition. The recorded value of p^H was 7.0 ± 0.45, DO 6.40 ± 0.72 mg/l and total hardness 108.0 ± 5.65 as $CaCO_3$. The fishes were divided into five groups each containing 5 fishes by using standard technique. The fishes of Group I was untreated and served as normal control, Group II was solvent (DMSO) exposed whereas the fishes of Group III, IV and V were treated with low, medium and heavy doses of pendimethalin i.e. 0.220 mg/L, 0.440 mg/L and 0.660 mg/L (10%, 20% and 30% of 96 h LC_{50} value of pendimethalin). During the course of experiment no food was given to the fishes as recommended by Ward, Parrish (1982) and Reish, Oshida (1987). Frequent monitoring was made to observe mortality upto 96 hours after which mean mortality from a particular dose and its replicate was calculated. The 96 h LC_{50} value of pendimethalin was calculated from the data obtained in acute toxicity bioassay by Finney's method (1971) of "probit analysis" and was calculated at 95% confidence limits using the formula of Mohapatra and Rengarajan (1995) on the basis of which sub-lethal concentrations were determined.

The specimens of *Channa punctata*

were treated for 96 hours. After exposure for 96 hours and before sacrifice signs of toxicity like inactive movement, increase in ventilation and decrease in sensibility to external stimulus were recorded. Then the non-anaesthetized specimens were sacrificed under laboratory condition and blood samples were collected from the caudal vein with the help of 1 ml non-heparinized syringe. The blood samples were stored in blood collecting appendorf tubes. Blood samples were centrifuged for the separation of serum at 10,000 rpm for 10 minutes at 10^0c and the supernatant serum was used for biochemical analysis. The serum samples of each group were mixed separately with the chemicals provided by the respective enzyme assay kits by adopting the standard procedure as mentioned in the kit.

In case of GOT and GPT, enzyme activity was measured by Reitman and Frankel's method at wavelength 505 nm, temperature at 37^0c (serum required only 0.10 ml). Again in case of ALP, enzyme activity was measured by Mod. Kind and King's method at wavelength 510 nm, temperature at 37^0c (serum required only 0.05 ml). For Hepato-somatic Index (HSI), the weight of fishes of each groups i.e. control and treated groups were taken before sacrifice and then the weight of the liver tissues were taken after sacrifice. Determination of hepato-somatic index (HSI) of the treated and controlled fishes was done by using the following formula

$$HSI = \frac{\text{Wet weight of liver}}{\text{Body weight of the fish}} \times 100\%$$

The data obtained were expressed as Mean ± SEM. The means were subjected to Student's t-test to determine the significant differences at 95% and 99% ($P<0.05$ & 0.01) confidence level using SPSS computer statistical software (version 21).

Results

The treatment of fishes with different sublethal concentrations of pendimethalin for 96 hours showed significant increase in liver enzyme levels (GOT, GPT and ALP) levels over the controlled ones. Result also showed decrease in liver wet weight indicating decrease in hepato-somatic index (HSI) in the treated fishes when compared to control.

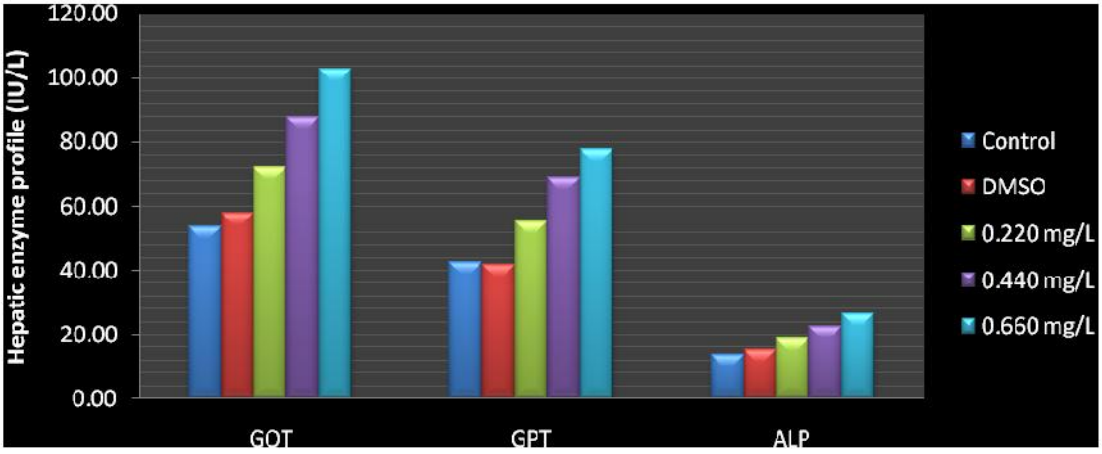

Figure 1: Alteration in liver enzyme (GOT, SGPT and SALP) levels exposed to sublethal concentrations of pendimethalin in *Channa punctata* after 96 hours treatment

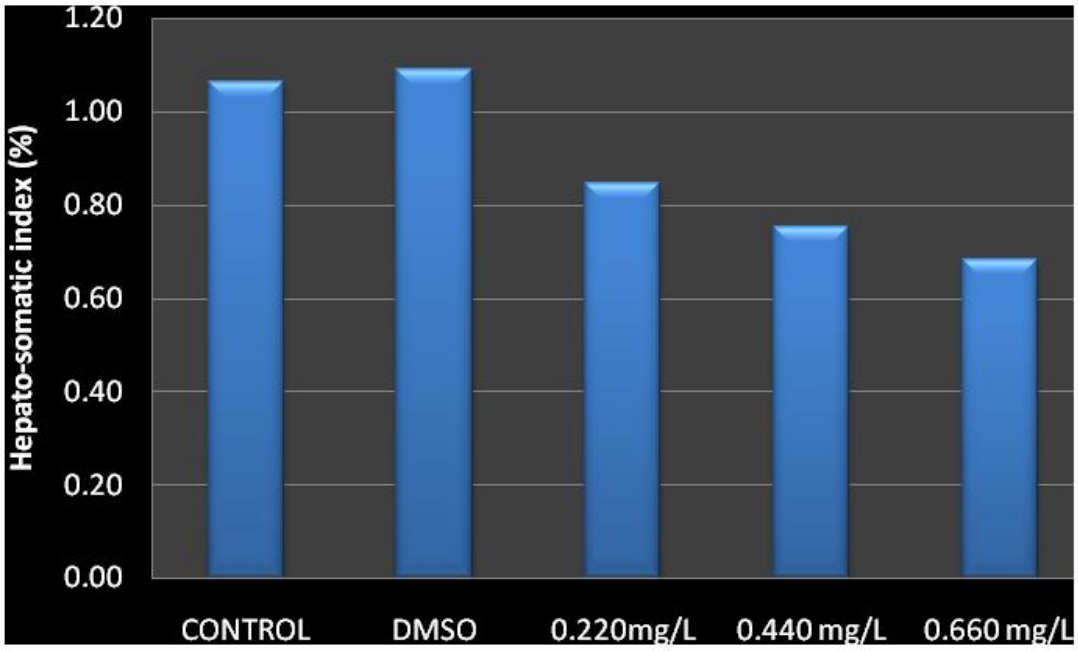

Figure 2: Alteration in Hepato-somatic index (HSI) of *Channa punctata* exposed to sublethal concentrations of pendimethalin after 96 hours treatment
Results are expressed as Mean±SEM ,*Statistically significant to the control at P<0.05 , **Statistically significant to the control at P<0.01

The results presented in Graph 1 shows the activity of GOT, GPT and ALP after 96 hours treatment. The concentration of GOT was 53.71, 72.19, 87.92 and 102.85 IUL^{-1} after 96 hours exposure for control, low dose, medium dose and heavy dose treated respectively with percentage increase of 34.33%, 63.60% and 91.38%. The level of GPT increased slightly in low dose treated group in comparison to the control whereas in case of heavy dose treated group, GPT level increased abruptly and was recorded to be almost double than that of the control. The level of ALP in control group was 13.91 IUL^{-1} whereas in treated groups it was increased by two to three folds. The maximum value was obtained in heavy dose treated group which was 26.53 IUL^{-1}. The present results revealed that pendimethalin induced alterations are dose dependent as it was gradually increase with increasing concentration. All these enzymes showed significant elevation ($p<0.05$) when compared to untreated control group. Result presented in Figure 2 shows significant decrease in hepato-somatc index (HSI) in pendimethalin treated groups over the control ones.

Discussion

Liver is the metabolic centre for detoxification of chemicals and changes in the activities of liver enzymes like GOT and GPT levels confirm liver damage (Asztalos and Hemesok, 1985). Transaminases are the important enzymes which take part both in

amino acid catabolism and biosynthesis (Martin *et al.*, 1983). The enzyme GPT is present in high concentrations in the liver whereas a very lesser extent in skeletal muscles, kidney and heart (Pappas, 1989) and therefore it is considered a good indicator of liver pathology (Neff, 1985). Since the concentration of SGPT is higher in liver, it is regarded to be one of the symptomatic of hepatic cytotoxic injury (Van Vuren *et al.*, 1994) and elevated levels are also thought to be associated with disease multiplicity (Wrobleski, 1959). SGOT is another liver guiding enzyme and changes in its level also reflects the functional state of the liver (Van Vuren *et al.*, 1994). In the present study, a significant increase ($P<0.05$) in GOT and GPT levels were observed in the serum of treated fishes which might be due to stress caused by exposure to herbicide pendimethalin, since stress in general is known to elevate aminotransferase levels. Some workers noticed that due to the exposure of cypermethrin transaminase levels in the liver of other fishes also increased. (Doss *et al.*, 2007; Prashant and Neelagund, 2008; Velisek *et al.*, 2006). Results of the present work are in agreement with the results of these workers. During stressed condition, fish need more energy to detoxify biotransform and excrete the toxicants with the view of minimizing the toxic effects. This is achieved by the use of carbohydrate, the principal and immediate energy source during chronic stress (Umminger, 1977). Under these conditions, generally animals use alternate metabolic pathways like gluconeogenesis which may produce energy. Enzyme Amino transferases play vital role in supplying certain amino acids for gluconeogenesis. Pyruvic acid accumulation in the cells occurs when the enzymes of the TCA cycle are inhibited (Das and Mukherjee, 2003; Kamalaveni *et al.*, 2001). The increase in the level of SGPT may be due to the increased level of pyruvate. According to Chandramohan (1979), high production of transaminases occurs due to the increased level of ammonia content.

Increase in the level of SGOT and SGPT may also signify some inflammatory disease or liver injury (Ayalogu *et al.*, 2001). These enzymes which are generally found in the functional organs (liver, heart, gill and kidney) and muscle tissue always leak into the blood when there is cellular damage (Heath, 1991; Pari & Amali, 2005). The increase might be as a result of hepatocellular damage due to the activity of various chemicals including different herbicides.

Alkaline phosphatase at an alkaline pH hydrolyses di sodium phenylphosphate to form phenol and mediates membrane transport (Goldfisher *et al.*, 1964). In the present study elevation in the level of ALP was observed in the pendimethalin treated fish groups. Increased level of SALP may be due to an accelerated membrane transport function (Jaroli and Sharma, 2005). Due to the exposure of various herbicides and pesticides hyperglycemic condition was observed in many fishes (Ansari and Kumar, 1988; Logaswamy and Remia, 2009) which may be another possibility for increasing the phosphatases level (Bhatia *et al.*, 1973). Rees and Sinha (1960), Sarkar *et al.*, (2005), Heath (1987) reported that due to the activity of various herbicides as well as different toxicant levels of transaminases and phosphatases in fishes increased which may leakage across damaged plasma membranes and increasing the synthesis of the enzymes by the liver.

In the present study, significant decrease ($P<0.05$) in hepato-somatic index (HSI) was

also observed in the treated fishes which might be due to the toxic effect of herbicide pendimethalin.

The present work indicates that the herbicide pendimethalin causes considerable alteration in liver enzyme concentrations as well as hepato-somatic index (HSI) and is likely to induce changes in intermediary metabolism in *Channa punctata* and may be considered useful in the assessment of environmental stress in the aquatic ecosystem.

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Optimisation of long term storage of eri silkworm *(Philosamia ricini)* hemolymph with special reference to phenoloxidase enzyme activity

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ABSTRACT

The study deals with the efficacy of storage buffer restoring phenoloxidase activity under preserved condition. During the study the efficacy of potassium phosphate, Tris-Cl, sodium phosphate and sodium cacodylate buffers were tested in presence of anticoagulant solution containing 30mM sodium citrate in various proportions to determine the maximum PPO activity of the extracts in presence of 4-methyl pyrocathechol and 4-hydroxyproline as substrate. Sodium cacodylate buffer restored the maximum PPO activity by inhibiting melanization of the hemolymph. We propose that sodium cacodylate buffer supplemented with anticoagulant solution containing 30mM

sodium citrate is best suited for determining PO activity and can be used for long-term storage of *Philosamia ricini* hemolymph *in-vitro*.

Keywords: Prophenoloxidase, *Philosamia ricini*, sodium cacodylate buffer, anticoagulant solution

Introduction:

Insects are the most abundant group of animals in nature which may be due to their ability to overcome the external challenges viz- Pathogens, parasites and other stress factors .They possesses a well developed immune system to eliminate the invading agents. (Lemaitre and Hoffmann, 2007). It is assumed that the population of the species can be restored via activation of the immune system which is germ line encoded and is classically divided into innate and adaptive immunity. The innate immune systems consists of effectors events viz. humeral and cellular (Schimid-Hempel, 2005). Humoral responses generally comprise the prophenoloxidase activating system (PPO-AS) while cellular responses include the involvement of hemocytes causing coagulation, phagocytosis, nodule formation and encapsulation (Gillespie *et. al.*, 1997).

Enzyme cascades, such as the phenoloxidase (PO) plays a very important role in the defence response to the foreign invaders, e.g., pathogens and parasites, in the formation of melanin as well as in cuticle sclerotization in insects (Ashida *et al.*,1990). The inactive form of PO i.e Prophenoloxidase (PPO), which circulates in insect hemolymph is activated by certain agents to PO through proteolytic cleavage.

Therefore the role of responses including activation of PPO cascade in response to various pathogens assumes great importance in insect immunological studies. It is even more significant in commercially important species like the *Philosamia ricini* which is intimately associated with the rural economy of the people. *P. ricini* is a multivoltine, silkworm species endemic to the state of Assam. *P. ricini* being a pampered species is subjected to many pathogenic diseases. So a comprehensive study on an effective approach to enhance PO activity of *P. ricini* shall go a long way in providing a possible clue to overcome infectious diseases which reportedly destroy large populations of the silkmoth. However, the study of PO cascade is not easy as it melanises quickly after activation of the enzyme resulting in hemolymph coagulation (Baruah *et al.*, 2018).

The present study therefore aims to identify a suitable buffer system for long term storage of eri silkworm hemolymph which will retain its phenoloxidase (PO) enzyme properties.

Materials and Methods

Preparation of anticoagulant solution: Anticoagulant solution was prepared following the methodology of Kwon *et al.*, (1997) with modifications by adding 30 mm trisodium citrate. P^H was adjusted to 8.0 with 1M NaOH and the solution was filtered with Whatman 0.45 mm syringe filter before use.

Collection of insects: 20 nos of healthy 5^{th} instar larva of eri silkworm (*P. ricini*) were collected from state sericulture farm, Assam, Khanapara. The study was carried out in the biotech hub Laboratory of B. Borooah college.

Storage of hemolymph fractions in tested buffers:

In order to test the efficacy of different buffer fractions for storing the collected hemolymph, four different buffer preparations were done following previously established

protocols (Feng & Fu, 2004). These included 0.2M Sodium cacodylate buffer, 50mM sodium phosphate buffer (pH 6.0), 80mM pottasium phosphate buffer (pH 6.5) & 0.2M Tris-Cl buffer (pH 7.2) (Shaiemma *et al.*, 2012).

The hemolymph collected was stored in the prepared buffers as fractions viz fraction A which comprises of 500µl of Sodium Cacodylate buffer, 500µl anticoagulant solution and 500µl hemolymph; fraction B which comprises 500µl of 80mM Pott. Phosphate buffer (pH 6.5), 500µl anticoagulant solution and 500µl hemolymph; fraction C which comprises of 500µl of 50mM Sodium phosphate buffer (pH 6.0), 500µl anticoagulant solution and 500µl hemolymph; fraction D which comprises of 500µl of 0.2M Tris-Cl buffer (pH 7.2), 500µl anticoagulant solution and 500µl hemolymph. After that, each separate tubes (fraction A to fraction D) were stored at -20 C freezer for 2 month.

Assay and determination of PO activity of separated hemolymph

Phenoloxidase activity was assayed by the spectrophotometric method of Pye (Pye, A.E,1974).After about 60 days of storage, measurement of PO activity was done using 4-methyl pyrocathechol and 4-hydroxyproline. For the same, 500µl 8mm methyl pyrocathechol and 500µl 8mm 4-hydroxyproline ethyl ester was added with 200µl 80 mm potassium phosphate buffer and 50µl hemolymph sample. Which was then incubated for 10 mins at room temperature and absorbance was recorded at 520 nm.

The PO enzyme activity was calculated by using the following formula-

$$b = \frac{\Delta A \times V}{E \times d \times v \times \Delta t} \times d.f$$

where, ΔA = change in absorbance

V = total volume assay mixture (in µl)

E = extinction coefficient (3.6 mM^{-1} cm^{-1})

d = light path

Δt = time for which the change was observed (in minutes)

v = Volume of sample (in µl)

d.f = dilution factor

Results:

Assay of Phenoloxidase activity: Among the four fractions phenoloxidase activity is highest in fraction A. From the table, we have found that among the four fractions phenoloxidase activity is highest in fraction A i.e,1.611. However in fraction B, significant difference were observed in PO activity i.e, PO activity decreased to 0.043. As compared to highest PO activity in fraction A, fraction C has significantly lowest PO activity that is 0.015, and in fraction D, PO activity was slightly less than fraction A i.e,1.184. No significant difference was observed among fraction A and Fraction D.

Table: Comparisions of PO activity in four different buffers.

Fraction	Replicates	O.D	Average	PO Activity
A	A_1	1.394	1.392	1.611±0.002a
	A_2	1.391		
	A_3	1.392		
B	B_1	0.038	0.038	0.043±0.001b
	B_2	0.039		
	B_3	0.037		
C	C_1	0.012	0.013	0.015±0.001b
	C_2	0.015		
	C_3	0.012		
D	D_1	1.022	1.023	1.184±0.002a
	D_2	1.022		
	D_3	1.025		

From our findings we can suggest that sodium cacodylate buffer is the best buffer for long term storage of eri hemolymph followed by Tris-cl buffer.

Discussion and conclusion:

Rearing and cultivation of *Philosamia ricini* has been a part of traditional practice of the region. Its cultivation contributes to economic stability, sustainable livelihood options and income generation. *P. ricini* has been widely exploited for commercial benefit and its wild populations has tremendously declined in recent past due to deforestations, rapid habitat fragmentations, depletion of host plant cover, climate change and anthropogenic pressure, whilst various tropical diseases caused by fungal, bacterial, viral and protozoan infestations have dwindled populations both in the wild and the cultivated forms. So a detailed study of its immune mechanism was long overdue.

We could overcome the process of rapid melanisation of larval hemolymph by addition of anticoagulant solutions to the buffer which was reported in other arthropods but was not reported in silk moths. Our experiments confirmed that sodium cacodylate buffer restored the maximum PPO activity by inhibiting melanisation of the hemolymph.

The result of this study is in conformation to reports in *Antherea assamensis* by Baruah *et.al.*, 2018. So tentatively we propose that sodium cacodylate buffer supplemented with anticoagulant solution containing 30 mM sodium citrate is best suited for determining PO activity and can be used for long-term storage of *Philosamia ricini* hemolymph *in vitro*.

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Can you find me again? Amphibians of Northeast India known from Type Locality

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ABSTRACT

Since 19th century, a number of amphibians have been described from the Northeast India; however, a lot of them - even after many years - are still known from their respective type localities only. Knowledge on the distribution of a species is the corner stone of most ecological research and helps in understating their natural history. However, species known only from its type locality poses a great risk to its survivability, as any destruction of its type locality can lead to the 'local' extinction of that species before we can understand the various aspects of its life. A cataloguing of any such list helps researchers to search for such species and further strengthen the knowledge base.

Keywords: endemic, lost amphibians, biodiversity hotspot, the Himalayas, Indo-Burma hotspot.

Introduction

Amphibians are one of the most diverse groups of vertebrates that dwell in varied habitats across the world. However, they are the most imperiled taxon with unprecedented declines having been documented lately. Linked to a number of factors like habitat destruction, pollution, climate change and emerging diseases, amphibian extinctions are being documented the world over (Sengupta, 2015). Currently, the Class Amphibia is represented by 3 living Orders, *viz.* Anurans characterized by tailless and limbed frogs and toads with 6971 species, Caudata characterized by tailed and limbed newts and salamanders with 722 species and Gymnophiona characterized by limbless caecilians with 209 species, totaling into 7902 species (Frost, 2018). In India, so far 438 species of amphibians [396 species of Anurans, 2 species of Caudata and 40 species of Gymnophiona] are known to occur (Frost, 2018).

Nestled in the remotest Northeastern corner of the country, and sandwiched between the Himalayas and Indo-Burma hotspots, the 7 states of North-east India, viz. Arunachal Pradesh, Assam, Nagaland, Manipur, Mizoram, Tripura and Meghalaya are home to 53 endemic species of amphibians (Saikia and Kharkongor, 2017), of which 33 species are known only from their respective type localities. Among these 33 species, 9 of them were described in the last century with a couple of species dating back to 1912 (Anandale, 1912) and yet to be seen again!

Despite the spurt of intensive survey efforts in these remote forest habitats coupled with modern molecular techniques enabling them to identify a huge number of new species; available pool of literature till date has still failed to report some of the species outside their respective type localities.

Material and Methods

This paper is a product of extensive literature studies carried out by the authors. Classification was followed after Frost (2018). We have ignored species which were described (and yet to be reported for the second time) based on collections from more than one location, for examples, *Megophrys oropedion, Odorrana arunachalensis*, etc. [Mahony *et. al.*, 2013; Saikia *et. al.*, 2017c].

Results

Of the 53 species reported as endemic by Saikia and Kharkongor (2017), 33 species are known only from their respective type localities (see Table 1). Although, 24 species among them have been described post 2000 and are relatively well described both in terms of their morphology and natural history; however, 9 species were briefly described during the last century and they lack details about their morphological characters as well as natural history.

Table 1: NE India amphibians known from the respective type localities

Sl. No.	Species	State	Type Locality	Year(s) of collection
Order: Gymnophiona				
Family: Chikilidae				
1	*Chikila alcocki* Kamei, Gower, Wilkinson & Biju, 2013	Nagaland	Dhyütere, New Sendenyu Kohima	2007, 2008 and 2009
Family: Ichthyophiidae				
2	*Ichthyophis alfredii* Mathew & Sen, 2009	Meghalaya	Nokrek BR, Daribokgre, East Garo Hills	2008
3	*Ichthyophis daribokensis* Mathew & Sen, 2009	Meghalaya	Nokrek BR, Rengsangre, West Garo Hills	2008
4	*Ichthyophis nokrekensis* Mathew & Sen, 2009	Meghalaya	Nokrek BR, Sasatgre, West Garo Hills	2008
5	*Ichthyophis sendenyu* Kamei, Wilkinson, Gower & Biju, 2009	Nagaland	Dhyütere, New Sendenyu Kohima	2007
Order: Anura				
Family: Bufonidae				
6	*Bufoides kempi* (Boulenger, 1919)	Meghalaya	Near Tura	No collection date
7	*Duttaphrynus kiphirensis* (Mathew & Sen, 2009)	Nagaland	Kiphire	2006
8	*Duttaphrynus mamitensis* (Mathew & Sen, 2009)	Mizoram	Mausen, Mamit	2006

9	*Duttaphrynus manipurensis* (Mathew & Sen, 2009)	Manipur	Chakpi, Penthakhuwphuw	2006
10	*Duttaphrynus mizoramensis* (Mathew & Sen, 2009)	Mizoram	Rekdekon, Kolasib	2005
11	*Duttaphrynus nagalandensis* (Mathew & Sen, 2009)	Nagaland	Wokha	2006
12	*Duttaphrynus wokhaensis* (Mathew & Sen, 2009)	Nagaland	Wokha	2006
Family: Dicroglossidae				
13	*Euphlyctis ghoshi* (Chanda, 1991)	Manipur	Khugairk Reserve Forest	1975
14	*Fejervarya sengupti* Purkyastha & Matsui, 2012	Meghalaya	Mawphlang, East Khasi Hills	2011
15	*Nanorana mokokchungensis* (Das & Chanda, 2000)	Nagaland	Mokokchung	1996
Family: Megophryidae				
16	*Leptobrachella khasiorum* (Das, Tron, Rangad & Hooroo, 2010)	Meghalaya	Mawphlang, East Khasi Hills	2009
17	*Leptobrachella nokrekensis* (Mathew & Sen, 2009)	Meghalaya	Nokrek BR, East Garo Hills	2008
18	*Leptobrachella tamdil* (Sengupta, Sailo, Lairemsanga, Das & Das, 2010)	Mizoram	Tamdil	2007
19	*Megophrys ancrae* Mahony, Teeling & Biju, 2013	Arunachal Pradesh	Namdapha National Park and Tiger Reserve, Changlang	2009 and 2011

20	*Megophrys serchhipii* (Mathew & Sen, 2007)	Mizoram	Serchhip	2006
21	*Megophrys vegrandis* Mahony, Teeling & Biju, 2013	Arunachal Pradesh	Sessa, West Kameng	2009
	Family: Ranidae			
22	*Amolops kohimaensis* Biju, Mahony & Kamei, 2010	Nagaland	Jotsoma, Kohima	2007
23	*Amolops nidorbellus* Biju, Mahony & Kamei, 2010	Nagaland	Jotsoma, Kohima	2007 and 2009
	Family: Rhacophoridae			
24	*Chiromantis cherrapunjiae* (Roonwal & Kripalani, 1966)	Meghalaya	Cherrapunji	1959
25	*Chiromantis senapatiensis* (Mathew & Sen, 2009)	Manipur	Kangpokpi, Senapati	2006
26	*Chiromantis shyamrupus* (Chanda & Ghose, 1989)	Arunachal Pradesh	Namdapha Tiger Reserve	1983
27	*Philautus kempiae* (Boulenger, 1919)	Meghalaya	Tura	No collection date
28	*Philautus kempii* (Annandale, 1912)	Arunachal Pradesh	Upper Rotung	1912
29	*Philautus microdiscus* (Annandale, 1912)	Assam	Kobo, Dhemaji	1912
30	*Polypedates subansiriensis* Mathew & Sen, 2009	Arunachal Pradesh	Soro, Lower Subansiri	2007
31	*Raorchestes sahai* (Sarkar & Ray, 2006)	Arunachal Pradesh	Gandhigram, Changlang	1988
32	*Rhacophorus subansiriensis* Mathew & Sen, 2009	Arunachal Pradesh	Ziro-Hapoli, Lower Subansiri	2007
33	*Theloderma nagalandense* Orlov, Dutta, Ghate & Kent, 2006	Nagaland	Tseminyu village, Kohima	2000

Discussion

In 2010, the reported number of amphibians from Northeast India was 119 (Mathew and Sen, 2010a), which later saw an unprecedented increase of newly described species as well as re-discovery of species thought to be extinct, bringing the known number of reported species to 146 by 2017, with 53 of them being endemic to the region (Saikia and Kharkongor, 2017). Among the endemic species, 33 species are still known from their respective type localities. The oldest among them were described in 1912 [*Philautus kempii* and *Philautus microdiscus* (Annandale, 1912)] based on collections made during the Abor Expedition (Hamilton, 1912; Annandale, 1923). However, because these species were described from the collections made during the Abor Expedition in Arunachal Pradesh, the type locality of *Philautus microdiscus* was wrongly presumed to be in Arunachal Pradesh as in reality, the type locality of *Philautus microdiscus* falls under Assam, a mistake which has been rectified recently (Saikia and Sinha, 2017) after 105 years of its description. Another interesting facet of the species described from the Abor Expedition was that as per Frost (2018), *Philautus kempii* is known to occur in India and China, with the possibility of its occurrence in Myanmar based on the report of its occurrence in China by Fei *et. al.*,(2012). However, a perusal of Fei *et. al.*, (2012) leaves no doubt that they have misinterpreted that the type locality (Upper Rotung, Arunachal Pradesh) of *Philautus kempii* falls within China. In the light of this information, the range of *Philautus kempii* must be restricted to its type locality only, under the territorial jurisdiction of India.

In 1919, George A. Boulenger described 3 species of frogs from Meghalaya without specifying their collection localities (other than Garo Hills) or collection dates. Among them, 2 species [*Bufoides kempi* and *Philautus kempiae*] are still known from the type locality.

Roonwal & Kripalani (1966) described *Chiromantis cherrapunjiae*, a bush frog from Cherrapunjee, Meghalaya based on their collections made in 1959. However, repeated visit to the type locality by one of the authors (BS) has failed to spot this species again. Though, Sarkar and Ray (2006) have included this species from Aruanchal Pradesh, but with a lot of contradictions; a thorough perusal of that report leaves no doubt that they had reported a different species as *Chiromantis cherrapunjiae*. Mathew and Sen (2010a), had reported the range of *Chiromantis cherrapunjiae* in Assam, without any supporting voucher specimen and hence, it is prudent to restrict this species to its type locality only.

Chanda (1991) described an aquatic frog species, *Euphlyctis ghoshi* from Manipur based on a single specimen collected in 1975. Since then, there has been no second report of that species.

Chiromantis shyamrupus was described from Arunachal Pradesh (Chanda & Ghose, 1989) based on the collection made in 1983. Similarly, another bush frog from the state, *Raorchestes sahai* was described by Sarkar & Ray (2006) based on a collection made 18 years before it description, in 1988. Both are yet to be seen again.

In 2000, Das and Chanda described a dicroglossid frog, *Nanorana mokokchungensis* from Nagaland based on a single male specimen collected in 1996 after which there has been no second sighting. Another frog from

Nagaland, *Theloderma nagalandense* was described by Orlov *et al.*, (2006) based on a single male specimen collected in 2000. Literature is yet to confirm its second sighting since their descriptions.

Post 2000, there has been a number of species which were described relatively in greater details [Biju *et. al.*, 2010; Das *et. al.*, 2010; Kamei *et. al.*, 2009 & 2013; Mahony *et al.*, 2013; Mathew and Sen, 2007, 2009 a & b, 2010b; Purkyastha and Matsui, 2012; Sengupta *et al.*, 2010], however, still they are known from their respective type localities.

A number of recently described species, *Amolops assamensis*, *Polypedates assamensis*, *Megophrys zunhebotoensis* and *Leptobrachium bompu* have already been reported from additional locations [Saikia, 2012; Saikia and Sen, 2012; Saikia *et al.*, 2017a&b]. While describing *Megophrys vegrandis* by Mahony *et al.*, (2013) from Sessa, Arunachal Pradesh, they have reported about its probable occurrence in the nearby Eaglenest Wildlife Sanctuary (WLS), located about 12.4 km from the type locality. Despite that, we have still keep this species in this list, as there has been no confirmed report of its occurrence from Eaglenest WLS.

Amphibian conservation has not attained the much required momentum because of the primary lack of follow up work after their initial taxonomic descriptions. Given that many species are habitat specific, they are prone for 'local' extinctions when their habitat undergoes minor perturbations. As such, understanding their ecology and distribution can help in implementing robust conservation action plans taking them as indicator species. Impending works that need to be addressed for these species is to collect quantitivate data on their populations and habitat in their type localities. Predictive species distribution models can also be put to use but interpreted with caution.

Amphibian fauna of Northeast India have characteristic gateway features with Indo-Chinese and Indo-Malayan origin. This compiled list presented here presents future thrust areas of targeted amphibian research. We highly recommend that extensive explorative work should be carried out in and around the type localities to find them again if at all they are there still.

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Study of the diversity of endoparasitic protozoans in the common cat fishes of Assam

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ABSTRACT

Endoparasitic protozoans consist of a major proportion of numerous parasitic organisms that harbour the bodies of various food fishes causing damage to fish health and fishery industry as a whole. This study was conducted on three main species of cat fishes of Assam which include *H.fossilis, C.batrachus* and *M.vittatus* to identify the possible endoparasitic protozoans present in the body of these fishes. Microscopic examinations of the blood smears and flushed out contents of the intestines revealed the presence of four groups of endoparasitic protozoans- 4 ciliates, 1 flagellate and 1 coccidian. Blastocysts, a Sarcodian was also found in the study. Most parasites were found to be localized in the intestinal regions and the most common protozooan among the 7 different species was Opalina,which

was found to be present in almost each individual fish taken up for study. As all the fishes selected for the study were freshwater fishes, a certain pattern of homogeneity was observed in the variety of endoparasites identified.

Keywords : endoparasitic, protozooans, cat fishes, fish diseases, freshwater

Introduction

Fish parasitology is a rapidly developing field of aquatic science. This is due to the growing importance of aquaculture, concerns on pollution effects on fish health and a generally increasing interest in environmental biology (Moller and Anders,1986). Fish protein is the most easily affordable animal protein and accounts for more than 40 % of the protein diet of two- third of the world's population (Eyo., 1992). In recent times, there has been tremendous increase in the development of fish farming and culture attributable to the increased need for affordable animal protein especially in the tropics (Davies *et al.*, 2006).

Among all vertebrates, fishes are the most parasite prone. (Klinger and Floyd, 2000) and the importance of parasitic infection on fish production has largely remained an issue of concern to fish farming industry (Dogiel.,1961). Fish parasites are numerous and many phyla in the animal kingdom have representative that are parasitic to fish. In case of aquaculture or fish farming, parasites may be highly pathogenic and contribute to high fish mortalities and economic loss, while in natural systems they may threaten the abundance anddiversity of various indigenous fish species. Parasites are of concern since they often produce a weakening of the host immune system thereby increasing their susceptibility to secondary infections, resulting in the nutritive devaluation of fish and subsequent economic loss (Onyedineke *et al.*, 2010).

Parasites affecting fishes may be of various types ranging from single celled organisms like the protozoans to multicellular organisms like the helminthes. These parasites maybe ectoparasites or endoparasites depending on the site of occurrence within the body or outside the body. One of the most important classes of endoparasitic organisms infecting fishes are single celled protozoans. They are mostly found in digestive and respiratory systems, muscles, blood and faeces of their hosts and cause diseases that affect the normal health conditions and cause reduction of growth, abnormal metabolic activities and even death of the affected fish. The factors that directly influence the abundance and prevalence of endoparasitic protozoan fauna of fishes include- diet, age, environment of fishes and season (Doglel,1964). Protozoan parasitic infection results in a variety of problems ranging from mild irritations to rupture or perforations of intestine, weakening of immune system and various other diseases causing innumerable damage to fishery industry.

Thus protozoans are a major sector of fish parasites which can cause serious damage and prove to be a hazardous threat to fish health. Accordingly a study was carried on the diversity of common endoparasitic protozoans of certain catfishes of Assam. Three species of cat fishes were selected for this study. The reason behind selection of these species were easy availability and popularity, high food value and their ability to sustain in captivity for longer periods of time.

The objectives of this study was to mostly study the diversity of the endoparasitic protozooans if present in the body of the

selected fishes and also to identify them so as to adapt better measures for prevention of fish diseases and improvement of fish health.

Materials and Method:

Collection of fishes:

For the conducted study, three common catfishes, easily available in Assam were selected namely: *Heteropneustes fossilis* commonly called 'xingi', *Clarius batrachus* commonly called 'magur' and *Mystus vittatus* commonly known as 'xingora'. Fishes were brought from the fish market and kept in aquariums till they were sacrificed as only one fish per day was used for the purpose of this study.Three fishes of each species was taken for the conducted study. Proper food was provided to the fishes during the period of their captivity and water was changed at regular intervals.

Collection of protozoan parasites:

For the purpose of study two basic methods were carried out. The first is the study of parasites in the blood of the fish and the next for the observation of parasites inside the viscera like stomach intestine, skin and gills.

Study of blood parasites among the protozoans were done by following the method by Kori - Siapere and Ake, 2005. For this purpose, a live fish was first desensitized by means of chloroform and blood was collected from the caudal vein with a 23 gauge plastic syringe. A thin blood smear was made from the blood collected, air dried and fixed in absolute methanol. The smear was then examined by placing it in a 10 x 40 magnification of electronic microscope. The observed parasites were compared with the keys of fresh water fish parasite pictorialguide by Deborah *et al.*, (2005) and recorded.

In the second method, the body cavity was opened with the aid of a dissecting kit starting from the genital papillae down to the gill region. The viscera, the gills and skin as well was lightly scraped and the contents were kept in a petri-dish with 0.9% saline solution. The intestine and the stomach were flushed out of its contents by means of a syringe into the petri-dish containing 0.9% saline solution, placed in a separate dish. Each drop of the residue was placed on the slide and viewed under 10x and 40x objective light microscope. For better visualization, vital staining technique was used to stain the parasites since they are almost transparent. The observed parasites were compared with the keys of fresh water fish parasite pictorial guide by Deborah *et al.*, (2005) and recorded.

The same procedure was repeated for all the experimental fishes.

Results:

The results obtained in this study revealed the presence of various endoparasites. The endoparasitic protozoans obtained are grouped into four main classes as done by Klinger and Floyd, 2000. They are :

- Ciliates
- Flagellates
- Coccidian
- Sarcodina

Ciliates :

Four species of ciliates were observed in the study. They include

1. *Ichthyophthirius multifilis* which is dark in colour having a horse-shoe shaped macronucleus and more translucent in appearance.
2. *Tetrahymena* is a teardrop-shaped ciliate and its entire body is covered by cilia and it is free living.
3. *Opalina* is a leaf-like in shape and is covered by flagelliform cilia. It has presence of numerous nuclei, the

appearance of two short sickle-shaped rows of cilia.

4. *Ambiphyra*, is a sedentary ciliate that is found on the skin, fins, or gills of host fish. Its cylindrical shape, row of oralcilia, and middle bank of cilia identify *Ambiphyra*.

Flagellates :

One species of flagellate was observed in the study namely Cryptobia which is a drop-shaped,with two flagella, one on each end.It consists of single nucleus and is seen in blood, gills as well as intestine.

Coccidian :

The only coccidian found in the study was *Sarcocystis* which is small and rounded containing single nucleus and remains in groups.

Sarcodina:

Balastocystis is a single celled parasite belonging to class sarcodina found in the study. Two forms of this parasite were seen - the vacuolar form having the presence of a large vacuole and the granular form with the presence of distinct granules.

The parasites were found and identified from various parts of the body of the fishes. The viscera, the vital internal organs, gills, skin and the blood were all seen to contain one or more of the listed parasites. However the intestinal region of all the seven fishes showed highest diversity of parasites in contrast to the other regions of the body. The location of the different groups of parasites were also seen to be different.

Table 1: Distribution of the various groups of endoparasiticprotozooans within the body of the fishes

Class of parasites	Location in the body
Ciliates	Skin, blood, gills, viscera, gut
Flagellates	Blood, gut
Coccidians	Viscera
Sarcodina	Gut

Discussion:

The conducted study revealed the presence of 7 different species of protozoan parasites in the body of the selected fishes although not each species of fish was seen to possess all the 7 different types of protozoan species. Similar studies on the diversity of endoparasitic protozoans in freshwater fishes was previously conducted by Abolarin,1996, Omeji *et al.*, 2011, Hoffman *et al.*,1967 and Kim *et al.*, 2002.,which reports the presence of various protozooans inside the body of various fishes including some endoparasites that are similar to the ones reported in our study. The intestine was found to harbour the highest diversity of parasites in the body of fishes. The reason behind this may be the reason that the most of the digestion and activity of enzymes in the break down of food takes place in the intestine which cause the liberation of the protozoan cysts or spores gets liberated from the ingested food which serves as the medium for the entry of the fishes. This result is supported strongly by the works of Omeji *et al.*, 2011 and Lom J, 1992 and Paprna *et al.*,1996.

Conclusion:

Thus the study conducted on the endoparasitic protozoans of catfishes showed a variety of species and this study was supported by various studies conducted previously and such studies will help in recognition of the fish parasites which will in turn lead to improved measures in their infestations and disease of fishes. This will contribute in the improvement of fish health and the fishery industry as a whole and also quality production of fish protein which in turn will result in better diet and human health.

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Effects of ethanolic leaf extract of *Hibiscus rosa-sinensis* on gonadosomatic index and testicular histology of cadmium induced male albino mice

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ABSTRACT

Infertility due to the toxic effect of drugs and environmental chemicals has become a major problem for the modern society. Cadmium (Cd) is a toxic heavy metal and an important pollutant, present widely in our environment and workplaces. It is well known that cadmium plays some negative role on male reproductive system. The present study examined the efficacy of leaf extract of *Hibiscus rosa-sinensis* plant, a natural herb, with androgenic properties, against infertility in male mice induced by cadmium (Cd). Twelve mature albino male mice were used as a mammalian model. The statistical analysis shows a highly significant ($P<0.01$) decrease in the gonadosomatic index in cadmium chloride treated group when compared with control group. There was a significant increase in gonadosomatic index in the cotreated ($CdCl_2$+leaf extract) group as

compared to cadmium chloride treated group. The cadmium treated mice showed significant alterations in the testicular tissue including decrease in diameter of seminiferous tubules and wide interstitial spaces. Absence of spermatozoa was also noted in the seminiferous tubules of this group of mice. But cadmium and *Hibiscus rosa-sinensis* leaf extract treated group showed normal structures although interstitial spaces still remain in reduced forms. The findings suggest that cadmium, like all other heavy metals, could induce infertility and administration of leaf extract of *Hibiscus rosa-sinensis* can reduce the antiandrogenic toxic effect of cadmium in male mice.

Keywords: *Hibiscus rosa-sinensis*, Cadmium Chloride, Gonadosomatic Index

Introduction:

The therapeutic use of plants and their extracts may be a promising approach for the treatment of different diseases. *Hibiscus rosa-sinensis* (Linn.) belongs to family Malvaceae and grows as an evergreen herbaceous ornamental shrub throughout the world. *Hibiscus rosa-sinensis* is widely used in folk medicine to treat a wide range of diseases and has numerous pharmacological activities. The roots are cylindrical, 5 - 15 cm in length and 2 cm in diameter, off white and with light brown transverse lenticles. The roots taste sweet and are mucilaginous.The leaves are simple ovate or ovate lancolate, and are at the base and coarsely toothed at the apex. The flowers are pedicillate, actinomorphic, pentamerous and complete. The corolla consists of 5 petals, red coloured and about 8 cm in diameter. It is well established that the leaves, stems, roots and flowers of *H. rosa-sinensis* have various pharmacological properties. Some of the chemical constituents isolated from this plant are cyanidin, quercetin, hentriacontane, calcium oxalate, thiamine, riboflavin, niacin and ascorbic acid, flavonoids (Nair *et al.*, 2005). *H. rosa-sinensis* has been used for the treatment of a variety of diseases as well as to promote wound healing (Shivananda *et al.*, 2007). Cold aqueous extract of *H. rosa-sinensis* leaves causes aphrodisiac activity (Dada *et al.*, 2007). In traditional medicine, the leaves of the plant are used in fatigue and skin disease.

Infertility is one of the major problems of the modern society. In general, approximately half of the infertility cases are caused by factors related to the male partner. A major portion of male infertility has a genetic basis, eg. Gonadotrophin- releasing hormone (GnRH) deficiency, spermatogenic failure, both obstructive and non-obstructive azoospermia (Mak, 1996). Besides these, the toxic effect of drugs and environmental chemicals has caused a major concern on male reproductive system (Barltrop *et al.*, 2003). Epidemiological studies have shown correlation between heavy metals concentrations in the body and human health. The body absorbs these toxic substances which are distributed into body systems and lead to different diseases. Cadmium (Cd) is one of the most important heavy metals present in the soil, water, air, food and in cigarette smoke. It shows high toxicity to different biological systems. It is a toxicant that has a long biological half-life (15-20 years) mainly due to its low rate of excretion from the body and accumulates over time within the blood, kidneys, liver and reproductive organs (Waisberg *et al.*, 2003). Gonad is the main target organ for environmental toxins and rodent testes are especially sensitive to the toxic effects of Cd exposure. Cd stimulates the production of reactive oxygen species (ROS)

in association with its inhibitory effect on mitochondrial electron transport. As a result, lipids are oxidized resulting in damage to membranes (Galazyn *et al.*, 2009). Cd reduces reproductive capacity by causing severe testicular degeneration, seminiferous tubule damage and necrosis in rats (Lafuente and Esquifino, 1999). Different levels of cadmium in seminal fluid may cause abnormal function of spermatozoa and their fertilizing capacity. In the present study we examined the androgenic activity of leaf extract of *Hibiscus rosa-sinensis* plant on cadmium induced albino mice.

Materials and methods:

Housing of animals: Adult male albino mice weighting 25-40 g were brought from the College of Veterinary Science, Khanapara, Assam. The animals were adapted to the laboratory condition for 1 week prior to experiment. Standard animal feed composing of wheat, bran, maize, biscuit and gram along with multi vitamins (agrimin forte) was given to the mice. Water was provided to the animals *ad libitum*.

Leaf extract preparation: Leaves of *Hibiscus rosa-sinensis* were collected from Nagaon district. The leaves were washed with distilled water thoroughly and kept for drying in shade. After drying, the leaves were crushed in mixture grinder into powdered form. 10 gram of leaf powder was dissolved in 100 ml of ethanol and kept at 27 °C for 2 days and filtered through Whatmann number l filter paper. The filtrate was then allowed to evaporate to get concentrated filtrate which was again reconstituted in small volumes of same solvent. Finally content was evaporated to dryness in a vacuum evaporator. The dry extract was then used in preparation of various required concentration with ethanol for transfer.

Cadmium Chloride Solution: Cadmium Chloride Dried Anhydrous manufactured by Sisco Research Laboratories Pvt. Ltd. was used for this experiment. Cadmium chloride was dissolved in 0.9% normal saline solution.

Experimental Design: 12 Albino mice of average body weight 30 g were randomly divided into 4 groups and each group contained 3 mice each (n=3). Group I served as control, group II served as Cadmium treated(4mg/kg), group III served as only *Hibiscus rosa-sinensis* leaf extract(1g/kg) treated and group IV served as both Cadmium Chloride(4mg/kg) and *Hibiscus rosa-sinensis* leaf extract(1g/kg) treated (cotreated).

Weight of the testes with epididymis: The mice were sacrificed under anaesthesia and their testes and epididymis were removed. The weight of the testes with epididymis was taken using single pan balance.

Gonadosomatic Index: To find the gonadosomatic index(GSI), the whole body weight of organism was taken and the testes weight of the organism was taken. Then the GSI was calculated by the following standard method.

GSI=(testes weight / total body weight)×100

Histological study: Animals were dissected and their testes were removed. For histological preparations, the testes were fixed in Bouin's fluid, dehydrated, cleared and embedded in paraffin wax. Five-micrometer thick sections were prepared and stained with Ehrlich's haematoxylin and eosin (Lillie *et al.*, 1976).

Statistical Analysis: All the gonadosomatic index and sperm count values were given as mean ± standard error of mean. Differences were tested for statistical

significance by one way analysis of variance (ANOVA) with the help of Microsoft EXCEL and were considered statistically significant when $p \leq 0.05$.

Result: Daily administration of cadmium chloride ($CdCl_2$) and leaf extract of *Hibiscus rosa-sinensis* plant for 3 days to the male albino mice causes some changes including:

Gonadosomatic Index: Gonadosomatic index variation in four groups of mice viz. control, cadmium chloride treated group, *Hibiscus rosa-sinensis* leaf extract treated group, cadmium chloride and Hibiscus rosa-sinensis leaf extract treated group are given below---

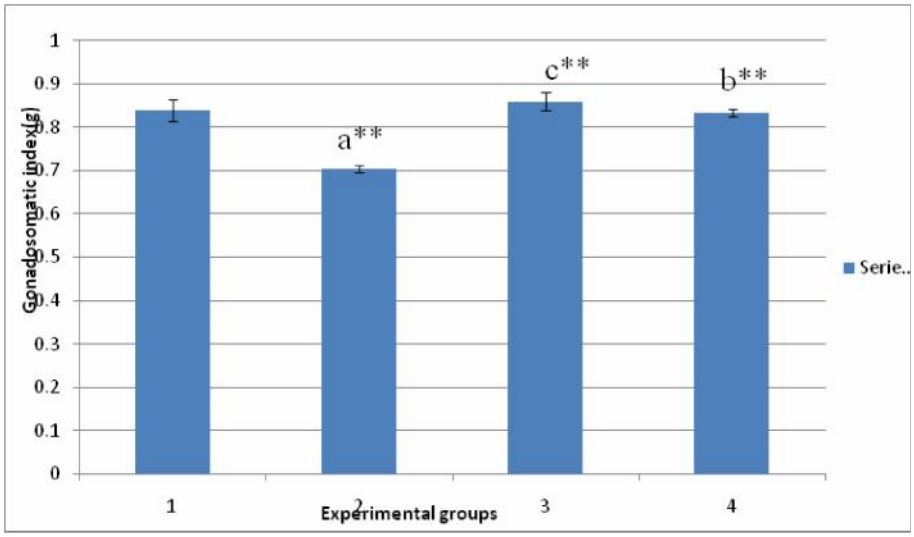


Fig 1: Graphical representation of the gonadosomatic index variation among different experimental groups viz. 1=Control, 2=Cadmium Chloride treated, 3= *Hibiscus rosa-sinensis* leaf extract treated, 4= Cadmium Chloride and leaf extract treated (Cotreatment)

$p \leq 0.001$=***, $p \leq 0.01$=**, $p \leq 0.05$=*

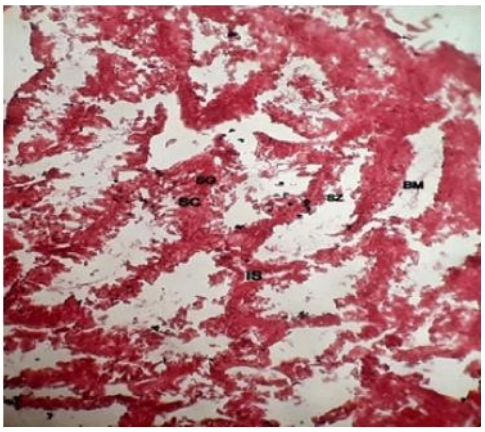
a. Group I vs. Group II

b. Group II vs. Group IV

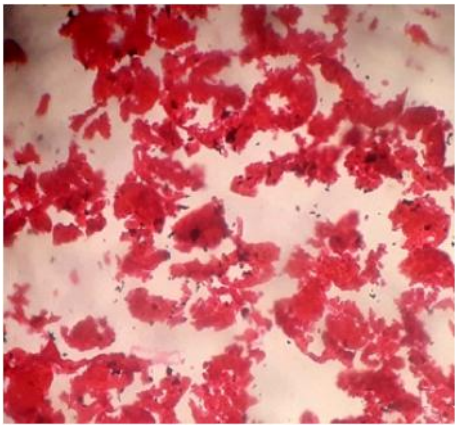
c. Group III vs Group IV

In the present study it was found that gonadosomatic index decreased with a significant ($p \leq 0.01$) difference in cadmium chloride treated animals (Group II) in comparison to control group (Group I). Gonadosomatic index increased with a significant ($p \leq 0.01$) difference in animals receiving leaf extract with cadmium chloride (Group IV) (cotreatment) as compared to cadmium chloride induced group (Group II). Gonadosomatic index of animals (Group III) treated with only leaf extract was also increased with a significant ($p \leq 0.01$) difference in comparison to cotreatment group (Group IV) (Fig.1).

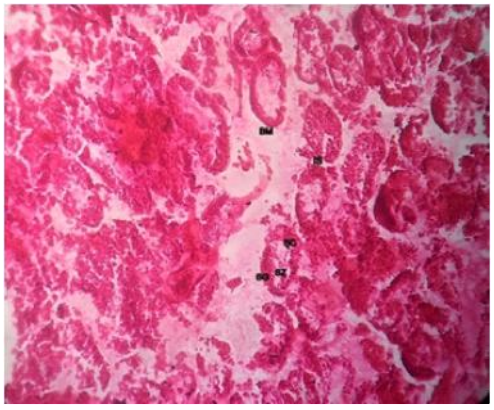
Histological Studies:



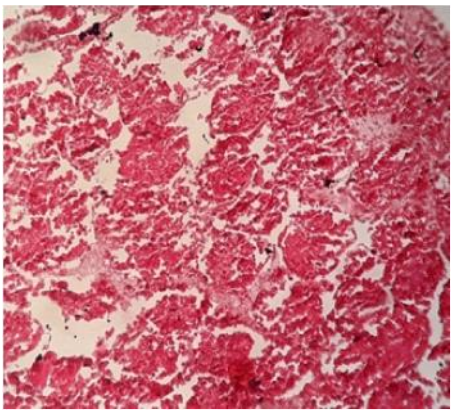
Photoplate: 1



Photoplate: 2



Photoplate: 3



Photoplate: 4

Photoplate:1 Control group showing normal structure of seminiferous tubules (ST). Spermatogonia(SG), spermatocyte (SC), spermatozoa (SZ) and basement membrane were seen; **Photoplate:2** Treated with Cadmium Chloride showing decrease in diameter of seminiferous tubule(ST), destruction of some seminiferous tubules and increase in interstitial space(IS); **Photoplate:3** Treated with only *H. rosa-sinensis* leaf extract showing the normality in diameter of seminiferous tubules (ST) and in interstitial space (SP); **Photoplate:4** Treated with Cadmium Chloride+ *H. rosa-sinensis* leaf extract showing normal seminiferous tubules (SP).

Discussion:

Result of the present investigation showed that Cadmium chloride exposure to mice resulted in decreased gonadosomatic index and significant detoriation in the histology. It was found that Cadmium has caused

changes in histology of the testis, seminal vesicle and restraint of spermatogenesis statistically significant increase in the percentage of the sperm abnormalities.

Similar result of decrease in body and testis weight was observed by Sakr and Nooh (2013) after the exposure of rats to cadmium. Histological analysis in his work revealed intertubular hemorrhage, degeneration of spermatogenic cells and interstitial tissue. A reduction in seminiferous tubule diameter and germinal epithelial height was also observed. Elgawish and Ghanem (2014) have observed same effect of cadmium on male reproductive system of albino rats. Akinloye *et al.*, (2005) reported that cadmium exhibits a deleterious effect on the reproductive system of Nigerian males. It has been reported that as low as 1-2 mg Cd/kg body wt. can cause testicular damage without pathological changes to other organs (Prozialeck *et al.*, 2006). Acute doses of cadmium are known to have a destructive action on testicular tissue (Parizek, 1960; Gunn and Gould, 1970; Friberg *et al.*, 1974).

In the present study we found that *Hibiscus rosa-sinensis* leaf extract can reduce the degenerative effect of cadmium chloride. We found that after administration of leaf extract of *Hibiscus rosa-sinesis* leaf extract, body weight and testes weight along with the epididymis of albino mice increased significantly which is the similar result with the work done by Dada *et al.*, (2007) by using *Hibiscus rosa-sinensis* Linn. leaf extract on immature albino male rats. He found that after the treatment with the alcoholic leaf extract there is an increase in the weight of the testis, epididymis, seminal vesicles and prostate of the albino rats which is the similar result with us.

Many other researchers have found the androgenic effect of leaf extract of *Hibiscus rosa-sinensis* albino mice. Moundipa *et al.*, (2006) carried out an experiment to observe the effect of different plant extracts of *Hibiscus macranthus* and *Basella alba*. Use of these extracts produced higher levels of testosterone in the incubation medium containing methylene chloride or methanol extracts. The seed extract of *Hibiscus cannabinus* showed the aphrodisiac activity by increasing the blood testosterone level in male albino mice (Zade and Dabhadkar, 2013). Results of the present study support their observations.

But the result of present study has contradicted to the findings of Mishra *et al.*, 2009 and Jana *et al.*, 2013. They found that after the administration of flower extract of *Hibiscus rosa-sinensis* body weight, testis and epididymis weight were reduced. The crude extract of the *Hibiscus-rosa-sinensis* flowers can lead to changes in germinal epithelium of the testes.

Conclusion:

From the present study it is concluded that, the exposure of Cadmium Chloride induces histopathological and biochemical effects in mouse testes. *Hibiscus rosa-sinensis* leaf extract has protective effect against cadmium toxicity evidenced by increase of gonadosomatic index, sperm concentration, sperm count and minimal histopathological changes in the testes of mice treated with cadmium+ leaf extract of *Hibiscus rosa-sinensis*.

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A study of water quality in some wetlands of the Brahmaputra valley, Assam, India

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ABSTRACT

In the present study, surface water quality of wetlands has been studied considering seasonal variation. Water samples from ten different wetlands of the Brahmaputra valley were collected and analyzed for pH, electrical conductivity, total dissolved solids, bicarbonate, chloride, sulphate, nitrate, calcium, magnesium, sodium, potassium and total hardness. The order of the abundance of the major cation and anion is as follows: HCO_3->SO_4^{2}->Cl^->Na^+>Ca^{2+}>K^+>Mg^{2+}>NO_3^- in premonsoon and HCO_3->SO_4^{2-}>Cl^->Na^+>Ca^{2+}>Mg^{2+}>K^+>NO^{3-}in postmonsoon. Based on the water quality index (WQI), Deepor Beel and wetlands of Kaziranga National Park shows poor water quality. Principal component analysis used

for source apportionment of the parameters indicated pH, HCO_3^-, TH, EC, TDS, Cl^-, Ca^{2+}, Mg^{2+}, Na^+ and K^+ as the mineral component of the wetlands whereas Mg^{2+}, NO_3^- and SO_4^{2-} originated from anthropogenic sources such as agricultural run-offs, nearby tea gardens and sewage sludge.

Keywords: Wetlands, Water quality index, Principal component analysis, physico-chemical properties

Introduction

Wetlands are landscapes, either temporarily or permanently covered with water and exhibits vast diversity according to their geographical location, genesis, water regime and chemistry. Assam is endowed with many natural lentic water bodies locally known as beel (Jhingran and Pathak, 1987). Assam has 3,513 wetlands covering a total area of 1012.32 ha. It constitutes 1.29% of the total geographical area of the state. This includes the natural and artificial wetlands, rivers, lakes and agricultural fields. Of these, 3388 wetlands are natural and dominate the state. Assam occupies seventh place according to geographic area under the wetlands of the country (National Wetland Atlas, MOEF, 2011). In Assam, the entire valley stretches from the western Brahmaputra valley covering the regions of Goalpara and Kamrup; the central Brahmaputra valley region covering Darang, Nagaon and Sonitpur districts; and, the eastern Brahmaputra valley covering the districts of Lakhimpur, Dibrugarh and Sibsagar with an entire stretch of 914 km^2.Wetlands in Assam are one of the most productive ecosystems of the region and plays an important role in the hydrological cycle. The wetlands studied in the work are situated in the floodplain areas of the Brahmaputra and are directly or indirectly linked with the major river system and its tributaries. Besides, it also has a link with the ground water aquifers. The chemistry of surface water is an important factor determining its use for domestic, irrigation and industrial purposes. The quality of surface water within a region is governed by both natural processes (such as precipitation rate, weathering processes and soil erosion, hydrological processes; physical, chemical and biological processes) (Khalil & Ouarda, 2009; Pejman, 2009) and anthropogenic effects (such as urban, industrial and agricultural activities and the human exploitation of water resources (Nouri, 2008; Noori, 2010)

The main objective of the work is to define the quality of water in the wetlands of the Brahmaputra valley with special reference to their physicochemical properties by calculating their water quality index (WQI).

Materials and methods

Description of the Study area

The following wetlands from different districts of Assam were selected for the study.

Table 1. Coordinates of the sampling locations

Sl.no	Wetland	District	Latitude	Longitude
1	Deepor Beel(DB)	Kamrup	26° 11' N	91°35'E
2	KNPw	Golaghat&Nagaon	26°46'N	93°08'E
3	Maguri	Dibrugarh	27°47'N	95°28'E
4	Saran	Morigaon	26°14'N	92°19'E
5	Samaguri	Nagaon	26°25' N	92° 51'E.
6	Hahila	Nagaon	27°27'N	92°53'E
7	Jengdia	Kamrup	26°16' N	91°46'E
8	Pobitora	Morigaon	26°12'N	91°59'E
9	Laukhoa	Dhubri	26°06' N	89°57'E
10	Sareswar	Dhubri	26°08'N	89°55' E

Sample collection and analytical methods

Water sample from the following wetlands were collected and analyzed for physicochemical parameters following the established procedures of (APHA, 2005). The parameters pH, EC and TDS were monitored at the sampling site with the (pHTestr20), (ECTestr11+), and (TDSTestr11+), and other parameters like total alkalinity and total hardness (titrimetric method), chloride(silver nitrate method), nitrate (phenoldisulphonic acid method)and sulphate (turbidimetric method) were analyzed in the laboratory. Calcium, magnesium, sodium and potassium were analyzed in Systronics Flame photometer128.

Water Quality Rating and Weightage

Water Quality Index was first formulated by the method given by Horton (1965) with slight modifications (Tiwari & Mishra, 1985; Trivedy and Goel, 1986).The weighted arithmetic index method has been used for the calculation of W.Q.I. Further quality rating or sub index (Qn) was calculated using the following expression:

$$Qn = 100 \times (Vn-Vo) / (Sn-Vo) \quad \text{(Eq.1)}$$

Where, Qn = Quality rating for the nth water quality parameter

Vn = Estimated value of the nth parameter at a given sampling station

Sn = Standard permissible value of the nth parameter

Vo = Ideal value of nth parameter in a pure water. (i.e. 0 for all other parameters except the parameter pH)

Unit weight was calculated by a value inversely proportional to the recommended standard values Sn of the corresponding parameters.

$$Wn = K/Sn \quad \text{(Eq.2)}$$

Where, Wn = Unit weight for the nth parameter.

Sn = Standard value for nth parameter.

K = Constant for proportionality

The overall Water Quality Index (W.Q.I) was calculated by aggregating the quality rating with the unit weight linearly.

$$WQI = \sum Qn\, Wn / \sum W \quad \text{(Eq.3)}$$

Statistical Analysis

The statistical software package SPSS 15 window was used for correlation coefficient and multivariate analysis of the data.

Pearson's correlation

The correlation matrix of the data was built to find out the associations between the variables. Significant positive correlations can be explained in terms of common source or chemical similarity (Knudson, 1977). Thus, significant positive correlation could indicate a common source for the pairs.

Principal Component Analysis (PCA)

PCA provides information on the most meaningful parameters which describe the whole data set interpretation, data reduction and summarize the statistical correlation among constituents in the water with minimal loss of original information (Helena *et al.,* 2000; Wunderlin *et al.*,2001). In this study, PCA of the normalized variables were executed to extract significant principal components (PCs) and to further reduce the contribution of variables with minor significance; these PCs were subjected to varimax rotation generating factors (Shrestha and Kazama, 2007).

Results and discussion

Physico chemical properties

pH is the indicator of acidic and alkaline condition of water status. BIS 1983, have suggested 6.5-8.5 range of pH for water for any purposes in that respect; the ranges indicate moderately alkaline water of the wetlands. In all the wetlands, pH in pre monsoon was lower than post monsoon due to the water levels and concentration of nutrients in water (Narayana, *et al.,* 2008).

Electrical conductivity is the water capability to transmit electric current and serves as tool to assess the purity of water (Murugesan *et al.,* 2006). This ability depends on the presence of ions, their total concentration, mobility, valence, relative concentrations and temperature of measurement (Shinde *et al.,* 2011). Conductivity of the wetlands was significantly different among sampling sites, varying from 22 to 169 µS/cm. High conductivity at DB and KNPw in post monsoon indicates the mixing of sewerage in river water.

TDS further indicates the salinity behavior of river water. TDS in water originates from natural sources, sewage, urban runoff, industrial wastewater andchemicals used in the water treatment process. The highest TDS is recorded in DB and KNPw during post monsoon due to the addition of organic matter and solid waste into the lake (Moss, 2014).

Bicarbonates in surface water is primarily a function of carbonate, hydroxide content and also includes the contributions from borates, phosphates, silicates and other bases. Highest HCO_3^- in DB indicates the sewerage mixing in the wetland. Post monsoon season recorded higher HCO_3^- due to high nutrients in water (Uduma, 2014).

TH is a very important property of water from its domestic application point of view. Hardness in water is due to the natural accumulation of salts of mainly calcium and magnesium. There is a moderate variation in TH contents among different sampling sites and a trend of higher TH was found at major polluted site in DB due to mixing of domestic effluents in the wetland water. Also, anthropogenic activities might be responsible for higher TH at this site. The similar trend of TH increasing in summer was established by (Moharana & Patra, 2014)

Calcium is one of the most abundant ions in fresh water and is important in shell

construction, bone building and plant precipitation of lime. The higher calcium content may be due to mixing of urban runoff and industrial wastewater. The lowest amount of calcium in water was recorded during post-monsoon due to calcium absorbed by the large number of organisms for shell construction, bone building and plant precipitation of lime (Solanki, 2012). Similar trend observed by Moharana & Patra, 2014.

Magnesium is often associated with calcium in all kinds of waters, but its concentration remains generally lower than the calcium. Magnesium is essential for chlorophyll growth and acts as a limiting factor for the growth of phytoplankton.The lowest value was recorded during pre-monsoon due to the magnesium essentiality for chlorophyll bearing plant for photosynthesis (Pawar and Pulle, 2005).

The high chloride reported in post-monsoon may be due to frequent run-off loaded with contaminated water from the surrounding area and evaporation of water (Moharana & Patra, 2014). Similar to our present observation, (Mishra & Tripathi, 2003; Zafar & Sultanab, 2008) also reported high chloride in post-monsoon. The high content of chloride may also be due to storage of the accumulated sewage during rainy season coupled with decaying process that accomplished by the microbes (Imnatoshi & Ahmed, 2010).

Sulphate and nitrate are important parameters of surface water showing the pollution status and anthropogenic load in any water. The highest amount of nitrate was recorded during post-monsoon because of high vegetation during winter which supported the growth of plankton (Pandit & Solanki, 2012). The lowest amount of nitrate in water was recorded during pre-monsoon due to the utilization by plankton and aquatic plants (Verma *et al.*, 2010).

High sulphate in Maguri beel and KNPw may be due to application of sulphur based fertilizers in tea gardens and agricultural runoffs.

Table 2 Average concentration of parameters in pre monsoon

	db	knp	maguri	hahila	samaguri	saran	pobitora	jengdia	laokhoa	sareswar
Ph	7.63	7.56	7.2	7.57	7.36	7.32	7.55	7.1	7.12	7.16
HCO3-(mg/l)	31.45	27.01	18	15	10.21	12.11	17.63	15.7	13.33	12.47
TH(mg/l)	34.48	29.36	20.89	17	16.28	22.45	21.6	16.5	19.29	15.77
EC(µS/cm)	158.75	146.57	66	57	128	90	100	122	22	48
TDS(mg/l)	101.6	81.64	42.24	36.48	81.92	57.6	64	78.08	25.89	30.63
Ca(mg/l)	8.15	9.01	1.5	2.9	5.5	5	4.78	1.9	1.2	3
Mg(mg/l)	3.04	2.66	2.82	2.56	2.13	2.34	3	1.6	1	1.89
Cl(mg/l)	12.97	12.95	6	5.8	7.6	8.2	7.5	6.9	6.5	5.9
SO_4(mg/l)	4.86	12.7	34.8	3.7	5.4	12.4	11.3	2.3	1.1	1.7
NO_3(mg/l)	0.26	0.16	0.09	0.68	0.1	1.56	0.16	0.25	0.16	0.07
Na (mg/l)	12.71	5.69	2.54	0.5	6.5	3.3	2	3.76	3	3.9
K (mg/l)	3.9	4.67	1.2	1.1	2.56	2.89	1.5	2.9	2.73	1.1

Table 3. Average concentration of parameters in post monsoon

	db	knp	maguri	hahila	samaguri	saran	pobitora	jengdia	Laokhoa	sareswar
Ph	7.97	7.88	7.17	7.6	7.5	7.4	7.59	7.2	7.17	7.24
HCO3-(mg/l)	45.83	31.87	19.62	21.3	13.97	16.83	21.5	20.8	15.2	14.56
TH(mg/l)	33.26	26.98	17.82	14.34	13.46	18.9	20.11	15.67	15.45	12.82
EC(µS/cm)	164.12	169.71	69	70	132	101	112	100	45	56
TDS(mg/l)	145.66	103.01	56.52	45.67	69.1	40.2	72.81	80.42	34.89	42
Ca(mg/l)	6.87	7.03	1.21	1.76	5.1	4.34	4.08	2	1.44	2.82
Mg(mg/l)	3.23	2.98	2.5	3	3	2.56	2.76	1.3	1.2	2.1
Cl(mg/l)	13.31	14.42	6	5.8	7.6	8.2	7.5	6.9	6.5	5.9
SO_4(mg/l)	12.4	14.5	12.5	4	8.45	17.81	15.56	3.2	3.33	4.5
NO_3(mg/l)	0.55	0.17	0.11	0.7	0.12	2	0.19	0.28	0.17	0.19
Na (mg/l)	26.42	7.81	3.2	1.4	7	3.8	2.34	4	3.5	4.3
K (mg/l)	3.87	3.99	1.67	0.7	2.1	2.2	1.32	1.3	2.34	1

Water Quality Index (WQI)

WQI of the wetlands is established from various important physicochemical parameters in two different seasons, (Fig.1). WQI showed excellent water quality in Maguri beel, Laokhoa beel and Sareswar beel; good water quality in Hahila, Samaguri, Saran, Pobitora and Jengdia; and poor water quality in DB and KNPw in pre monsoon season. In post monsoon DB and KNPw showed very poor water quality; whereas Maguri, Laokhoa and Sareswar have excellent water quality; in Hahila, Saran and Jengdia water quality was good and lastly poor water quality was recorded in Samaguri and Pobitora. It is also observed that pollution load is higher in post-monsoon season. Deepor beel and KNPw recorded the first and second highest values of WQI respectively in both the seasons, indicating the most polluted of all the wetlands studied. The common source of pollution for both the wetlands may be the surrounding agricultural activities, where lots of agrochemicals are used and the nearby National highway from where anthropogenic chemicals are released to the wetland through surface runoffs.

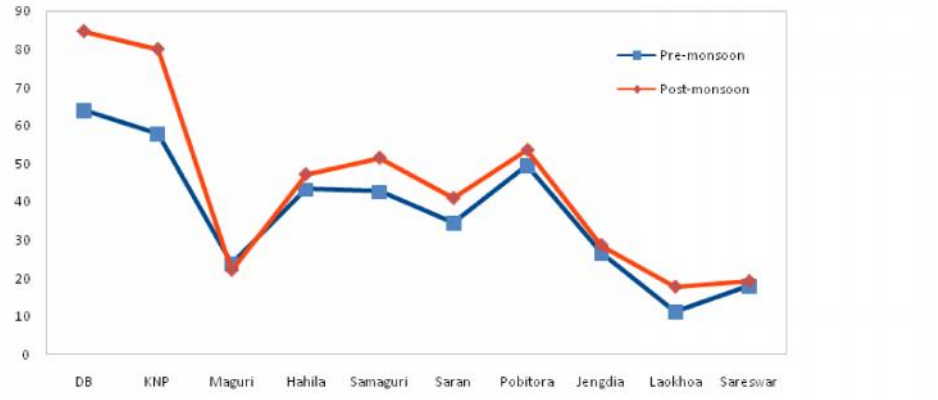

Figure 1. WQI of the wetlands in both the season.

Pearson's correlation

As shown in (Table: 4), a positive correlation is found among pH, HCO_3^-, TH, EC, TDS, Ca^{2+}, Mg^{2+}, Na^+, K^+ and Cl^-, which may indicate a common source of origin like weathering of rocks. Mg^{2+}also shows a positive correlation with SO_4^{2-} and NO_3^-, depicting a common anthropogenic source like fertilizers and pesticides. There is no significant correlation among SO_4^{2-} and NO_3^- which may be due to a different nature of anthropogenic source of nitrate like untreated sewage in addition to runoff from surrounding agricultural fields.

Table 4. Correlation matrix of the parameters

	pH	HCO_3^-	TH	EC	TDS	Ca	Mg	Cl	SO_4	NO_3	Na	K
pH	1											
HCO_3^-	.758**	1										
TH	.636**	.805**	1									
EC	.696**	.661**	.653**	1								
TDS	.682**	.808**	.698**	.900**	1							
Ca^{2+}	.717**	.563**	.758**	.828**	.688**	1						
Mg^{2+}	.779**	.525*	.485*	.567**	.457*	.575**	1					
Cl^-	.735**	.803**	.880**	.837**	.796**	.878**	.469*	1				
SO_4^{2-}	.179	.21	.294	.188	.095	.133	.512*	.170	1			
NO_3^-	.105	-.059	.044	-.007	-.128	.072	.434*	.018	.162	1		
Na^+	.589**	.800**	.676**	.625**	.815**	.564**	.360	.696**	.036	-.031	1	
K^+	.413	.557*	.772**	.686**	.642**	.744**	.143	.869**	.032	.022	.588**	1

** Correlation is significant at the 0.01 level

* Correlation is significant at the 0.05 level

Principal component analysis:

PC1 (eigenvalue 7.15) represent 59.65% of the total variability in one axis (VF1) which has strong positive loadings on pH, HCO_3^-, TH, EC, TDS, Cl^- , Ca^{2+}, Mg^{2+}, Na^+ and K^+. PC1 can be interpreted as a mineral component of the wetland water and is associated with weathering and solute acquisition processes in the catchment. PC2 (eigenvalue 1.51) accounts for 12.58% of the total variance and has strong positive loading on Mg^{2+}, NO_3^- and SO_4^{2-}. This factor accounts for anthropogenic origin, particularly from fertilizer application in the agricultural fields and tea gardens in the vicinity of the wetlands (Reimann & Caritat, 2005).

Conclusion

Physico chemical properties of the surface water of the wetlands with seasonal variation are studied. The order of the abundance of the major cation and anion is as follows: HCO_3^->SO_4^{2-}>Cl^->Na^+>Ca^{2+} >K^+>Mg^{2+}>NO_3^- in pre-monsoon and HCO_3^- >SO_4^{2-}>Cl^->Na^+> Ca^{2+}>Mg^{2+}>K^++>NO_3^- in post-monsoon. Based on the WQI classification, DB and KNPw are found to be of poor water quality and are the most polluted wetlands of the Brahmaputra valley. PCA source apportionment indicated pH, HCO_3^-, TH, EC, TDS, Cl^- , Ca^{2+}, Mg^{2+}, Na^+ and K^+ as a mineral component of the wetland water whereas Mg^{2+}, NO_3^- and SO_4^{2-} originated from anthropogenic sources.

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Hematological Profiling of Albino mice after oral exposure of banned Antibiotic Chloramphenicol

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ABSTRACT

Chloramphenicol (CAP) is one of the old broad spectrum antimicrobial agents used against various infection. Use of Chloramphenicol is banned in food producing animals due to its side effects. In the present study, two groups of albino mice were treated with Chloramphenicol twice a day for 8 days in two different doses (300mg/kg b.wt and 500mg/kg b.wt, respectively) and compared with a control group. The hematological profiling of the animals was done in all the three groups of animal and the results showed a decreasing value of erythrocytes concentration ($p \leq 0.01$) and hemoglobin concentration ($p \leq 0.001$) and an increase in leucocytes concentration ($p \leq 0.01$). It was concluded that CAP is a dose dependent antibiotic with controversial use which directly leads to hematotoxicity.

Keywords: *Chloramphenicol, hematological, hematotoxicity, albino mice*

Introduction:

A drug is a chemical substance used to cure or prevent the risk of a disease. A banned drug is not allowed to intake due to various adverse side effects more than clinical effects.In India, 344 drugs are banned by Ministry of Health and Family Welfare (2016) in their fixed combination and Chloramphenicol (CAP) is one of common banned drug. Chloramphenicol (CAP) was first quarantined from bacterium *Streptomyces venezualae* in the year 1947 by Bartz. In 1948, it was prescribed in mass following an outbreak of enteric fever in USA. CAP is used as an eye drop, ear drop, eye ointment, capsule, injection and from 1949 it was used extensively as a potent inhibitor of protein synthesis. CAP is historically used in veterinary for food-producing animals and in human. Chemically, CAP is a white to greyish white or yellowish white coloured fine crystalline powder or crystal with a molecular weight of 323.126 g/mol.

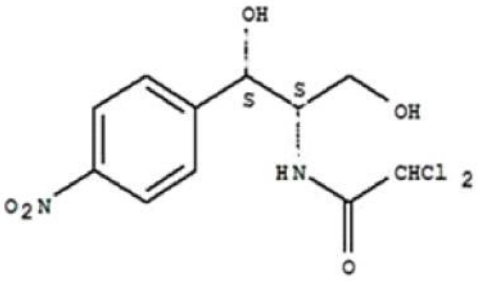

Fig1: Molecular structure of Chloramphenicol

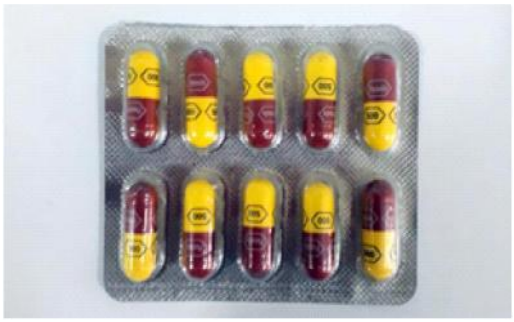

Fig 2: Chloramphenicol I.P. Capsule

CAP is limited in use due to association with aplastic anemia(AA) (Rich *et al.*, 1950). It is a unique condition when body fails to produce blood cells. AA is a dose independent and irreversible symptom of CAP which in most cases are seen years after the treatment (Yunis, 1989) and sometimes show fatal results (Turton *et al.*, 2002). AA is caused by the oral administration of CAP and this has made the CAP to be prescribed parenterally by many physicians. There is no such certainty that this parenteral administration lowers the incidence of AA butthe risk is lowered. CAP also used as ophthalmic preparations (Rosenthal *et al.*, 1965; Carpenter, 1975 and Abram *et al.*, 1980).

CAP directly induces apoptosis in hematopoietic stem cellsleading to AA (Kong, *et al.*, 2000).Early reports estimated that approximately 5% of aplastic anemia cases evolved to leukemia (NTP Board of Scientific Counselors Report on Carcinogens Subcommittee, 2000). The risk of developing AAafter CAP administration is 1:30000 to 1:50000 (Li *et al.*, 2010). In a previous study by Dubey *et al.*, (2011), Chloramphenicol was administered at 150 mg/kg dose in rats for 14 days which causedsignificant hematotoxicity characterized by decrease in erythrocyteconcentration, leucocyte concentration and Hemoglobin concentration which were indicatorsof anemia.

Hematological profiling is the preliminary diagnosis of any disease or any side effects of

a drug. Abnormal increase or decrease in blood cells from normal state proved important explanation of a disease (Solanke *et al.*, 2000; Das *et al.*, 2003; Jee, *et al.*, 2005;Rahman *et al.*, 2006; Uboh *et al.*, 2005, Shukla *et al.*, 2013).

Materials and methodology:

Experimental animal:

Adult albino mice were brought from College of Veterinary Science, Assam; weighted between 20-40 g. The animals were adopted to the laboratory condition for 1 week prior to experiment. Standard animal feed composition of wheat, bran, maize were given to the mice along with attached water bottles. They were examined routinely for their body weight.

Administration of material:

The Chloramphenicol capsule IP manufactured by Abbott Healthcare Pvt. Ltd. (mfg Lic. No.-MB/06/296) were used for this experimentation. Chloramphenicol suspension was freshly prepared and administrated by cannula twice a day orally for 8 days.

Albino mice were selected for the experiment and randomly assigned to three groups, each group consisting of 5 experimental animals. Group I contained the control group, Group II contained the low dose CAP treated group (300mg/kg b.wt.) and group III contained high dose CAP treated group (500mg/kg b.wt).

Hematological studies:

Blood collected from the experimental animals was assessed for the hematological parameters like erythrocytes (Red Blood Cell), leucocytes (White Blood Cell), Hemoglobin concentration. Erythrocytes and leucocytes count were done by Neubauer's Slide using Hayme's fluid and Turk's fluid (Dairo, 2008) and the counting was done under microscope 10x resolution. Hemoglobin concentration was determined using Sahli's hemometer.

Results:

Erythrocytes, leucocytes and Haemoglobin concentration showed variation in all the three group of animal. All the three parameters showed a highly significant value for high dose of Chloramphenicol (500mg/kg b. wt.) compared to control but at low dose (300 mg/kg b.wt.) only haemoglobin concentration showed highly significant result whereas erythrocytes concentration showed less significant.

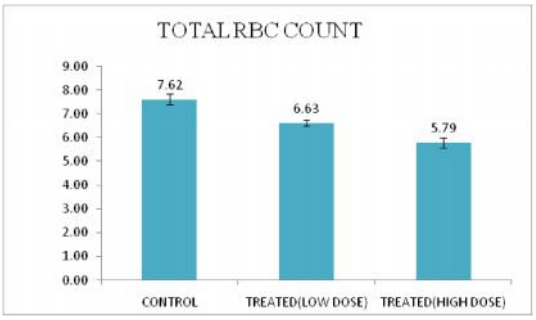

Fig 1: Graphical representation of total erythrocyte count for control, low dose (300mg/kg b.wt.) treated and high dose (500mg/kg b.wt.) treated. *=p ≤ 0.05, **=p ≤ 0.01

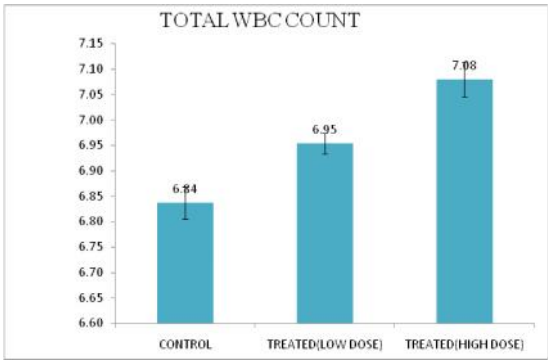

Fig 2: Graphical representation of total leucocyte count for control , low dose (300mg/kg b.wt.) treated and high dose (500mg/kg b.wt.) treated group. ns = not significant, ** = p ≤ 0.01

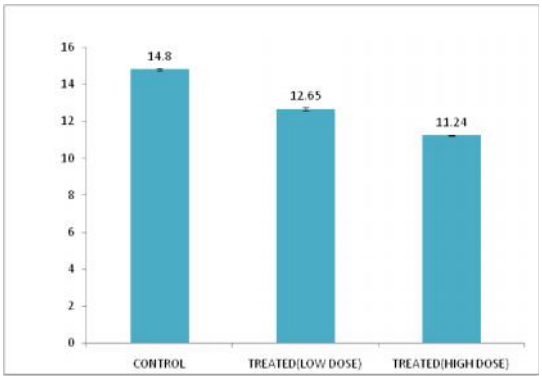

Fig 3: graphical representation of haemoglobin concentration for control, low dose (300mg/kg b.wt.) treated and high dose (500mg/kg b.wt.) treated. ***=$p \leq 0.001$

Discussion and Conclusion:

In the present study, hematological analysis released a highly significant reduction of erythrocytes and hemoglobin concentration in the treated groups. Leucocyte concentration gradually increase from control to low dose treated and high dose treated. Literature suggested that CAP induces and enhances some defects which results in suppression of undifferentiated bone marrow stem cells (Cronkite, 1964) which may lead to death. At a concentration of 2000-4000µg/ml CAP depressed phagocytosis of macrophage and burst activity of neutrophils (Paape, *et al.*, (1990). Other studies suggested that CAP directly induce apoptosis in hematopoietic stem cells, directly leading to hematotoxicity (Kong, *et al.*, 2000)

In another study it was revealed that actually the p-nitrosulfathiazole group is responsible for AA by inhibiting DNA synthesis in marrow stem cells. This observation is mainly based on a CAP derivative thiamphenicol which does not have a p-nitrosulfathiazole group and does not cause AA (Yunis,1973).

The enhancing lymphocyte demonstrated toxicity in lymphoid tissues.The increase in total white blood cells observed may be due to acceleration of lymphopoiesis and increasing discharge of lymphocytes from myeloid tissue of lymph (Das *et al.*, 2003). The abnormal increase of leukocytes gave non-specific immune response and drug induced tissue damage. Shukla *et al.*, (2013) reported similar results of erythrocytes , leucocytes and Haemoglobin concentration. The observed decrease in erythrocytes concentration may be assumed to be associated with retarded hemopoeisis, destruction and shrinkage of erythrocytes.

It was concluded that the Chloramphenicol is a potent antibiotic but still it has some adverse health hazard such as decrease in erythrocyte concentration, Hemoglobin concentration and increase in leucocytes concentration. It is a dose dependent hematotoxic. It is still used in some remote areas. However, its use is controversial in the light of such a long list of alternative antibiotics.

Acknowledgement:

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Effect of certain air pollutant and pesticide residues on phenoloxidase (PO) of *Antheraea assamensis Helfer*

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ABSTRACT

The enzyme phenoloxidase (PO) is an important component of insect defense mechanism. Successful inhibition of PO activity could be effective against insects and be used in their biological control. The present study was therefore conducted to observe inhibition of PO activity on the Muga silkworm *Antheraea assamensis Helfer*. Four different chemical residues i.e, Benzoic acid, Sodium sulphite, Thiourea and Dithiothretol (DTT) were used at 1mM, 2mM, 3mM and 4mM concentration to see their inhibitory effect on L-DOPA oxidation by PO. It was found that with increasing concentration, all the tested inhibitors exhibited decrease in PO activity, maximum inhibition being recorded at 4mM concentration.

Among the inhibitors, DTT resulted in maximum inhibition followed by Thiourea and Benzoic acid. Comparison of different inhibition on enzyme activity at 4mM concentration too proved DTT to be the best inhibitor (2.02±0.81) whereas Sodium sulphite exerted the least inhibitory effect (6.21±1.17).

Keywords: *Antheraeaassamensis*, Benzoic acid, Sodium sulphite, Thiourea, Dithiothretol

Introduction:

Insects are the most diverse group of animals. They are exposed to hostile environment which consists of microorganisms and parasites on a regular basis. Tosurvive in a world full of microorganisms and parasites, insects developed a potent defense mechanism that recognizes and removes microbial threats. The reason of their abundancy is their success in adapting themselves to varying conditions. They depend on innate immunity for their survival.

Insect immunity consists of both cellular and humoral mechanisms (Gillespie *et al.*, 1997; Lavine and Strand, 2002). Cellular immunity is mediated by haemocytes through phagocytosis, encapsulation and nodule formation (Lavine and Strand, 2002). Humoral mechanism on the other hand includes various Antimicrobial peptides (AMP), Pattern Recognition Properties (PRP) and the Phenoloxidase (PO) system.

Among the innate immune system factors, PO is critical for the insect's defence mechanism (Ajamhassani *et al.*, 2012). It is involved in multiple processes, such as cuticular sclerotization, melanization and wound healing. Sclerotized cuticle have been reported to block pathogen entry into the body, while melanization destroy invading pathogens (Ashida,M. & Brey, P 1997). PO which is a copper-3-polypeptide[2] is usually synthesized and released into the haemolymph as the inactive zymogen Pro-phenoloxidase (PPO). Once activated (by challenges) PO catalyses the initial step of the melanin biosynthesis pathway by hydroxylation of monophenols into o-diphenols and then further oxidation of p-diphenols into o-quinones. o-quinones can non-specifically crosslink neighbouring molecules to form insoluble melanin (Nappi & Christensen, 2005). Quinones generated by PO go through a cascade of enzymatic and non-enzymatic reactions leading to polymerization. Further, quinones crosslink with cuticular structural proteins and chitin which results in the hardening of the cuticle (Hall *et al.*, 1995). The hardened cuticle provides the first line of defence against external challenges, melanization around invading pathogens provide a further mechanism of immunity (Soderhall, & Cerenius, 1998; Kanost & Gormen, 2008).

So, it can be hypothesised that enhanced PO activity could provide the insects with protection against invading pathogens and inhibition of PO activity could render them susceptible to external challenges. Therefore to test this hypothesis the present study aims to inhibit PO activity on a test insect using selected inhibitors (copper-chelators). Such inhibitions, if proven to affect the insects immune system, could pave the way for the development of a novel method of insect control in future.

With this objective, the Muga silkworm *Antheraea assamensis Helfera* sericigenous insect, native to the North-East region of India (Bardoloi & Hazarika, 1992) was selected as our test insect.

Materials and methods:

Insects: Healthy and disease free Muga silkworm (*Antheraea assamensis helfer*) larvae of 5th instar reared on Som plants (*Machilus bombycina* King) were collected from the Central Silk Board Farm, Boko, Guwahati, Assam.

Collection of haemolymph: Haemolymph from the larvae were collected by excising one of the prolegs and immediately diluted with an anticoagulant (containing trisodium citrate, P^H = 7.0) and with a buffer (TrisHcl, P^H = 7.1) in the ratio of 1:1:1 and homogenized in a ice cooled potter homogenizer and the lysate was centrifuged at 17000rpm for 30 minutes. Supernatant was stored at -20^{0}C freezer.

Inhibition Study: Four copper-chelators, *viz.* Benzoic acid, Sodium sulphite, Thiourea and Dithiothretol, at different concentrations (1mM, 2mM, 3mM and 4mM) were tested for their inhibitory effect on L-DOPA oxidation by PO in the haemolymph of *Antheraea assamensis*, following the methodology of Goudru et *al*., 2013 with slight modifications. 6mM L-DOPA concentration was taken as substrate for the inhibition process as maximum PO activity occurs in this concentration (Goudru et *al*., 2013).

A solution containing 20µl of haemolymph supernatant obtained from the stored sample after homogenization and centrifugation at 17000rpm, containing PPO was incubated for 5min with 10µl of 10% CPC to obtain the active enzyme PO. To this, 940µl of buffer with specific concentrations of one particular inhibitor was added and incubated for 10min at 30^{0}C. After adding the substrate (6mM L-DOPA), the reaction was triggered and absorbance was recorded for 2min under standard assay conditions. Same procedure

$$b = \frac{\Delta A \times V}{E \times d \times v \times \Delta t} \times d.f$$

was followed for all the inhibitors at different specified concentrations (1mM, 2mM, 3mM and 4mM).

The absorbance was measured at 475nm and the enzyme activity was calculated by using the following formula-

where, ΔA = change in absorbance

V = total volume of assay mixture

E = extinction coefficient ($3.6mM^{-1}cm^{-1}$)

d = light path

Δt = time for which the change was observed (in minute)

V = volume of sample(in µl)

d.f = dilution factor

Statistical analysis: Experimental data for PO activity as affected by inhibitors were analyzed using one way analysis of variance (ANOVA). Significance of inhibition by the different inhibitors was tested at $p \leq 0.05$ determining the LSD.

Result:

In the present study, inhibition of PO activity was observed with all the 4 chemical residues *viz.* Benzoic acid, Sodium sulphite, Thiourea and Dithiothretol at 1mM, 2mM, 3mM and 4mM concentration. The inhibition was determined in terms of their inhibitory effect on L-DOPA oxidation by PO. 6mM L-DOPA concentration was used as a substrate for the inhibition process as it has been reported that maximum PO activity occurs in this concentration (Goudru et *al*., 2013).

Our results showed that in 6mM DOPA all the inhibitors at their different concentrations showed active inhibition of PO activity, albeit

at different rates (Table 1). All the tested inhibitors exhibited a linear decrease in PO activity with increasing concentrations; being highest at 4mM (Table:1). Dithiothretol (DTT) was observed to show the maximum inhibition followed by Thiourea and Benzoic acid.

Table 1:Effect of selected inhibitors on enzyme activity at different concentration.

Inhibitors	1mM	2mM	3mM	4mM
Benzoic acid	7.02±1.82	6.42±0.90	5.85±1.09	5.76±0.54
Thiourea	5.53±1.68	4.68±1.29	4.41±1.16	4.05±1.33
Sodium sulphite	6.39±1.89	6.25±1.01	6.21±1.61	6.21±1.17
Dithiothretol	5.85±1.55	3.29±1.15	3.19±0.54	2.02±0.81

* Enzyme activity (Mean ± SD)

Table 2: Comparison of the effects of different inhibitors on enzyme activity at 4mM concentration.

Inhibitors	4mM conc.
Benzoic acid	5.76±0.54a
Thiourea	4.05±1.33a
Sodium sulphite	6.21±1.17ac
Dithiothretol	2.02±0.81b

*Same alphabets at the end of the numbers indicate no difference

DTT exhibited the best inhibitory effect particularly at 4mM concentration (2.02±0.81). At lower doses too DTT proved to be more effective as an inhibitor (5.85±1.55 at 1mM, 3.29±1.15 at 2mM and 3.19±0.54 at 3mM) than the others (Table 1). Thiourea too followed the same linear pattern of inhibition from lower to higher concentration; best effect being recorded at 4mM concentration (4.05±1.33). Benzoic acid did not show a reduction in enzyme activity with an increase in molar concentration although a gradual decrease in PO activity was evident from 1mM to 3mM concentration. Incidently no differences could be interpreted between 3mM and 4mM concentrations indicating an optimal range of inhibition not exceeding 4mM concentration. Sodium sulphite showed the least inhibition among the four inhibitors used.

Oneway ANOVA performed on the dataset obtained from the study helped in the comparison of PO inhibition potential of all the inhibitors at 4mM concentration (highest inhibition as suggested by the results). The comparison proved DTT to be the best inhibitor among the four tested (Table 2), followed by

Thiourea and Benzoic acid, which among them showed no significant variation in their inhibitory potential. Sodium sulphite proved to exert the least inhibitory effect among all the inhibitors.

Discussion:

The results suggest the inhibitory potential of all the inhibitors tested. All the tested inhibitors exhibited a linear decrease in PO activity with increasing concentrations. Similar trends in PO activity have also been reported by Popham *et al.,* 2004 working on *Heliothis virescens*. Insect PO being a typical copper-3-protein, it is not surprising that copper-chelators (the tested inhibitors) would prove to be good inhibitors. Similar justifications were offered by Lerner *et al.*, 1950 and Prabhakaran *et al.,* 1969 working on *Mycobacterium leprae*. The chelating properties of such inhibitors have also been reported by Li and Kubo, 2004. The inhibitors used in the study, viz, DTT, Benzoic acid, Thiourea, and Sodium sulphite are all reported to be competitive inhibitors. Therefore they compete with the substrate for binding with the active sites of the enzyme (PO). Increased concentration of the same enables them to outcompete the substrate in terms of binding at the active sites. The substrate failed to bind to the active sites, enzyme-substrate complex formation reduces and as a result enzyme-substrate reaction goes down exhibiting less enzyme activity in turn.

Another explanation regarding inhibition of PO activity may be cited from the findings of Lu *et al.*, 2014. According to their reports, PPO of most insects have one or two disulfide bonds at the c-terminus. Deletion of the disulphide bonds prove to decrease or inhibit PPO activity greatly. The same argument may also be sighted in support of our results, although tentatively. It is probable that the inhibitor used in our study, in addition to blocking the active sites of the enzyme (PO), might also affect the disulfide bonds leading to the observed reduced activity of theenzyme. However to prove this hypothesis, further extensive research on inhibition mechanism of PO is absolutely essential.

Conclusion:

The results of our study conclusively prove that PO inhibition by chemical residues is possible. This information in all probability has the potential to lead to the development of a novel way of environment friendly, non-toxic insect control mechanism in the near future.

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Effects of *Andrographis paniculata* Nees crude extract on Alloxan Induced Hyperglycaemic Mice

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ABSTRACT

Diabetes mellitus has been considered as one of the major health concerns all around the world today. For the control of diabetes mellitus, *Andrographis paniculate*, a herbaceous plant has been effectively used in traditional Asian medicines for centuries. To evaluate this, we have obtained the aqueous extract of the plant *Andrographis paniculata* Nees. at a dose of 0.05 mg/kg b.w. which was tested for its efficiency against alloxan induced hyperglycaemic mice. In the present investigation, aqueous extract of *Andrographis paniculate* was used to test its efficiency against alloxan induced hyperglycaemic mice. The hypoglycaemic action of the plant extract was detected through IPGTT and IPITT. Initially hyperglycaemia was induced in a group of mice by alloxan. It was followed by IPGTT and IPITT of hyperglycaemic mice. Results of IPGTT showed that 15 minutes after administration of exogenous glucose, the blood glucose level increased significantly except in Group I. This rise in blood glucose level was evident until 30 minutes indicating the hyperglycaemic activity of exogenous glucose. This was

followed by a decrease in the blood glucose level until 120 minutes in control alloxan induced and co- treated mice. During the IPITT, it has been observed that, 15 minutes after the administration of insulin, the blood glucose level dropped significantly except in water injected mice. This fall in blood glucose level was evident for the subsequent 30 to 45 minutes indicating hypoglycaemic activity of exogenous insulin hormone. Eventually, from 60 minutes onwards, a shoot-up in the blood glucose level has been recorded in cotrol, alloxan induced and co-treated mice. During both IPGTT and IPITT, it is found that there is a decrease in the blood glucose level of the co-treated mice when compared to the alloxan induced mice since the diabetic mice were treated with *Andrographis paniculata* crude extract which indicates the hypoglycaemic activity of *Andrographis paniculata*.

Keywords: *Andrographis paniculata*, Alloxan, Glucose tolerance, hyperglycaemia, Insulin tolerance, hypoglycaemia.

Introduction

Diabetes mellitus is a disease caused by increased blood glucose level as a result of impaired insulin secretion. Diabetes mellitus has been considered as one of the major health concerns all around the world today. Experimental animal models are one of the best strategies for the understanding of patho-physiology of any disease in order to design and develop the drugs for its treatment. Numerous animal models have been developed for the past few decades for studying diabetes mellitus and testing anti-diabetic agents that include chemical, surgical and genetic manipulations. One of the most potent methods to induce experimental diabetes mellitus is chemical induction by Alloxan. Alloxan is a urea derivative which causes selective necrosis of the β cells of pancreatic islets. Hence, it is used to induce diabetes in laboratory animals.

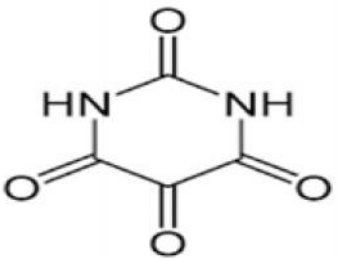

Fig: Structure of Alloxan

Until present, there is no treatment that can cure diabetes mellitus completely. That is why efficient research have been done to search for new hypoglycaemic agent from plants and herbs that can be used to cure or control diabetes mellitus traditionally by certain community. One such plant is *Andrographis paniculata*, locally known as 'Kalmegh' or 'Chirata' that has been effectively used in traditional Asian medicines for centuries. *Andrographis paniculata* has been reported as having antibacterial, antifungal, antiviral, choleretic, hypoglycaemic, hypocholesterolemic, and adaptogenic effects (Bhatnagar *et al.*,1961). It contains diterpenes, lactones, and flavonoids. The leaves contained two bitter principles - andrographolide and a compound named kalmeghin. It is found that andrographolide is the main phytoconstituent having hypoglycaemic activity.

Intra-peritoneal glucose tolerance test (IPGTT) is a standard procedure that addresses how quickly exogenous glucose can be cleared from the blood. In animal research, the IPGTT is used to assess the degree of

diabetes and also to test the desired effects of insulin or other drugs on the body's ability to process glucose. It can also be used to detect the unintended side effects of drugs intended to treat other unrelated diseases.

Intra-peritoneal insulin tolerance test (IPITT) is designed to determine the sensitivity of insulin receptors in tissue by measuring blood glucose levels before and after insulin administration. This is a standard test to determine the diabetic status in humans and experimental animals.

Materials and Methods

Test animals: Albino mice (*Mus musculus*) procured from the Department of Pharmacology, Veterinary College, Khanapara were taken for the experiments. The animals were acclimatized prior to treatment and were housed in a lab temperature (22°C) on a natural light-dark cycle. All animal experiments were conducted according to institutional guidelines congruent with the guide for the care and use of laboratory animals. The animals were divided into four groups.

Experimental induction of diabetes: The mice were injected with alloxan monohydrate (Sigma®) dissolved in 0.9% sterile normal saline at a dose of 75 mg/kg body weight. Alloxan was injected intra-peritoneally. Before the injection, all the mice were fasted for 18 hours. After 48 hours of injection, mice with normal blood glucose level (150-160 mg/dl) were used for the experiment.

Intra-peritoneal glucose tolerance test (IPGTT): For IPGTT, mice were allowed to fast for O/N for 16 hours (approximately) by taking away food only, while giving them free access to water *ad libitum*. Fasting blood glucose level was measured in each of the mice before administration of glucose load. For measuring blood glucose, blood was collected by scoring the tip of the tail using a fresh sterilized scalpel. This served as the baseline (t = 0). This was followed by intra-peritoneal administration of freshly prepared 20% glucose solution at the concentration of 2 gm/kg body weight, using a 27 G needle. After administration of glucose load, the blood glucose was measured at 15 minutes, 30 minutes, 60 minutes, and 120 minutes (t= 15, t=30, t=60, t=120) during the post administration period. At the end of the experiment, the animals were supplied with food and water.

Intra-peritoneal insulin tolerance test (IPITT) : For IPITT, fed mice were fasted for 4 hours by taking away food only, while giving them free access to water *ad libitum*. Fasting blood glucose was measured in each of the mice before administration of insulin. For measuring blood glucose level, blood was collected by scoring the tip of the tail using a fresh sterilized scalpel. This served as the baseline (t = 0). This was followed by intra-peritoneal administration of insulin @ 0.10/kg body weight, using a 27 G needle. After administration of insulin, the blood glucose level was measured at 15minutes, 30 minutes, 45 minutes, 60 minutes and 120 minutes (t = 15, t = 30, t = 45, t = 60 and t = 120), during the post administration period. The mice were then observed and monitored constantly for the onset of hypoglycaemia.

Preparation of aqueous extract of *Andrographis paniculata*: The *Andrographis paniculate* plants were collected locally from six mile, Guwahati. Leaves and tender stem of *Andrographis paniculata* were shaded dried and grinded using a grinder machine. To obtain crude extract, the leaves of *Andrographis*

paniculata were blended with distilled water at the rate of 10gm/100 ml of distilled water. The mixture was stirred using magnetic stirrer for 30 minutes. After 72 hours, the mixture was filtered and the filtrate was evaporated on a petridish. After complete evaporation, the dry extract was used to prepare different concentrations for treatment.

Treatment of *Andrographis paniculata* extract : The mice were treated with *Andrographis paniculata* crude extract at the dose of 0.05 mg/kg body weight for 7 days. The crude extract was administered orally using feeding tube.

Statistical analysis : Data are expressed as mean ± S.E.M. Level of statistical significance was determined by performing t-test (at 95% confidence level) using MS- excel package.

Result

Hypoglycaemic activity of Andrographis paniculata Nees. in alloxan induced diabetic mice was evaluated for seven (7) days. For seven days the mice were treated with the test substance in accordance with their respective groups. The observational data from the blood glucose levels of mice are presented by mean ± S.E.M. Effect of treatments given in blood glucose level are shown in Table I.

Table I : Blood glucose level (in mg/dl) in different groups of mice during the treatment period.

Data are presented as means + S.E.M

* Significantly different from blood glucose in normal control group ($p<0.05$)

Significantly different from blood glucose in alloxan treated group ($p<0.05$)

No. of days	GROUP I Normal control	GROUP II Alloxan treated(@75mg/kg body wt.)	GROUP III Alloxan + *A. paniculata* (@0.05 mg/kg body wt.)
Day 1	96.60±1.78	140.00±2.12*	138.2±2.31*
Day 2	97.00±0.95	143.20±0.92*	137.40±0.81*#
Day 3	94.80±1.39	145.40±1.86*	138.80±0.37*#
Day 4	95.00±1.64	149.40±2.34*	136.00±0.45*#
Day 5	95.04±1.03	153.40±2.18*	133.60±0.40*#
Day 6	96.60±0.93	156.80±1.02*	131.40±0.51*#
Day 7	95.20±1.28	158.80±0.58*	129.40±0.40*#

From Table I it is observed that, throughout the treatment period there is a significant increase in the glucose level of the alloxan treated diabetic (Group II) mice when compared to the normal control group (Group I). From the above table it can also be seen

that, although there is no significant decrease in the blood glucose level of the alloxan treated + *Andrographis paniculata* treated (Group III) mice when compared to the normal conrol group but, there is a significant decrease in the blood glucose level of the alloxan treated + *Andrographis paniculata* treated (Group III) mice when compared to the alloxan treated diabetic(Group II) mice.

Table II: Blood glucose level (in mg/dl) during Intra-peritoneal glucose tolerance test (after 07 days of Andrographis paniculata treatment)

Data are presented as means + S.E.M

* Significantly different from blood glucose at 0 minutes after 16 hour fasting ($p<0.05$)

Significantly different from blood glucose at respective time interval of normal control group ($p<0.05$)

@ Significantly different from blood glucose at respective time interval of alloxan treated group ($p<0.05$)

Different groups	Blood glucose level recorded at different time intervals after administration of glucose load (in minutes)				
	t=0	t=15	t=30	t=60	t=120
GROUP I (Control for IPGTT (water injected)	93.80 ± 0.58	92.80 ± 1.16	93.80 ± 0.86	92.20 ± 0.86	92.20 ± 0.86
GROUP II (Normal control)	90.80 ± 0.73	232.20 ± 1.02*	191.40 ± 1.03*	153.00 ± 1.55*	103.20 ± 1.07*
GROUP III (Alloxan treated (@ 75mg/kg body weight)	148.80 ± 2.82#	345.20 ± 2.82*#	432.20 ± 4.73*#	406.20 ± 2.58*#	329.20 ± 3.28*#
GROUP IV (Alloxan treated + *Andrographis paniculata* (@ 0.05mg/kg body weight)	124.80 ± 1.93#@	290.20 ± 3.54*#@	255.40 ± 2.48*#@	198.20 ± 3.90*#@	140.60 ± 2.52*#@

Table II shows the data for IPGTT. The mice were divided into 4 groups. Immediately after 15 minutes of intraperitoneal glucose administration, hyperglycaemia has been observed in Group II, Group III and Group IV. In these groups we can see that there is a significant rise in the blood glucose level until 30 minutes from the basal level. However, this was followed by a decrease in the blood glucose level until 120 minutes in all the groups. The observation reveals that, the Group III and Group IV mice confirmed diabetes as their blood glucose level was beyond 120 mg/dl even after 120 minutes of glucose administration.

From the above table we can also see that there is a reduction in the blood glucose level of Group IV mice when compared to Group III mice.

Table III: Blood glucose level (in mg/dl) during Intra-peritoneal insulin tolerance test (after 07 days of *Andrographis paniculata* treatment)

Data are presented as means + S.E.M

*** Significantly different from blood glucose at 0 minutes after 4 hour fasting (p<0.05)**

 # Significantly different from blood glucose at respective time interval of normal control group(p<0.05)

@ Significantly different from blood glucose at respective time interval of alloxan treated group(p<0.05).

Different groups	Blood glucose level recorded at different time intervals after administration of insulin load (in minutes)					
	t=0	t=15	t=30	t=45	t=60	t=120
GROUP I (Control, saline injected)	98.40 ± 0.93	98.20 ± 1.28	98.20 ± 1.71	97.00 ± 1.22	97.20 ± 0.86	98.60± 0.75
GROUP II (Normal control)	100.40 ± 1.36	93.00 ± 1.30*	83.00 ± 0.84*	75.40 ± 1.36*	82.00 ± 0.71*	95.00 ± 0.84*
GROUP III (Alloxan treated (@ 75mg/kg body weight)	154.40± 0.87#	143.00 ± 1.05*#	146.20 ± 0.58*#	148.00 ± 0.77*#	150.80 ± 0.37*#	152.00 ± 1.22#
GROUP IV (Alloxan treated + *Andrographis paniculata* (@ 0.05mg/kg body weight)	131.60± 0.93#@	119.40 ± 1.47*#@	109.40 ± 1.17*#@	101.80 ± 0.66*#@	107.80 ± 0.80*#@	114.80 ± 0.86*#@

Table III shows the data for IPITT. The mice were divided into 4 groups. Immediately after 15 minutes of intraperitoneal insulin administration, hypoglycaemia has been observed in the Group II, Group III and Group IV. In these groups we can see that there is a

significant fall in the blood glucose level until 45 minutes from the basal level. However, this was followed by a significant elevation in the blood glucose level until 120 minutes indicating gradual loss of insulin tolerance in the target cells. Infact, it has been observed that, this rise in blood glucose level towards the later period of IPITT (60 minutes and 120 minutes) marking the loss of insulin tolerance in the target cells is statistically significant in the different groups.

Discussion

During the treatment period a significant increase in the blood glucose level of the mice was found. Same result was also reported by Federiuk et al. 2004 who also used alloxan for diabetes induction in mice. When mice were treated with the herbs of Andrographis paniculata (@ 0.05mg/kg body weight) for seven (7) days, a significant ($p<0.05$) reduction in the blood glucose levels of diabetic mice were seen when compared with the diabetic control mice. Similar findings were also reported by Mulja Hadi Santosa *et al.*, (2013), who observed a decrease in blood glucose level of the diabetic mice when treated with *Andrographis paniculata* (@ 28 mg/20 gm body weight) for 7 days. From Table I it is seen that on treatment with *Andrographis paniculata* (@0.05 mg/kg body weight) there was a significant reduction in the blood glucose level within 7 days. Also on the 2nd day of treatment, there was a decrease in the blood glucose level from (143.20 ± 0.92 to 137.40 ± 0.81). From the third day onwards, a gradual and significant reduction in the blood glucose level was seen. By comparing the results of the 1st day of treatment with the 7th day of treatment, a marked difference in the reduction of the blood glucose level (from 140 ± 2.12 to 129.40 ± 0.40) was found. Thus, treatment of the diabetic mice with *Andrographis paniculata* Nees. Crude extract produces a significant ($p<0.05$) reduction in the blood glucose level of diabetic mice. This observation supports the work of Aty Widyawaruyanti et al.(2013).

During IPGTT it is seen that initially after 15 minutes of administration of exogenous glucose, the concentration of glucose in the blood increases causing hyperglycaemia which prevents the body from converting glucose into glycogen, which in turn makes it difficult or impossible to remove excess glucose from the blood. From Table II, a significant rise in the blood glucose level until 30 minutes from the basal level in all the groups were found. However, this was followed by a decrease in the blood glucose level until 120 minutes which indicates glucose tolerance in the target cells of Group II mice as the level of blood glucose is below 120 mg/dl. But, in the Group III and Group IV, it was seen that even after 120 minutes of glucose administration, the level of blood glucose is beyond 120 mg/dl (329.20 mg/dl in Group III and 140.60 mg/dl in Group IV) which indicates "impaired glucose tolerance" and confirms a diagnosis of diabetes. Again, when the blood glucose level of the Group III and Group IV mice were compared, a decrease in the blood glucose level in the Group IV mice was seen because the Group IV mice were treated with crude extract of *Andrographis paniculata* which decreases the blood glucose level of diabetic mice. Thus, significant relationship is observed between basal blood glucose level, its increase and decrease during IPGTT in the different groups of mice. This observation supports the work of Peichuan Zhang (2011).

During IPITT, it was seen from Table III that initially after 15 minutes of administration

of exogenous insulin, the concentration of insulin in the blood increases causing hypoglycaemia by increasing the rate of glycolysis and cellular glucose uptake in the target cells. Similar reports were also reported by Georgios *et al*., 2013. It has also been suggested that the increased activity in glycolysis and cellular glucose uptake may in turn lead to stress of the hormone receptors of the target cells thereby decreasing the activity of insulin which is marked by significant rise in blood glucose level indicating gradual loss of insulin tolerance in the cells. Similar finding was also found from the present study as we can see that there is a gradual increase in the blood glucose level in the Group II, Group III and in Group IV until 120 minutes indicating gradual loss of insulin tolerance in the target cells. Again, if the data of Group III mice with that of the Group IV mice were compared, it can be seen that there is a significant fall in the blood glucose level of Group IV mice in each of the time duration. This fall in blood glucose level is due to the hypoglycaemic effect of *Andrographis paniculata* Nees. crude extract in Group IV mice.

Conclusion

The study reveals that, treatment of the diabetic mice with *Andrographis paniculata* Nees. crude extract results in a significant reduction in the blood glucose level. So, *Andrographis paniculata* Nees. crude extract can be used to control diabetes and this would definitely poove beneficial to the mankind at large owing to the cost effectiveness and easy availability of the plant. From the present observation it is assumed that administration of exogenous glucose load in diabetic mice results in hyperglycaemia and administration of exogenous insulin load leads to a gradual loss of insulin tolerance of the cells with increase in time duration. However, further investigations are necessary in this regard to establish the co-relation of exogenously administered glucose and insulin action with the endogenous glucose and insulin during IPGTT and IPITT respectively.

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Therapeutic Analysis of Herbal Extract on Fish Disease

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ABSTRACT

Aquaculture has the potential to make significant contribution to the increasing demand for aquatic food in most world regions but their productivity greatly affected by different disease. Fish in freshwater systems are susceptible to a number of bacterial, viral and parasitic diseases. Diseased cat fish was collected for the present study. Infectious microbes have been isolated from the affected region of the fish and its pure culture was obtained. The microbial strain was identified by using different biochemical methods. Methanolic leaves extract of *Annona reticulata, Brucea javanica* and *Ricinus communis* was made and their phytochemical screening was performed. The methanolic extracts were screened for the anti-microbial action.After performing the biochemical screening the microbial strain was found to be of genus

Staphylococcus. The methanolic extract showed the common presence of alkaloid and flavanoids in all the three plant leaves extracts, whereas saponins and tannins were found only in *Annona reticulata* and *Brucea javanica* respectively. Anti-microbial property of *Annona reticulata* and *Ricinus communis* was found to be highest in 100% concentration with an effective zone of inhibition area of 1.13 cm^2 and 1.538 cm^2 respectively. In case of *Brucea javanica* it shows similar antibacterial property in 10%, 75% and 100% with the same effective zone of inhibition area of 0.78cm2.

Keywords: *Annona reticulata, Brucea javanica, Ricinus communis*, anti-microbial, cat fish.

Introduction

Aquaculture is now-a-days regarded to be one of the growing sectors that has the potential to make significant contribution to the increasing demand for aquatic food in most of the regions of the globe. However, this sector faces a large number of challenges including climate change, changes of water quality parameters, diseases etc. It is reported that the rapid expansion of commercial culture of fin fish is threatened by bacterial, fungal, viral and parasitic diseases affecting survival and growth of not only fish but also endanger to human health e.g. Pathogenic strains of *Vibrio parahaemolytics* can cause shrimp lethal disease (Ling *et al.*, 2007).

However infectious diseases are still an increasingly important public health issue in the world. It has been reported that about 2 million people died in 2000 due to diarrheal disease worldwide (Ling *et al.*, 2007).

In present day scenario scientists show much interest in the research and development of new strategies for disease control within the frame of good husbandry practices including adequate hygiene conditions, vaccination programme and the use of probiotics, prebiotics and immunostimulants. Recently novel strategies have emerged, such as specific killing of pathogenic bacteria by bacteriophages, growth inhibition of pathogen by short chain fatty acid and polyhydroxyalkanoates and interference with the regulation of virulence gene (Estefania *et al.*, 2013)

Inspite of the modern improvements in chemotherapeutic technique, infectious diseases are still an increasingly important public health issue. Nowadays the development of resistance by a pathogen to many of the commonly used antibiotics provides an impetus for future attempts to search for new antimicrobial agents to combat infectious. Research must be carried out to investigate drugs from natural sources and also drugs that can either inhibit the growth of pathogen or kill them and have no or least toxicity to the host cells.

In the current study attempts have been made to isolate culture and characterize microbial strain taken from external body surface of diseased fish (*Heteropneustes fossilis*). We made an initiative to make plant extract and screened them for presence of different phytochemicals. The plant extract was evaluated for antibacterial properties against the isolated pure bacterial strain obtained from diseased fish. This study includes identification of disease causing bacteria and its treatment approach with herbal formulation. The herbal formulation will pave many new routes towards the treatment of fish diseases in India as well as throughout the globe.

Materials and Methods:

Materials:

Collection of specimen: A live fish (*Heteropeustes fossilis*) with severe infections on its external body surface was collected from Morigaon beel, Morigaon, Assam in the month of February, 2017 and then brought to the departmental laboratory in live condition.

Methods:

Isolation of bacterial pure culture: A sterilized cotton swab was taken and the infected portion of the live fish was swabbed. Then it was used to inoculate the bacteria in the prepared agar plate. The plate was then incubated in the incubator at 30°C for 24 hours. Afterwards, the primary cultured colonies were observed and they were again subcultured to obtain pure bacterial colony.

Gram staining: An individual colony from the cultured agar plate was taken out by using inoculating loop. A drop of water was taken on a slide and the isolated individual colony was mixed with that drop of water and was allowed to heat dry. Then the bacterial colonies were subjected to gram staining procedure.

Biochemical analysis of the bacterial strain:

The following biochemical tests were performed following standard methods:

Indole production test: Indole production test is used to check the ability of an organism to split amino acid tryptophan to form indole.

Methyl red test: Methyl red test is used to identify bacteria that produce stable acid by mechanism of mixed acid fermentation of glucose.

Voges proskeur test: The test is used to detect the presence of acetoin, a metabolite (four carbon compound i.e used as an external energy stored in bacteria) in bacterial broth culture.

Citrate utilization test: This test is performed to check the ability of an organism to ferment citrate as a sole source of carbon.

Oxidase test: This test is performed to check whether an organism can produce Cytochrome C Oxidase; an enzyme used for bacterial electron transport system.

Catalase test: The test is used to determine the ability of some micro-organisms to degrade hydrogen peroxide by providing the enzyme catalase.

Litmus milk reaction: This test is performed to differentiate among micro-organisms that enzymatically transform different milk substrate into varied metabolic end products.

Hydrogen sulphide and motility test: This test is performed to check whether the microorganism can produce hydrogen sulphide gas and also to check whether the microorganism is motile or not.

Starch hydrolysis test: This test is performed to check the ability of the microorganism whether it can be able to hydrolyse starch or not.

Collection of Plant materials and preparation of extract:

In the current investigation, three plants were selected for studying antibacterial properties. They are as follows:

A. *Annona reticulata* B. *Ricinus communis* C. *Brucea javanica*

Methanolic extracts of the selected plants were prepared as follows:

Leaves of the three plants selected for the experiment were dried under shade for 14 days. When the leaves dried completely, they were cut into small pieces and finally grinded using a stainless steel grinder. The grinded leaves parts were allowed to pass through sieve

number 80 and were processed further. The leaves powders were soaked in methanol for 24 hours with continuous stiring using a magnetic stirer. After 24 hours the mixture of leaf powder and methanol were filtered using muslin broth and which were further filtered using whatman filter paper grade 1. The filtrates were further concentrated by evaporating the methanol using a rotary evaporator. Afterwards the concentrated extract thus obtained was stored at -20°C.

We have investigated the presence of various phytochemical in these three extracts using the standard test (Trease and Evans, 2002)

Analysis of Antimicrobial property:

The antimicrobial properties of the plant extract were assessed by using the standard disc diffusion method (Kirby burear method). Different concentration of the plant extracts i.e 10%, 25%, 50%, 75%, 100% were prepared. Circular discs made up of cellulose paper having similar diameter were prepared for the experiment. Ampicillin was taken as standard control for the experiment with a concentration of 100%. The Ampicillin loaded circular discs along with different concentration of the extract were placed in a pre-inoculated petriplate. Plates were then kept in BOD incubator for 24 hours and results were observed. Diameters of the zone of inhibition of bacterial colony shown by different concentration of all the three plant extracts were calculated.

Results and Discussion :

The results of the biochemical test used for analysis of microbial strain were given in table1.

Table 1: Summarized Results of Biochemical Test:

SL No	Name of the biochemical test	Results
01	Gram staining	+
02	Indole production test	-
03	Methyl red test	+
04	Voges proskeur test	+
05	Citrate utilization test	+
06	Oxidase test	-
07	Litmus Milk	Acid followed by reduction
08	Hydrogen sulphide and motility test	-
09	Catalase test	+
10	Starch hydrolysis	+

The results of phytochemical screening of the plants leaves extract were summarized in table 2.

Table 2: Summarized Results of Phytochemical Screening:

Sl.No.	Compounds	Annona reticulata	Riccinus communis	Brucea javanica
1	Alkaloids	Present	Present	Present
2	Carbohydrates	Absent	Absent	Present
3	Flavonoids	Present	Present	Present
4	Tannins	Absent	Absent	Present
5	Proteins	Absent	Absent	Absent
6	Reducing sugars	Absent	Absent	Absent
7	Saponins	Present	Absent	Absent

The results of antimicrobial properties of the three plant extracts were summarized in table 3

Table 3: Summarized Results of Antimicrobial Assay

Concentration (µg/ml)	*Annona reticulata*		*Ricinus communis*		*Brucea javanica*	
	Radius	Area	Radius	Area	Radius	Area
10	0	0	0.3	0.2826	0.5	0.78
25	0.3	0.2826	0.4	0.5024	0.25	0.196
50	0.4	0.5024	0.5	0.78	0.25	0.196
75	0.5	0.78	0.6	1.13	0.5	0.78
100	0.6	1.13	0.7	1.1538	0.5	0.78
Standard	2	12.56	2	12.56	2	12.56

In the present study we have collected a diseased fish (*H. fossilis*) having certain infections on its external surface. We then isolated bacteria from the site of infection by using cotton swab and made sub-culture and finally pure culture of isolated bacteria on nutrient agar plate.

In the current context we have performed gram staining procedure for characterizing the isolated bacteria. And we found it to be gram positive cocci. After confirmation we further executed certain tests as reported by Kreign and Holt (1984) like indole production test, methyl red test, voges proskeur test, catalase test, citrate utilization test, Hydrogen sulphide and motility test, oxidase test, starch hydrolysis test and litmus milk reaction test (Kreign and Holt,1984). The

outcome from the confirmation test were compared as per bergery's manual and the outcome from the very same suggest that the isolated bacteria may belongs to the genus *Staphylococcus*. Bujjamma and Padmavathi, 2015 reported *Staphylococcus* to be one of the causative agents for peripheral skin disorders in fish. Deka *et al.*, 2005 identified and characterize three bacterium namely *Shigella sp, Streptococcus faecalis* and *Aeromonus hydrophilla* on the basis of biochemical tests(Kreign and Holt,1984) and they were found to be the main causative agents of skin ulcers of *Cirrhinous mrigala* (Deka *et al.*, 2005)

Annona reticulata, Ricinus communis and *Brucea javanica* have been selected for the current study. Alkaloid and flavonoids were found in all the plant extract, carbohydrates and tannins were present only in *Brucea*, Saponins was found only in *Annona reticulata* whereas proteins and reducing sugar were absent in all the plant extracts.

Prescott *et al.*, 1990 reported that disc diffusion method can be used to screen the herbal extracts for antibiotic activity. The antibacterial property was checked by using disc diffusion method (Kirby-Bauer method) using different concentration and taking Ampicillin as standard test control. In the current study it is shown that the isolated bacteria belonging to the genus *Staphylococcus* have showed variable sensitivity to the three antibacterial agents. The results from the disc diffusion method clearly depicts antibacterial property of *Annona reticulata* to be highest in 100% concentration with an effective zone of inhibition area of 1.13 cm^2 and to be lowest at 10% with no zone of inhibition area.

In case of *Ricinus communis* it is found that it shows its highest antibacterial property in 100% concentration with an effective zone of inhibition area of 1.538 cm^2 and to be lowest at 10% with an effective zone of inhibition area of 0.2826. Again, in case of *Brucea javanica* it shows highest antibacterial property in 10%, 75% and 100% with the same effective zone of inhibition area of 0.78cm^2 and lowest in 25% and 50% with zone of inhibition area of 0.196 cm^2.

The research work conducted by Mastan, 2013 have screened sixty six crude extracts obtained by alcoholic and aqueous solvent of twenty two plants for its antibacterial property by using disc diffusion method. There was no inhibition zone for negative control (water). From the Results it was summarized that activity against gram positive bacteria was less frequent than against gram negative. It was also reported that out of the total twenty-two the medicinal herbs *N. lutea* and *V. minor* showed the best results.

Several studies reported that the bacteria *Aeromonus hydrophila* showed considerable levels of resistance against chloramphenicol, penicillin, amoxicillin, metronidazole, sulphamethoxazol- trimetroprim and amikacin (Maurel *et al.*, 2002; Hatha *et al.*, 2005; Jongjareanjai *et al.*, 2009; Kaskhedikar and Chhabra, 2010)

Gogoi *et al.*, 2015 reported that ethanolic extracts of aerial parts of *H. perforatum* show considerable activity against *S. aureus* and *P. aeruginosa* and methanolic extracts against *Klebsiellla oxytoca, E.coli, Proteus mirabilis, B. Cereus, S. aureus, L. monocytogenes*.

In the literature, thousands of plants derived compounds have been screened and their inhibitory effects against all types of micro-organisms have been confirmed. With

such a growing amount of data, Cowan *et al.*, (1999) emphasized that the methods of extraction and *in vivo* testing should be standardized to facilitate the interpretation of the results.

From the performed study, the antibacterial property of the mentioned plant can be further evaluated using different high-end technologies as well as could be considered a promising source of new drug candidates in aquaculture industry. In addition to that the isolation of phytochemicals can be a new route for antimicrobial agents' discovery against prevailing fish diseases. Moreover, further research needs to include *in-vivo* tests to determine the effectiveness, stability and impact of studied extracts on fish and on the environment.

Conclusion:

From the present study we can conclude that the genus *Staphylococcus* to be one of the major causative microorganism responsible for peripheral skin disease prevailing in the state. Moreover, the green plant can act as an alternate source of antimicrobial for their treatment.

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