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Original Articles

Review Article

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Original Article

Status and Conservation of Asian Elephant in Chandaka-Damapada Elephant Sanctuary

Prafulla Kumar Mohanty*, Bandana Khuntia**

Received on 13.08.2016, **Accepted on** 22.08.2016

Abstract

Chandaka- Damapada sanctuary is situated 20 km from the center of temple city of Bhubaneswar. Chandaka forest got the status of sanctuary by the Government of Odisha in 1982. It was established to provide inviolate refuge for elephant with a view to minimize their depredation. The aim was for overall protection of forest ecosystem, particularly for providing a safe heaven for resident elephants. It is a dense forest covering an area of 193.39 sq. kilometres where elephants roam freely. The sanctuary is abutting both Cuttack and Khordha district. Deras, Jhumka and Kumar Khunti are reservoir surrounded by forest hillocks. Flora is moderately diverse with intimate mixture of evergreen and deciduous forests. According to the Geological Survey of India (GSI), it was found that 37 species of mammals, 167 species of birds, 13 species of amphibians and 33 species of reptiles are present but due to some anthropogenic activities the life of wild animals are in danger. In Chandaka, elephant is a flagship species. According to 2012 census, the member of elephants was 24 but in 2015, it was found surprisingly declined to 8. The major causes of declining number of elephant in Chandaka are the scarcity of foodand water sources, human settlement in forest area, urban development, construction of road, noise pollution, and corridor destructions. For this reason elephants are forced to come out from their original habitat and migrate towards connected forest to Nayagarh, Ganjam and Athagarh. Sometimes elephant also are entering nearby town, destroy the crop damages the house and kill the human beings. Conservation and protection of this key stone species in Chandaka is essential. Conservation priorities for this endangered species includerevival of the corridors, conservation of forests, maintenance of habitat and social development along with avoidance of noise and light.

Keywords: Elephant; Key Stone Species; Conservation; Chandaka.

Introduction

Chandaka- Damapada sanctuary is situated 20 km away from the centre of temple city of Bhubaneswar. Chandaka forest got the status of sanctuary by the Government of Odisha in 1982. It was established to provide inviolate refuge for elephants with a view to minimize their depredation. The aim was for overall protection of forest ecosystem, particularly for providing a safe heaven for resident elephants. Godibari is the main gate of the sanctuary (Figure 1). This is located in between Daspur and Bhola reserve forest on both of sides of Old Grand Trunk Road. The road has significance of creation during Shershah Suri having historical importance. During 16th centuryBuliagarh was an ancient fort of king Chodaganga Deva. In Chandakasanctury Deras, Jhumka and Kumar Khunti are the reservoirs surrounded by the forest hillock. Chandaka sanctuary enjoys undulating topography with rich floral and faunal bio-diversity. Flora is moderately diverse with intimate mixture of evergreen and deciduous type. Chandaka forest covers and dominates mainly Sal, Piasal, Asan, Bamboo trees etc. which offers a

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congenial habitat of elephants (Figure 2). A good variety of wild animals and birds are distributed of which the Asian elephant is the flag ship species (Anonymous, 2014) along with chital, barking deer, mouse deer, wild pig, common langur, common Indian mongoose, hyena, sloth bear, monitor lizard and rock python. Among the birds are red jungle fowl, peafowl, crested serpent eagle, great hornes owl etc. (Tiwari, 2000). Fragmentation of forest and destruction of habitats caused by farming, expansionof roads, railways, shortage of food and water have led elephants to move towards the nearby urban areas. This situation has percolated into destruction of houses and crops, human casualties and end in death of elephants (Khuntia, 2010). According to 2012 elephant census, 24 elephants were seen but in 2015 number of elephants in Chandaka surprisingly declined to eight (Anonymous, 2015). To prevent the migration of elephants from the sanctuary to nearby linked forest, management initiatives like educating villagers, pre-hand information about elephants' movement, installation of scare away devices, habitat improvement, providing adequate food and water in the forest, creation of an effective barrier around sanctuary, alternative crop production to deter elephant man interaction to a minimum extent are desirable (Kar and Lahiri, 2002; Swain, 2004; Menon et al., 2005). Compassionate payment is one of the most important measures in the field of animal depredation. The amount for compassionate payment has been revised for better mitigation (Khuntia and Mohanty, 2013; Pal 2015). Since there is a regular man-elephant conflict in Bhubaneswar(the capital of Odisha), the objective of this paper is to suggest the measures to overcome this alarming situation and maintaining the population of elephant.

Materials and Method

Data utilized in this investigation were collected from various locations depredated by elephants. Report of the Government of Odisha, newspaper highlights and interaction with affected people, crop, house and property affected by elephants in the areas have been taken into account. Moreover, human and elephants' casualties were recorded in different years. Scare away devices to drive away elephants to their original habitat and their effectiveness were also studied.

Findings

Chandaka sanctuary is situated in the city of Bhubaneswar which amalgamates both in Cuttack and Khordha district. It is located in latitude 20^0-12'to 20^0-26' N and longitude 85^0- 34'to 85^0-49'E (Figure 3). The sanctuary enjoys a dense forest covering area of 193.39 sq. km where elephants roam freely. There are 47 villagers nearby Chandaka forest. The average temperature is 40^0 C in summer and 10^0C in winter. The annual rainfall is 1200 mm to 1400 mm having humidity 80%. According to Geological Survey of India (GSI), there are 37 species of mammals and 167 species of reptiles where elephants are considered as a key stone or flag ship species of the ecosystem. In other words, the sustenance and development of the forests is hinged with elephants. But the population of elephant is decreasing in an alarming rate regularly chiefly because of anthropogenic activities. According to 2015 census, the number of elephant is eight (Tables 1 and 2). It was found that during harvest season of paddy crop, the elephant depredation is at the peak and many villages get affected in the month of November, December and January. Elephants do not move normally during the day time and take shelter in place where there is tree cover and stay in the particular area for food, water and shelter (Anonymous, 2010). In 2007, the number of elephant in Chandaka sanctuary was 67, but in 2015 census, it was found to be eight. (Figure4) in which male, female and young was 3, 3 and 2 respectively. According to the report of Forest Department of Odisha, only two elephants were dead due to different reasons in last three years (Table 3). 18 elephants moved towards linked forest area Athagarh-Narshingpur forest and 8 towards Nayagarh forest. Bhubaneswar, the capital city of Odisha, is magical combination of ethnic heritage and modern town planning. The city is rapidly growing with population and space. The Chandaka wildlife sanctuary is the only green patch and protected area near the city. Being an ecologically sensitive area, the rapid growth, expansion and development of Bhubaneswar at the cost of its fringe comprising areas of high conservation value have caused emerging environmental concern (Khuntia and Mohanty, 2015). The haphazard mushrooming growth of the real estate business in Bhubaneswar is also the biggest threat to Chandaka and Bharatpur sanctuary. Large institutions, apartment complexes, closed colonies etc. are being built on boundary of the sanctuary and their powerful lights, construction, noise and increased human movement restrict the movement of pachyderms (Anonymous, 2016). Because of all these, they lead a wretched life through drinking water from leaking pipe lines, improper habitat and stroll outside the sanctuary at night. Elephants have been observed to leave their habitat and corridor to the human

habitation and institutions for various reasons especially for food and drink leading to varieties of casualties (Table 4). Therefore, the Government is forced to pay compensation under various aspects of damage, loss and causalties (Table 5).

Conclusion

Chandaka- Damapada is now a popular destination for solitude seekers, haunt of eco tourists, laboratory for researchers and temple of learning for all. The major cause of decreasing number of elephants in Chandaka has been observed to be due to fragmentation of corridors, increased human activities in the forest, vehicular noise and disturbance and scarcity of food and water. To overcome such conflicts, some measures like implementation of forest laws providing alternative source of income to the villagers, restriction of tourists and their vehicles in the reserve forest and use of bio-gas fuel instead of forest wood are suggested to be introduced. People living in villages located on high ground or on the migration path of elephants or habitat of elephants need to adopt a few preventive steps. An important aspect in the management of elephant-human conflict is to enable the people residing in elephant zones to live in harmony with elephants. Government of Odisha has taken many preventive measures to overcome the problems like erection of trenches, elephant proof barriers, solar fencing deploying anti depredation squads etc. (Figures 5 and 6). In order to restrict the movement, elephants belonging to Chandaka-Damapada sanctuary works have been initiated for restoration of the elephant proof trenches, water passages, for whichRs 590.00 lakh have been sanctioned during the current year.

Appropriate conservation measures, alternative food source and restricted anthropogenic activities are strongly suggested to maintain the corridor, habitat and population of this keystone species.

Acknowledgements

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Table 1: Population of elephant in Chandaka 2002-2015

S. No	Year	No of elephant
1	2002	62
2	2007	67
3	2010	23
4	2012	24
5	2015	08

Source: Department of Forest and Environment, Govt. of Odisha.

Table 2: Census of elephant in Chandaka, 2002-2015

S. No.	Year	Male	Female	Calf	Total
1	2001	13	35	10	58
2	2002	13	40	09	62
3	2005	13	40	12	65
4	2007	14	39	14	67
5	2010	7	9	7	23
6	2012	9	11	7	27
7	2015	04	01	03	08

Source: Department of Forest and Environment, Govt. of Odisha.

Table 3: Death of elephants from 2010-2015

S. No	Year	Tusker	Female	Calf	Total
1	2009-10	-	01	-	01
2	2010-11	-	-	-	-
3	2011-12	01	-	-	01
4	2012-13	-	-	-	-
5	2013-14	-	-	-	-
6	2014-15	-	-	-	-
				Total	02

Source: Department of Forest and Environment, Govt. of Odisha.

Table 4: Human death and injury by wild elephants 2010-2015

S. No	Year	No of Death	No of injury
1	2010-11	-	-
2	2011-12	02	-
3	2012-13	-	-
4	2013-14	-	-
5	2014-15	-	-

Source: Department of Forest and Environment, Govt. of Odisha.

Table 5: The rate of compassionate payment by the government

S. No	Type of loss	Amount in Rs
1	Human kill	3 Lakhs
2	Permanent injury	1 Lakhs
3	Temporary injury	5, 000 / -
4	Crop Damage	10, 000 / ha
5	Vegetable and cash crop	12, 000 / ha
6	House damage	
	Full damage	10,000 / -
	Partial	2,000 / -

Source: Department of Forest and Environment, Govt. of Odisha.

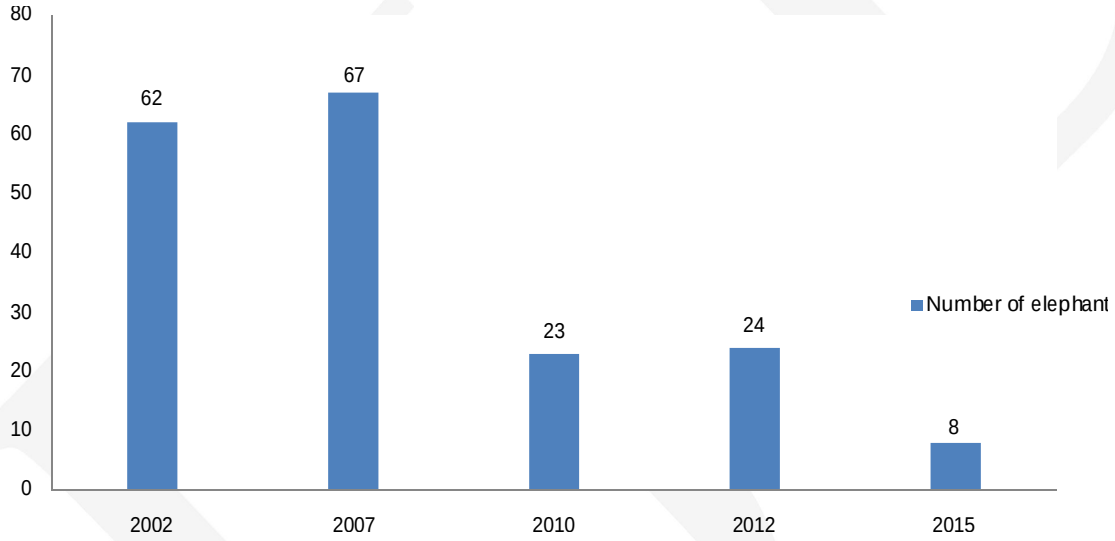

Fig. 4: Population of elephant from 2002-2015.

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Original Article

A Preliminary Survey of Fresh Water Fishes in Muntjibpur Pond of Allahabad (U.P.)

Ashok Kumar Verma

Received on 12.09.2016, **Accepted on** 11.10.2016

Abstract

The present study is undertaken to find out the distribution of fresh water fishes naturally occurring in Muntjibpur pond of Allahabad. The survey was centralised mainly on distribution and diversity of fishes and conducted during a period of one year from Jan 2014 to Dec 2014. A total of 11 species of fishes belonging to 10 genera, 7 families and 4 orders were identified as result of preliminary survey. This was the first ever systematic survey on the fish diversity of this pond. Siluriformes were found most dominant order represented by 5 genera followed by Cypriniformes with 3 genera.

Keywords: Preliminary Survey; Fish Biodiversity; Fish Fauna; Muntjibpur Pond; Conservation.

Introduction

Hydrobiology is the study of life in water while limnology is the study of the physical, chemical, geological and biological aspects of all naturally occurring fresh water. Freshwater habitats such as lakes, ponds, dams, reservoirs are known as lentic (still) while running water such as rivers, mountain streams are known as lotic (flowing). The term 'pond' refers to a relatively shallow body of water usually smaller than a lake, contained in an earthen basin retaining sewage or organic wastes.

In India, a number of ponds, lakes and reservoirs are naturally found but they are not being utilized properly due to lack of insufficient study of their hydrobiology. The study of different water parameters is very important for understanding of the metabolic events in the aquatic ecosystem. One of the most important features of ponds is the presence of standing water, which provides habitat for wetland plants and animals.

Fishes are cold-blooded, gill-bearing aquatic craniate vertebrates that include both the bony and the cartilaginous fishes but sometimes jawless fishes too. They belong to phylum: Chordata, subphylum: Vertebrata and super class: Pisces. The fishes are not only used as good source of food for mankind, having economic importance from medicinal point of view but also play a crucial role in the second tropic level of the aquatic ecosystem.

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Prakash *et al* (2015a, 2015b and 2016) and Verma *et al* (2016a, 2016b, 2016c and 2016d) conducted the limnological studies as well as studies on fish biodiversity in a fresh water body. The present study is undertaken as a preliminary survey to find out the distribution and diversity of fresh water fishes naturally occurring in Muntjibpur pond of Allahabad. This survey was conducted during a period of one year from Jan 2014 to Dec 2014.

Study Area

Muntjibpur pond is a natural pond, located on north side of village Muntjibpur. This pond is surrounded by agricultural fields and covers more than 5000 square meters. It is located in Pratappur block of Phoolpur tahsil of Allahabad district of Uttar Pradesh. This village is surrounded by Miraipur in east, Fatuhan in west, Saidpur in north and Fulahan in south.

Picture 1: A view of Muntjibpur pond

Material and Methods

The pond was surveyed for fishes once in a month for the period of one year from January 2014 to December 2014. The fishes were caught and collected for present survey from Muntjibpur pond by hand-nets, gill nets, cast nets, hooks, drag nets with the help of local people and fisherman.

Fishes were identified using the standard keys of Mishra (1959), Day (1989), Jhingran (1991), Jayaram (1999) and Srivastava (1998).

Result and Discussion

The summer, monsoon and winter season show different seasonal fluctuation in various hydrobiological parameters in this pond. The water present in the said pond is useful for irrigation as well as fish culture. The water quality of the this pond is although having some pollution but is suitable for agricultural purposes also, as it is rich in organic humus, planktons and nutrients.

Table 1: Showing fishes reported from Muntjibpur pond in the year 2014

S. No.	Zoological name	Family	Order
1.	*Catla catla*	Cyprinidae	Cypriniformes
2.	*Labeo rohita*	Cyprinidae	Cypriniformes
3.	*Labeo calbasu*	Cyprinidae	Cypriniformes
4.	*Cyprinus carpio*	Cyprinidae	Cypriniformes
5.	Mystus seenghala	Bagridae	Siluriformes
6.	*Rita rita*	Bagridae	Siluriformes
7.	*Wallago attu*	Siluridae	Siluriformes
8.	*Clarias batrachus*	Clariidae	Siluriformes
9.	*Heteropneustes fossilis*	Saccobranchidae	Siluriformes
10.	*Channa punctatus*	Ophiocephalidae	Ophiocephaliformes
11.	*Gudusia chapra*	Clupeidae	Clupeiformes

During the study period, a total of 11 species of freshwater fishes belonging to 4 orders, 7 families and 10 genera were recorded from the Muntjibpur pond. The collected fish species including their zoological names, family and order are shown in the table given.

Fish fauna of the pond studied belong to 4 orders namely Siluriformes, Cypriniformes, Ophiocephaliformes and Clupeiformes. In present investigation Cyprinidae family was the most dominant group representing 4 species followed by Bagaridae family representing 2 species. The families Siluridae, Clariidae, Clupeidae, Saccobranchidae and Ophiocephalidae were represented by one species each. In this way, authors recorded 11 different species.

Conclusion

A total of 11 species of freshwater fishes belonging to 4 orders, 7 families and 10 genera were recorded from the Muntjibpur pond during its preliminary survey. A detailed study of this pond is recommended to understand its fish biodiversity and conservation status.

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Erratum

Article Titled **"Variation in Net Radiation over Wheat (*Triticum aestivum* L.) in Different Phenophase Intercropped with Dalbergia Sissoo in Nelder Wheel Design"** Publsihed in Indian Journal of Biology, Volume 2 Number 2, July - December 2015 pages 141-146

A wrong value in the sentence in Abstract and Materials & Methods has published in said article and this sentence is now to be read as

"Tree spokes were pruned 0%, 30%, 45%, 60% and 75% starting from north direction respectively"

Mistake is regretted.

Editor-in-Chief

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Original Article

Antibacterial and Toxicity of *Dillenia indica* L. Based Green Colloidal Silver Nanoparticle (CSNP)

Jyoti Prasad Saikia*, Rajesh Saikia, Yutika Nath*, Alak Kumar Buragohain*,***, Bhaskarjyoti Gogoi***

Received on 04.06.2016, **Accepted on** 16.06.2016

Abstract

D. indica fruit juice based SNP synthesis has been carried out using previously established method. The SNP were characterized using UV-Vis, furrier transform infrared spectroscopy (FTIR) and transmission electron microscopy (TEM). The CSNP were found to be antimicrobial and chemotactic positive. The cytotoxicity against RAW 246.7 (murine macrophage) cell line is found to be moderate at 10% (v/v) of CSNP in culture medium. Moderate toxicity has been observed against *Vigna radiata* Linn. seed and *Oryza sativa* (*variety Ranjit*) seeds. $NaBH_4$ alone and $NaBH_4$ based CSNP is found to be promoting germination of plant seeds.

Keywords: Siver Nanoparticle; Dillenia Indica; Antibacterial; Cytotoxicity; Phytotoxicity.

Introduction

Colloidal SNP is a requirement of the present day. Green methods are only hope for CSNP synthesis and supply with respect to its increasing demand. Out of the recent CSNP synthesis research a major amount of publications are focused on green CSNP synthesis. Comparison of these green CSNP with respect to the chemically synthesized CSNP is essential. Some of the green CSNP are found to be better in some aspects compared to their chemical counterpart. The variation in green CSNP is due to mixture of chemicals present in plant, animal and microbial extract. These chemicals sometimes act as reducer or stabilizer or both.

Researchers also suggested that green CSNP will be less toxic and hazardous for the environment as part of their precursor is from a living organism. The toxicity should be tested for scientific validity of the claims.

Dillenia indica L. fruits are sour in taste and are used in the preparation of jam and jellies and as flavouring agent for curries. Traditionally the juice of leaves, bark and fruits are mixed and given orally for the treatment of cancer and diarrhoea [1]. The plant is reported to contain betulinic acid, betulin, cycloartenone, n-hentriacontanol and α-sitosterol [1]. Fruit of *D. indica* also contain about 34% of total phenolics in methanolic extract and polysaccharide like an arabinogalactan [2]. These literatures suggest that *D. indica* fruit juice contain all the needed chemicals required for synthesis of CSNP. In our previous publication we have already synthesized the CSNP using *D. indica* fruit juice [3].

Cytotoxicity of nanomaterial is always evaluated using animal cell line only [4, 5]. Research on effect of nanomaterials on plant system is scanty. In many cases the plants also get exposed to these nanomaterials. Any effect on them can adversely affect the ecological balance and productivity in crop field. Therefore in the present study the cytotoxicity is evaluated on animal cell line as well as plant seeds.

In the light of above information, a project has been design to synthesized, characterize, test antimicrobial, chemotaxis and toxicity properties of the colloidal silver nanoparticle.

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Materials and Methods

Materials and Chemicals

D. indica is collected from Sonitpur, Assam, India. $AgNO_3$ (A.R.) is obtained from Merck, India. Sodium borohydride is obtained from Fluka Chemicals, USA.

D. Indica Extract Preparation

The process for extract preparation is followed from Singh *et al.* [3] with modifications. Ripped *D. Indica* fruits were collected from the tree, Sonitpur, Assam. The fruit is washed properly to remove all debris and 25g calyx washed gently with sterile distilled water. The calyx was cut into 0.5 cm^3 pieces and homogenized using mixer grinder (Philips HL1629 Mixer grinder, Bajaj Electronics India) with 100 ml sterile distilled water. The grinding was done until liquefied mixture get formed. Liquefied mixture was filtered using double-layered cheese cloth to remove solid debris. Filtrate was centrifuged at 5000 rpm for 30 min (Spinwin MC-02, Tarsons) and supernatant was collected. The supernatant was further subjected to 0.45 mm filtration followed by 0.22 mm filtration. The filtrate is used for synthesis of colloidal silver nanoparticle (CSNP) and will be referred further as *D. indica* extract.

Synthesis and Characterization of the Silver Nanoparticle

Synthesis was performed following the method described by Singh *et al.* [3] with minor modifications. 20 ml of $AgNO_3$ (0.5 M) was mixed with 50 ml of the extract in 4 ml distilled water. The solution was made slightly basic by adding 100 ml 0.01 M KOH and the final volume was adjusted to 5 ml with distilled water. Control silver nanoparticle was synthesized using $NaBH_4$ following the method described by Phukon et al. [6] and labelled as 'PC'. Different types of silver nanoparticle and negative controls synthesized are given in Table 1.

The characterization of the CSNP was performed using UV-Vis wavelength scanning from 200-800 nm, transmission electron microscopy (TEM) JEOL JEM 2100, Japan. Fourier transform infrared spectroscopy (FTIR) of Nicolet Impact 410 spectrometer.

Antibacterial Activity

The antibacterial assay was done by using the Agar Well Diffusion method described by Bharali et al. [7]. Briefly the bacterial culture was adjusted to the McFarland standard No. 0.5 before the tests. The media used for the assay was Mueller Hinton Agar (Himedia). The test was performed against *Bacillus subtilis* (MTCC 121) and *Escherichia coli* (MTCC 40). Streptomycin (STR) (50 mg / ml, Sigma) was taken as the positive control. The test was performed in triplicates.

Chemotaxis Analysis

Chemotaxis analysis was performed following the method described by Bharali et al. [7]. The bacteria used for analysis were *Staphylococcus aeurius* (MTCC 3160) and *Klepsiala pneunomonia* (MTCC 618).

Cytotoxicity Assay

Cytotoxicity assay was performed on RAW 246.7 (murine macrophage) cell line and *Vigna radiata* Linn. and *Oryza sativa* (*variety Ranjit*) seeds.

Mouse macrophage cells were plated in 96 well plates ($1x10^4$ cells / well) and kept for 24 h using DMEM media supplemented with 10% FBS (fetal bovine serum) 200 U / mL penicillin, 100 μg / mL streptomycin, 0.3 g / mL L-glutamine and 2 mM $NaHCO_3$ in 5% CO_2 atmosphere at 37°C so that cells can reach confluency. The cells were then treated with $AgNO_3$ (0.001M), distilled water (control), DISNP and $AgNO_3$(0.001M) solutions of volume 1, 5 and 10 mL and then incubated for 24h. After incubation 20 mL of 3(4, 5dimethyl-2-thiazolyl)2,5-diphenyl-2H-tetrazoliumbromide (MTT) is poured to each well and incubated. After incubation for 4 h the MTT solution was discarded without disturbing the formed formazone crystal and 100 mL of MTT solvent was added to each well to dissolve the complex. The optical density of the solution was measured at 580 nm using Multiskan Go equipment (Thermo Scientific, India).

The toxicity on plant cells were evaluated using germination process. In short, 2.50 g (dry weight) of *Vigna radiata* Linn. and *Oryza sativa* (*variety Ranjit*) seeds were surface sterilized and then soaked in 50 mL volume of distilled water, $AgNO_3$, PC, DISNP and DIEX for 24h. After imbibitions the seeds were washed thoroughly under sterile condition and allowed to germinate in germination chamber under ambient temperature and light / dark phase using plant tissue culture facility. Number of seeds germinated was recorded after 7 days.

Results

Silver Nanoparticle Synthesis and Characterization

Synthesis of colloidal silver nanoparticle using *D.*

indica extract is successful. Visually the colour of the DISNP colloid is golden yellow. Formation of CSNP in DISNP sample is confirmed using UV-Vis wavelength scanning 200-800 nm (Figure 1, A). The peak of absorption for DISNP and PC are at 420 and 410 nm, respectively. Less intensity of DISNP (after 1 day) compared to PC might be due to less amount of CSNP formation in the previous. The phenomenon might be due to weak organic reducers present in *D. indica* compared to sodium borohydride [1]. The intensity of the DISNP peak after 1 day is comparable to results obtained by Grishchenko *et al.* [8] using arabinogalactan. The DISNP intensity increases with time (Figure 1, B and C; after 27 days) and become comparable with PC. Intensity of the negative controls (Figure 1, A) are low and no signature of CSNP has been observed during the study. The symmetry of the peak is not disturbed during 27 days of storing at room temperature and slight increase in width is observed, suggest that DISNP nanoparticles are uniform [9].

DISNP peak shifted from 420 to 440 nm during 27 days period. This might be because of slight increase in the particle size due to slight agglomeration or increase in the particle size (Figure 1, C).

Table 1: Composition of different samples

Name	$AgNO_3$ 0.5M (ml)	*D. indicia* extract (ml)	KOH 0.01M (ml)	Distilled water (ml)
DISNP	20	50	100	4830
AGDI	20	50	0	4930
DIKOH	0	50	100	4850
AGKOH	20	0	100	4980

NB: PC was prepared using 10 ml 0.001M $AgNO_3$ and 30 ml 0.002M $NaBH_4$.

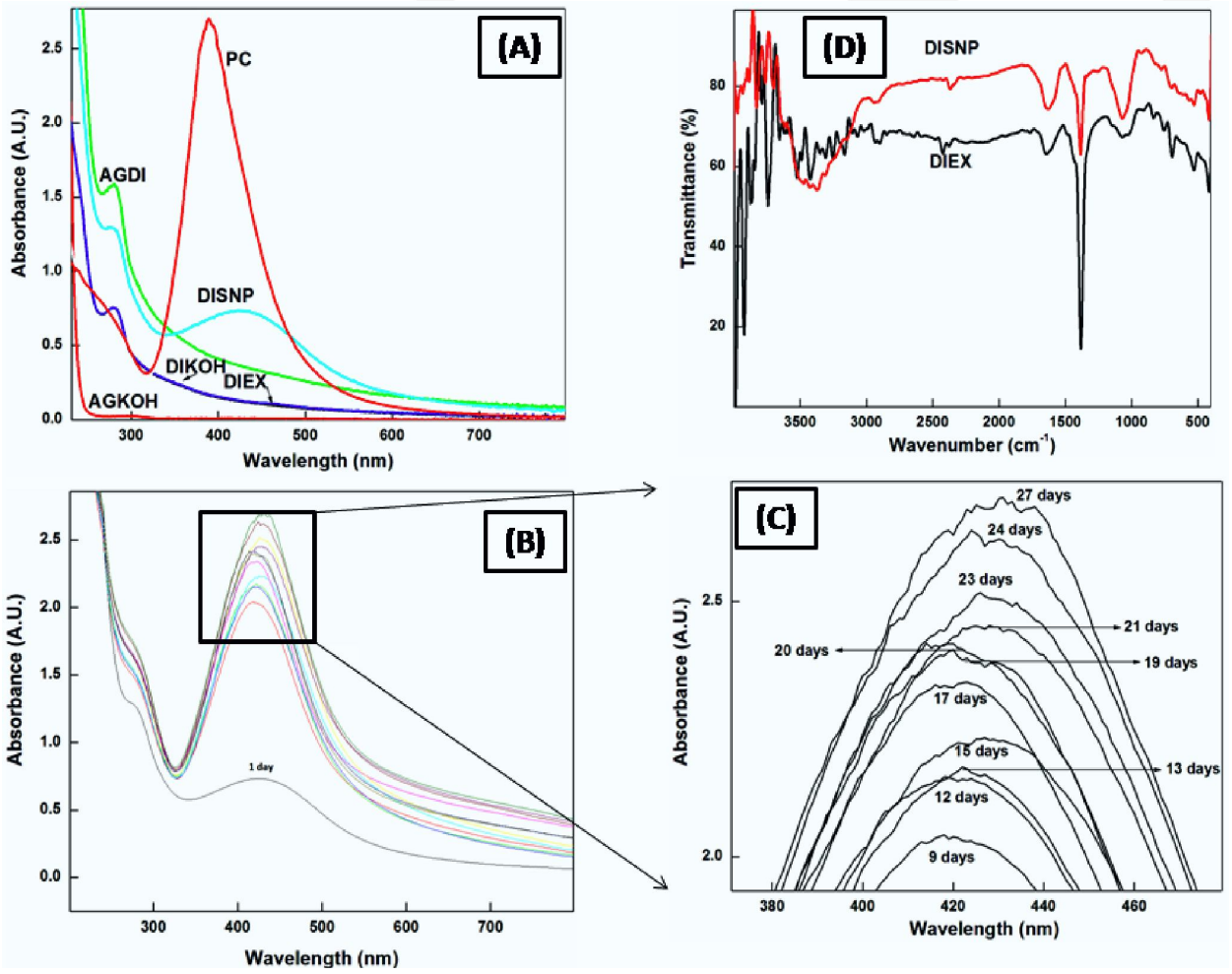

Fig. 1: Spectroscopic characterization of colloidal silver nanoparticle, **(A)** UV-Vis absorption spectra of different samples after 24 h (1 day) of incubation, **(B)** UV-Vis absorption spectra of DISNP with respect to different days (up to 27th day), **(C)** Enlarged and day wise labelled UV-Vis absorption spectra of DISNP, **(D)** FTIR analysis of DISNP and DIEX

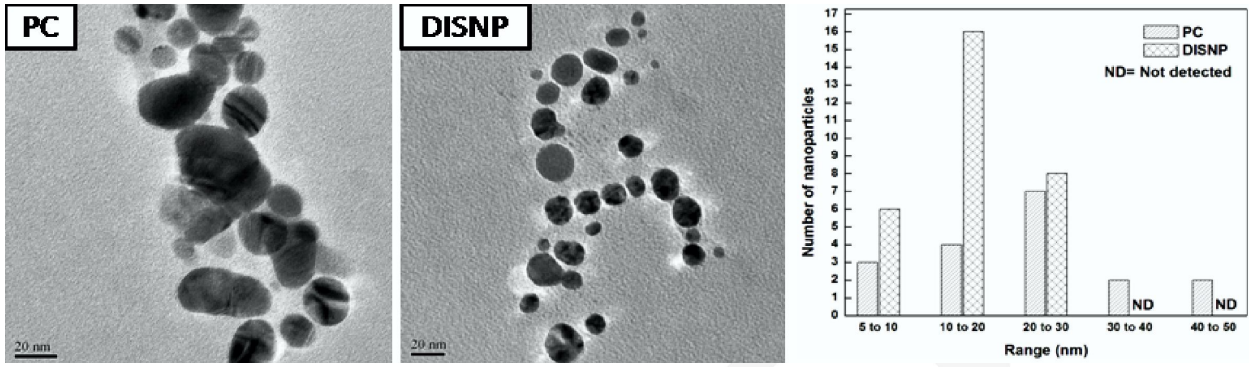

Fig. 2: TEM images of PC and DISNP. Size range wise distribution of nanoparticles as calculated from the figure is presented as bar diagram (right)

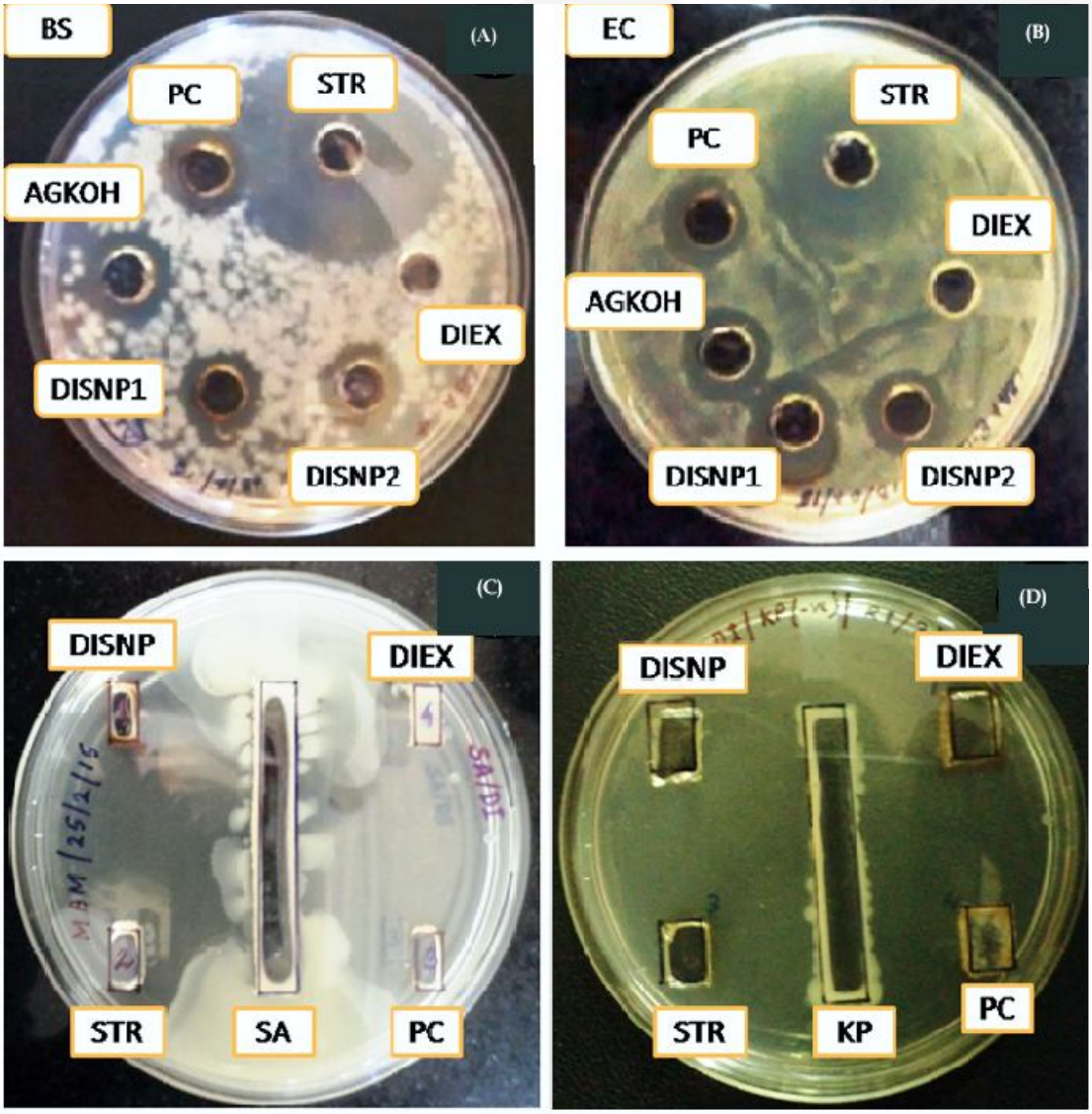

Fig. 3: Antibacterial **(a and b)** and chemotaxis **(c and d)** activity of nanoparticles, **(a)** Antimicrobial activity of silver nanoparticles against *B. Subtillis* (BS), **(b)** Antimicrobial activity of silver nanoparticles against *E. coli* (EC), **(c)** Chemotaxis activity of *S. aeurius* (SA) towards silver nanoparticles, **(d)** Chemotaxis activity of *K. pneunomonia* (KP) towards silver nanoparticles, DISNP1=27 days old DISNP, DISNP2=30 days old DISNP, STR= Streptomycin. Graphical representation of antibacterial assay is presented as bar diagram.

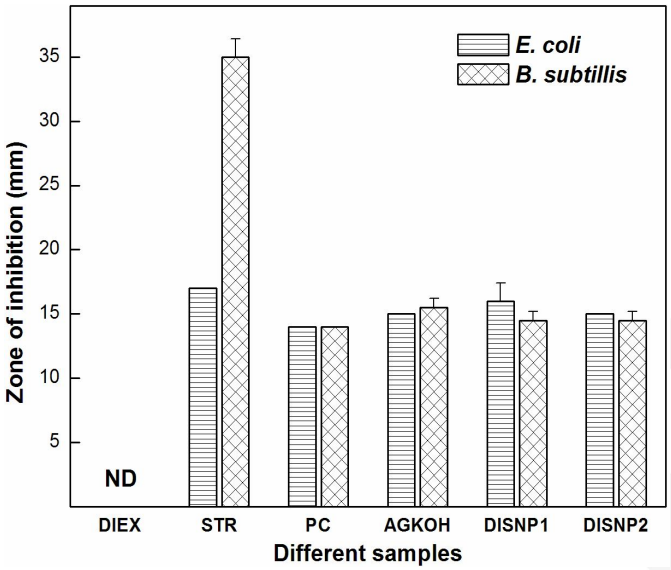

Fig. 3 (graph)

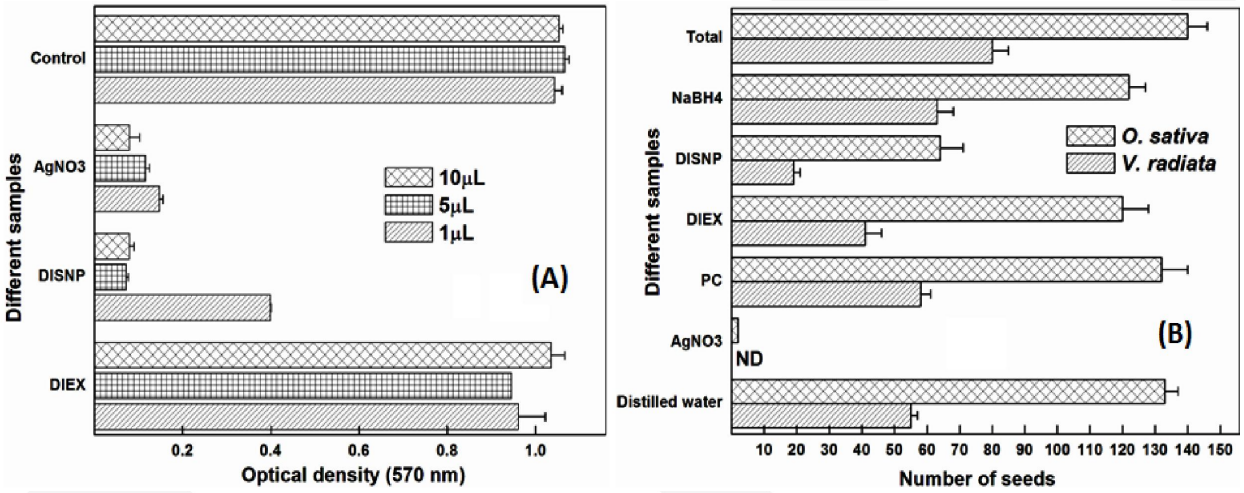

Fig. 4: Toxicity assay of silver nanoparticles in **(A)** RAW 246.7 (murine macrophage) cell line, **(B)** seeds of *V. radiata* and *O. sativa.*

Figure 1, D shows the FTIR analysis of 27 day old DISNP and DIEX. DISNP follows the pattern of DIEX except in the region of 4000 to 3000 cm^{-1}. This might be related to bound water to the compounds and suggest DISNP have more bound water compared to the *D. indica* extract (DIEX). TEM analysis presented in Figure 2 shows 10-50 nm size range for PC and 5-30 nm for DISNP. This image further confirms the formation of CSNP using *D. indica* extract. The bar diagram presented in Figure 2 shows that maximum number of particle in TEM image of PC are in the range of 20-30 nm whereas the same for DISNP is in the range of 10-20 nm.

Antibacterial and Chemotaxis Analysis

Figure 3 (a & b) shows antibacterial activity of all samples against *B. subtillis* and *E. coli*, except DIEX. PC is well known for its antibacterial activity, AGKOH might be showing antibacterial activity due to toxicity of $AgNO_3$ and alkaline KOH. DISNP1 (27 days old DISNP) and DISNP2 (30 days old DISNP) do not have any significance difference in their antimicrobial activity.

Figure 3 (c and d) shows the chemotaxis activity of the *S. aeurius* and *K. pneunomonia* towards DISNP, DIEX, STR and PC. *K. pneunomonia* does not show any growth outside the well. *S. aeurius* show positive growth towards *D. indica* extract (DIEX). Growth of *S. aeurius* towards PC and STR are comparable. DISNP is found to be chemo-attractant for *S. aeurius*. The result for PC coincides with our previously reported finding Bharali *et al.* [7]. Figure 4 (B), shows the number of seeds germinated with respect to different treatment and figure 4 (A) viability of the mouse macrophage cells with respect different treatment, represented in the form of optical density. The seed germination

number was found to be varying significantly with respect to different pre-germination soaking medium for a time period of 24 h. From figure 4 (B) it was clear that soaking seeds in 0.001M $AgNO_3$ solution is detrimental and prevent the germination. Distilled water and PC are found to be non toxic to the germination process. DIEX was found to be slightly preventing the germination. DISNP was found to an intermediate toxic in the process of germination.

In case of mouse macrophage cell line, the toxicity corresponds directly to lower optical density as observed for $AgNO_3$ and DISNP. 1mL DISNP was found to be significantly less toxic compared to other two higher concentrations whereas DIEX does not show any toxic effect for the applied concentration as the absorbance of the solution was found to be equal to the control cells.

Discussions

In our previous research the data has been observed for about 6 days with absorption of 1.2 units [3]. Presently the analysis was done further up to 27 days and the observed absorbance is equivalent to PC, suggest that all $AgNO_3$ got reduced to Ag^0, then clump and formed nanoparticle. Shifting of the DISNP peak (Figure 1, B and C) towards longer wavelength and slight broadening, suggest increase in the size of the nanoparticle [10].

The TEM images of the nanoparticle presented in Figure 2 propose that DISNP particle are smaller compared to PC (chemically synthesized particles). The bar diagram presented in Figure 2 clearly suggests that most of the PC particles fall under the size range of 20-30 nm. The data is slightly higher compared to previous result presented by Solomon *et al.* [10] as 12 nm. The size range observed in the present study 5-50 nm for PC is also found to bigger compare to Song *et al.*'s [11] report of 30-40 nm. In case of DISNP the maximum particle fall under the range of 10-20 nm and show a narrow size distribution (5-30 nm) compared to PC (5-50 nm). These suggest that DISNP based synthesis is better compared to chemical synthesis method (using $NaBH_4$) for obtaining smaller sized nanoparticle with a narrow size distribution. The credit of narrow size distribution of DISNP might be attributed to natural surfactant molecules present in DIEX [12]. The polysaccharide like an arabinogalactan present in the DIEX might be acting surfactants and preventing clumping and thereby preventing formation of larger particles [13, 2]. During filter sterilization of the DIEX a huge amount of mucilaginous substances might get separated from the *D. indica* extract. Presence of those might further stabilize the nanoparticle. A future study can be conducted in unfiltered DIEX and additional stability provided therefore.

Antimicrobial activity presented in Figure 3 (a & b) suggest that DIEX do not have any antimicrobial property. Abdille *et al.* [14] suggested that lowest amount of total phenolics (1.4%) from *D. indica* fruit is recovered using water as solvent. The low amount of polyphenolic constituents might be responsible for not having antibacterial property. In case of *B. subtillis* treated with streptomycin (STR) the antimicrobial property was found to be highest (35.0 ± 1.4 mm). The same in case of PC (14.01.2), AGKOH (15.50.7), DISNP1 and DISNP 2 (14.50.8) are not significantly different from each other. PC is well known for its antibacterial activity against most of the bacteria with exceptions *B. subtillis* (MTCC 441) [7]. Similarly, in case of *E. coli* with streptomycin (STR) treatment show highest zone of inhibition (17.0ND, ND= not detected). Same in case of PC (14.0ND), AGKOH (15.0ND), DISNP1 (15.0ND) and DISNP 2 (15.01.4). In sample AGKOH the zone of inhibition might be due to the combine action of AgNO3 and un-reacted alkaline KOH. DIEX do not show any antibacterial activity against *E. coli*. These results suggest that antibacterial value addition to the DIEX can be done by synthesizing silver nanoparticle in fruit extract and it might be useful in increasing the shelf life of the fruit sap.

Literature on chemotaxis assay using silver nanoparticle and bacteria are scanty. In our previous research we found the same *S. aeurius* (MTCC 3160) was found to be highly chemotactic towards PC. In the present research a moderate chemotaxis was observed towards the PC [7]. Movement of the *S. aeurius* towars the well containing streptomycin (STR) is further less compared to PC (Figure 3, c). Movement of bacteria towards DISNP and DIEX is prominent. This suggests that DISNP might have the capacity to attract and then kill bacteria. This might be an added advantage over PC. In case of *K. pneunomonia* no movement was observed towards any of the well (Figure 3, d). Similar result was observed when *E. coli* (MTCC 40) is used for the assay (figure not presented). Since DIEX contain some amount of polysaccharide and other sugars a positive chemotaxis is expected at least towards it. The negative result might be due to the lack of mobility by the bacterial strains. During our literature search we have not found any literature suggesting lack of mobility by the above said strains.

To perform the toxicity of the DISNP towards mammalian cells, MTT assay was performed. From the Figure 4 (A) it has been found that the cells are

more sensitive to $AgNO_3$ and DISNP and hence only few cells can survive upon the treatment of $AgNO_3$ and DISNP. Even though at lower volume of given concentration i.e. 1 µl, toxicity reduced to half of the control cells. In case of DIEX, it did not show any toxic effect to the cultured macrophage cells. This happens only because of the presence of more numbers of live cells forming more numbers of formazone complexes by reacting with MTT solution. Henceforth the absorbance obtained in this case was more which was found equivalent to the absorbance of the control cells.

Germination of seeds follows a normal course of secretion of giberellic acid from embryo which give the signal to aleuron layer of cells to secret alpha-amylase. The alpha-amylase enzyme degrades the endosperm food reserve (major component starch) to produce reducing sugar. Therefore, the success of germination is depends a lot on degradation of starch. The data regarding toxicity of DISNP against plant seeds (*O. Sativa* and *V. mungo*) are presented in Figure 4B with respect to number of seeds germinated when DISNP, distilled water, DIEX, $AgNO_3$ and PC were used as imbibitions medium for 24h. The toxicity of DISNP was found to be moderate (Figure 4, B) against *O. Sativa* compared to *V. mungo* seeds. $AgNO_3$ in equal concentration with DISNP and PC is found to highly toxic to both seed types. It is a surprise to observe that PC is completely nontoxic (seen equal number of germination with distilled water) and even more number of seeds germinate in PC compared to distilled water in case of *V. mungo*. Chemically the PC contains Silver nanoparticle and excess of $NaBH_4$ (10 ml 0.001M $AgNO_3$ + 30 ml 0.002M $NaBH_4$). $NaBH_4$, being a strong reducer might be responsible for degradation of starch and doing so it promote germination. The hypothesis is yet to establish by performing an amylase assay for starch degradation.

Comparing Figure 4 (A) and (B) it can be summarised that DISNP is less toxic than $AgNO_3$, have comparable antimicrobial activity and size distribution of the nanoparticle is better than the $NaBH_4$ based nanoparticle (PC). Further research to evaluate the mechanism of stimulating germination by PC is necessary. *D. indica* based nanoparticle might be toxic to mammalian cells and plant seed. Therefore, one should be merely suggest a green nanoparticle will be always less toxic until the toxicity assay is performed and confirmed.

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Original Article

A New Formulation of Cow Urine based Polyherbal Hair Conditioner and its Antifungal Activity Against *Candida albicans*

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Abstract

Dandruff is a common embarrassing scalp disorder, affecting a large population.For the treatment of dandruff, shampoos containing imidazole derivatives and other chemicals are available but due to certain limitations such as poor clinical efficacy, compliance issues, side effects andinability to prevent recurrence, the people are attracted towards herbal products.Worldwide attraction and attention towardsherbal products for health care, health foods and natural cosmetic products including hair care formulations has increased. In the present work cow urine based polyherbal hair conditioner was developed as the medicinal properties of cow urine and herbs have been well described in ancient Indian medical science, Ayurveda and modern researchers have also reported their medicinal importance. Hair conditioner was formulated using coarse powder of *Sapindustrifoliatus, Acacia concinna,* mixture of *Emblicaofficinalis*, *Terminaliachebula* (retz.) and *Terminaliabelerica* in equal proportion, camphor, thymol and urine from indigenous cow. The developed formulation and available marketed products were compared for organoleptic characters such as pH, percent solids contents, dirt dispersion, detergency ability, ease of rinsing, luster of hair, foaming ability and stability. Evaluation of developed formulation for in-vitro anti-dandruff activity along with accelerated stability study was also carried out and presented.

Keywords: Cow Urine; Hair Conditioner; Candidaalbicans.

Introduction

History of mankind has witnessed the relationship between the beauty and cosmetics. Since ancient time, herbs are being used for maintenance and augmentation of human beauty. Hair care products are available in the marketin the form of shampoos, forcleansing the hair of accumulated sebum, scalp debris and residues of hair-grooming preparations [1]. Synthetic detergents have been claimedto be the most important factor in the growth of shampooproducts available in the market [2], in different forms such as liquid, lotion, cream, paste, gel, dry shampoo etc [3,4,5]. Sodium lauryl sulfate based detergents are most common but the concentration vary considerably from brand to brand and even within a manufacturer's product range [6,7]. For the treatment of dandruff, shampoos containing imidazole derivatives and other chemicals are also available in the market but due to their certain limitations, poor clinical efficacy or compliance issues or side effects andinability to prevent recurrence, people attracted towards other alternatives [8]. The hair conditioners or shampoos are toxic and found to have side effects like irritation of scalp, skin and mucous membrane of the eyes, dryness of scalp and hairs, discoloration, loss of hairs and variation in the individual response due to natural differences and chemicals used in them [9].

Medicinal plants due to their health benefit properties, areused as primary health care aid in the form of plant extracts or their active components [10].

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A remarkable increase in the use of medicinal plant products have been observed in the recent past decade. Today, herbs still found in 40% ofprescriptions, and the interest for use of herbal remediesinstead of chemical drugs is increasing because of lessside effects [11]. The importance of drugs of plant, animal and mineral origin for the treatment of various types of diseases has been well described in Ayurveda, the ancient Indian medical science.

The revered Indian cow, known as "Kamdhenu", in Indian Scripts, is believed to be a "Mobile Hospital", for most of the diseases. "Gou-Mutra", hereinafter referred as cow urine, has been used as a medicine in India, since ancient time. In Rigveda, cow urine has been compared with the nectar. Cow urine has clinical effects like appetizer, tranquilizer, anti-spasmodic and useful in swelling, inflammation, jaundice, anemia, leucoderma, dermatitis, itching and leprosy etc [11,12]. The U.S. patents granted for the "Gou-Mutra Ark"(cow urine distillate) to Council of Scientific and Industrial Research (CSIR) on "pharmaceutical preparation cow urine distillate" and "use of bio-active fraction from cow urine distillate as a bioenhancer of anti-infective, anti-cancer agents and nutrients" were the major breakthrough for the research on various medicinal properties of cow urine [13,14,15].

In this investigation an attempt has been made to develop an effective, economic and stable anti-dandruff hair conditioner by value addition of natural resources like herbs and cow urine, without using all traditionally incorporated synthetic active ingredients or preservative. The formulation was evaluated for its physicochemical properties and compared with marketed products andits efficacy against pathogen *Candida albicans*have been evaluated and discussed.

Materials and Methods

The herbal materials purchased from local market were identified and authenticated by Dr. S.K.Padoley, Ex. Head,Deptt. of Botany, Porwal College of Science, Kamptee. The fruits were dried under shade for 10–15 days. The seeds were removed from the fruits, powdered and passed through sieve (mesh no. 60) to get fine powder. Cow urine was collected from healthy indigenous cows (*Gir*), kept under veterinary supervision at "Sewadham", Go-Vigyan Anusandhan Kendra, Dewalapar, Nagpur. It was ensured that urine sample collected was the first urine micturated early morning once the cattle awakened. All the chemicals and reagents used from Hi-Media (India) Ltd. and Merck India Ltd.

Formulation of Hair Conditioner

Composition of the developedproduct (DP) is summarized in Table 1. The coarse powder of Sapindustrifoliatus (*Reetha*), Acacia concinna (*Shikekai*) and mixture of *Emblicaofficinalis*, *Terminaliachebula* (retz.) and *Terminaliabelerica* (roxb.) in equal proportion i.e. *Trifala* powder (mixture of *Amla, Harad and Baheda*)were soaked in Cow urine for twelve hours, then concentrated to half of its volume by gentle heating. The concentrated liquid was filtered with nano-bolt cloth and cooled. Powder of camphor and thymol mixed to get liquid in a beaker and added to concentrated liquid to obtain the product as hair conditioner.

Evaluation of Formulation

The developed product (DP) and three samples of the products available in the market (MS1, MS2, MS3) were compared for following physico-chemical tests.

Organoleptic Evaluation

Various parameters such as physical appearance, color, odour and transparency by sensory organs of the developed product have been evaluated and discussed [16,17].

Determination of pH

A sample with 10% concentration was prepared by diluting the developed productusing distilled water. pHwas recorded using digital pH meter at room temperature [17].

Solid Contents

A clean dry China dish was weighedand 4 grams of developed product (DP) taken in the dish, placed on the water-bath untilthe liquid portion evaporated,then placed in oven at 110^0 C and weight of the dry content was calculated [17].

Dirt Dispersion

Two drops of developed product added in a large test tubecontaining 10 ml of distilled water. A drop of India ink wasadded; the test tube was stoppered and shacked ten times. The amount of ink in the foam was estimated as None, Light, Moderate, or Heavy.

Detergency Ability

Thompson method[18] was used to evaluate the

detergency ability of the samples. Briefly, a crumple of hair were prewashed with a 5% sodium lauryl sulphate solution**,** then dried divided into 3g weight groups. The samples (3g) were suspended in 20mln-hexane solution containing 10% artificial sebum and mixture was placed on shaker for 15 minutes at room temperature. Thethe solvent was evaporated at room temperature and weighed for determination of their sebum content. In the next step, each sample divided into 2 equal parts of 1.5 g each, one washed with 0.1 ml of the 10% test sample (DP) [18], the other sample was considered as the internal control and left untreated. After drying at 60^{0}C for 4 hours, the sebum remained in treated and untreated samples, were then extracted using 20ml hexane for 30 minutes on a rotary shaker. The sebum extracted from both the samples was weighed after complete evaporation of hexane solution. Detergency was evaluated as a percentage of sebum removed after treating with formulations using equation; Detergency = 100-(T x 100 / C) where T is the weight of sebum in treated sample and C is the weight of sebum in control sample.

Rinsing

The time taken to remove the detergent was performed by applying 5 ml of the shampoo and time taken for complete removal of frothing from wash water was determined.

Combing (Wet)

Ease of combing was performed by passing a comb through the wet hair and checking whether the comb glides smoothly.

Combing (Dry)

Ease of combing was performed by passing a comb through the dry hair and checking whether the comb glides smoothly.

Luster of Hair

The luster of hair was tested by checking the shine and smoothness of hair after drying.

Foaming Ability and Stability

Cylinder shake method with slight modification used for determining foaming ability. 100ml of the 10% shampoo solution taken into a 250 ml graduated measuring cylinder shaken for 10 times. The total volume of the foam contents after each 1 minute was recorded up to 5 minutes and foam volume generated was recorded after 10 minutes.

Anti-dandruff Activity (In-vitro)

Candida albicans were employed for testing antifungal activity using well diffusion method. Sabouraud's agar medium prepared, autoclaved and 20ml of it was dispensed into sterilized petriplates. After solidification of the agar, 0.2 mL of 72 hour old suspension of *Candida albicans*poured in the centre of the sterilized petriplates using a micropipette and spread evenly on the agar using a sterilized glass spreader. Four wells (10 mm diameter) were made in to the agar at four corners of the petriplates taking care that the wells did not lie in close proximity to the edges of the petriplates or to each other. Well no. 1,2,3, and 4 werefilled with 0.05ml (100µL) of a solution of the 1g, 2g, 3g and 4g sample dissolved in 10 mL DMSO respectively. In other plate, four similar wells (10 mm diameter) were madeand filled with 0.05ml (100µL) of a solution of the 0.1g, 0.2 g, sample dissolved in 10 mL DMSO, pure sample and DMSO as controlrespectively.The plates were kept for diffusion at 4^{0}C for one hour and incubated at 30^{0}C for 48 hours. After incubation, the zone of inhibition measured in mm and compared [19].

Accelerated Stability

Accelerated stability of the developed product was evaluated at the 40±2^{0}C and 75±5% relative humidity for 90 days [20,21].

Results and Discussion

The knowledge about medicinal plants, their use seems to be well known to people of India. The recent interest of consumers in the herbal cosmetics has been stimulated due to decline in faith of modern synthetic cosmetics and belief that plant remedies are natural and thereby superior to synthetic cosmetics. These reasons have contributed to increased acceptance as well as manufacture of herbal cosmetics [22]. The ingredients used in this formulation were found rich source of novel drugs. The various quality control parameters were checked along with antifungal activity. The results obtained were encouraging and much better than the marketed products.

Organoleptic Evaluation

The developed formulation and marketed samples

were found to be semi liquid in nature with characteristic odour and various colours. The DP was not transparent whereas all the marketed samples were transparent (Table 2).

pH: The pH of the DP was 5.25 whereas that of marketed samples was in the range of 6-7.5. Though the pH of DP was lower than marketed samples, no harmful effect on scalp and hair was observed (Table 3).

Percent Solid Content

The percent solid content of marketed samples wasin the range of 15 to 17.5 but that of developed productwas 56.84 percent (Table 3).

Dirt Dispersion

The dirt should stay in water and should not concentrate in the foam, otherwise it is considered as of poor quality. The dirt stayed in the foam redeposits on the hair and is difficult to rinse. The developed formulation and all the marketed samples showed similar results [23,24] (Table 3).

Detergency

All the samples showed ability between 70 to 72.6 with highest detergency abilityrecorded for developed product andhence may be referred as active cleanser (Table 3).

Rinsing

In the case of ease of rinsing DP was rinsed out quickly, when compare to other marketed formulations(Table 3).

Combing (Wet and Dry)

In case of combing, all samples showed combing with friction in wet condition however at dry condition DP showed better results(Table 3).

Luster of Hair

In maintaining luster of hair, all the formulations showed fairly good luster but DF showed better results(Table 3).

Foaming Ability and Foam Stability

Normally it is considered that forming ability of natural shampoos or hair conditioners is not comparable with its synthetic counter parts. However the present investigation proves that natural formulation having combination of natural surfactants such as *Reetha* and *Shikakai* in optimized concentration can generate sufficient foam ability of shampoo. However, foam does not have much to do with the cleaning process. But it is of paramount importance to the consumer and is therefore an important criterion in evaluation of shampoos.[17]The DF showed better foaming ability as compared to marketed samples and unlike those of marketed samples, its foam remains as it is even after 10 minutes, which proves natural surfactants Reetha and Shikakai may be the best replacement for the harsh synthetic detergents which are commonly used in majority of shampoos (Table 4).

Table 1: Composition of Product

S. No.	Common Name of Ingredients	Botanical name/English name	Quantity
1.	Cow urine	----	5 L.
2.	Reetha	*Sapindustrifoliatus*	1.2 Kg.
3.	Shikekai	*Acacia concinna*	400g
4.	TrifalaChurna (Mixture of Amla, Harad and Baheda)	Mixture of *Embilicaofficinalis, Terminaliachebula (retz.)* and *Terminaliabelerica* (roxb.)	125 g (41.66g dried powder of each three)
5.	Kapoor	Camphor	40g
6.	Ajawain Sat	Thymol	25g

Table 2: Organoleptic Evaluation

S. No.	Specifications	DF	MS 1	MS 2	MS 3
1.	Physical appearance	Semi liquid	Semi liquid	Semi liquid	Semi liquid
2.	Colour	Brownish	Green	Brown	Whitish
3.	Odour	Characteristic	Characteristic	Characteristic	Characteristic
4.	Transparency	Not transparent	Transparent	Transparent	Transparent

Table 3: Physico-chemical and Generalobservations of DP and other marketed products

S. No.	Test	DF	MS 1	MS 2	MS 3
1	pH	5.44	6.1	6.02	7.28
2	Solid content (%)	56.84	16.04	15.72	17.24
	Dirt dispersion	None	None	None	None
3	Detergency ability	72.6	72.2	70.5	70.2
4	Cleaning action	96.94	89.37	91.58	90.15
5	Ease of rinsing	***	**	**	**
6	Ease of combing (wet)	**	**	**	**
7.	Ease of combing (dry)	**	*	*	*
8	Luster of Hair	***	**	**	**

* Good, ** Better, *** Best

Table 4: Foam formation and foaming stability

Time (minutes)	Foam volume (ml)			
	DF	MS 1	MS 2	MS 3
1	248	247	245	247
2	246	245	243	245
3	244	242	241	242
4	242	240	238	240
5	242	240	236	240
10	150	120	125	128

Table 5: Antimicrobial activity of DP against Candida albicans

	Concentration wise zone of inhibition (in mm)						
Conc.	1%	2%	10%	20%	30%	40%	Pure sample
Zone	Nil	13	18	20	23	25	31

Table 6: Accelerated Stability Testing

Test	Initial month	After 1 month	After 2 months	After 3 months
Physical appearance	Semi liquid	Semi liquid	Semi liquid	Semi liquid
Colour	Brownish	Brownish	Brownish	Brownish
Odour	Characteristic	Characteristic	Characteristic	Characteristic
Transparency	Not transparent	Not transparent	Not transparent	Not transparent
pH	5.44	5.45	5.46	5.46
Percent solid content	56.84	56.84	56.84	56.84
Dirt dispersion	None	None	None	None
Detergency ability	72.2	72.00	71.21	71.20
Foam volume	242	240	240	240
Thermal Stability	OK	OK	OK	OK
Degradation of product	Nil	Nil	Nil	Nil
Microbial count (cfu/g)	$0x10^20x10^2$	$0x10^20x10^2$	$0x10^20x10^2$	$0x10^20x10^2$

1. 10%, 2.20%, 3. 30% and 4.40% concentration

1.1%, 2.2% concentration, 3. Pure sample and 4. DMSO solvent

Fig. 1: Zone of inhibition (mm) of DP against one of the test isolates.

In-Vitro Anti-Dandruff Activity

Dandruff has worldwide occurrence. The dermatophilic fungi on the scalp utilizes the secretions of the sebaceous glands for its growth and causes infections like dandruff and associated secondary infections. Anti-dandruff shampoos based on chemicals are being used for its treatment but due to their side effects or recurrence problem, the people are attracted towards alternatives based on natural resources. Medicinal plants are increasingly of interest as antimicrobial agents and have been widely used in traditional medicines [25].

The antifungal activity of developed formulation showed positive resultsagainst the tested fungal pathogen *Candida albicans*, in all concentration except at 1% which may be considered as MIC, as 2% concentration showed zone of inhibition 13mm. Increase in concentration showed increase in zone of inhibition and the pure sample of DP showed maximum zone of inhibition 31 mm (Table 5). The higher antifungal activity of DP may be because of the synergistic activity of all herbal ingredients along with cow urine's enhancing properties.

Accelerated Stability Study

Accelerated stability testing of the developed formulation conducted at 40±2^{0}C temperature and 75±5% relative humidity for 90 days (Table 6).

Conclusion

In the present study, a new formulation of cow urine based polyherbal hair conditioner was developed and compared with three marketed products for various physico-chemical properties. The results obtained were encouraging and much better than the marketed products.The antifungal activity of developed formulation showed positive results against the tested fungal pathogen *Candida albicans*, in all concentration except at 1%. The main objective of this study was development of effective, economic and stable antidandruff formulation withoutusing any kind of synthetic additives, which are normally incorporated in such formulations. The technology of this value added product is ready for dissemination for entrepreneurship development and employment generation as well as societal benefit in the form of good health.

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Original Article

Vigna mungo Seed Non-Destructive Based Colloidal Silver Nanoparticle Synthesis and Toxicity

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Abstract

Silver nanoparticle synthesis needs reduction. Seeds of plant are known to exude reducing agent during imbibition. Combining these two ideas we synthesize novel red coloured colloidal silver nanoparticle without destroying the *Vigna mungo* seeds. Rather than going with the general idea that green method will produce less toxic nanoparticle, we evaluate the toxicity in germinating seeds and RAW cell line along with antimicrobial and chemotaxis test. The result suggests that silver nanoparticles are antibacterial positive without chemotactic property. The cytotoxicity against RAW 246.7 murine macrophage cell linewas found high. Phytotoxicity was also found potent in case of *Vigna mungo* (dicot) and *Oryza sativa* (monocot) seed with respect to inhibition of germination.

Keywords: Silver Nanoparticle; *Vigna Mungo*; Toxicity; Antibacterial; Chemotaxis.

Introduction

Why SNP?

Silver nanoparticle (SNP) synthesis, application and other related studies provide 43,300 different scientific papers in 'Google Scholar' search during last five years (2010-14). About half of these (21,600) are related to key word 'green silver nanoparticle'. The immense thrust on 'green' might be due the immense probability of novelty. Researcher try to categorise the CSNP synthesis with respect to the green precursor used [1]. Every plant species extract is unique to its composition though the major chemical compounds have similarity with other species. CSNP synthesis is a highly sensitive process and show great variation with slight change in reduction medium (including presence of minor chemicals).

Exploring the same opportunity during our literature survey we found many different plant enzymes / proteins, acids, polysaccharides, pectin and vitamins were exploited for the synthesis of colloidal silver nanoparticle [1-3]. Many of the methods used food items during synthesis. The logic behind might be edible will be less toxic compared to non edible. Industrialization of these CSNP synthesis methods might have impact on respective food prices. There are several reports of green silver nanoparticle synthesis from *Lantana camara*, *Plumeria alba*, *Nyctanthes arbor-tristis*, *Piper longum*, Sunflower like plant sources [4-8]. The literatures on application of food industry by-product like'seed exudates' for CSNP synthesis are scanty.

Black gram (*Vigna mungo*) seeds highly priced and common in Indian meals. The seed has been reported for hypolipidemic, immunostimolatoryetc [9,10]. Very few reports are available on *V. mungo* seeds exudates. Unlike other legume seeds the seed exudates of *V. mungo* do not contain detectable sugar [11]. Like all other legume seeds *V. mungo* seeds during imbibitions secrets flavonoids and nitrogenous metabolites such as alkaloids, terpenoids, peptides and amino acids [12].

Bioactivity of colloidal silver nanoparticle has been

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evaluated by number of researcher with respect to antibacterial,chemotaxisand cytotoxicity [13-16]. Antibacterial property of the green silver nanoparticle has been evaluated by many different researchers[7, 6, 4, 17]. Chemotaxis property of colloidal silver nanoparticle has been reported by Bharali et al., Kirschling,Sosenkova and Egorova, and Tran et al. [15, 18-20]. Silver nanoparticle application for UV-protection has been reported for textile fabrics [21-23]. Colloidal silver nanoparticle effect on Cys-Cysdisulfide bond is reported by López-Tobar et al. [24].

Cytotoxicity of colloidal silver nanoparticle is reported by Yu, Samberg et al. and Hatipoglu et al. [25-27].

Materials and Methods

Chemicals and consumables: *Vigna mungo* seeds were collected from Sonitpur, Assam, India. $AgNO_3$ (A.R.) is obtained from Merck, India. Sodium borohydride is obtained from Fluka Chemicals, USA.

Green Synthesis Method

V. mungo seed Exudates Preparation (VMEX)

Five gram (5.00 g) *V. mungo* seeds were surface sterilized with 70% ethanol in sterile distilled water (v / v) for 5 min and then washed with sterile distilled water to remove traces of ethanol. The seeds were soaked in 100 ml sterile distilled water for 24 h. The seed exudates obtained were filtered using Whattman no. 1 filter paper. The filtrate is further filter sterilized using 0.22 mm nitrocellulose filter paper assembly. The filtrate is stored at $4^{O}C$ for further use and labelled as VMEX.

Protein, Reducing Sugar and Polyphenol Estimation of VMEX

For the experiment 50 g seeds were surface sterilized as mentioned above and soaked in 200 ml sterile distilled water for 24h. Exudates of 5 ml for protein, 5 ml for reducing sugar and 5 ml for polyphenol estimation were collected at 3^{rd}, 6^{th}, 12^{th} and 24^{th} h of imbibitions. The total volume of the exudates is measured during every collection using a sterile measuring cylinder under sterile condition inside laminar air flow cabinet. These actual volumes were used later for calculating the total amount of protein, reducing sugar and polyphenol exude by seed at a particular time. Protein and reducing sugar estimation was performed using Bradford's andanthrone method respectively. Total polyphenol estimation was done using Cordenunsi*et al.*'s method [28]. In short 500 mL extract was mixed with 2.5 ml 0.2 N Folin's reagent and incubated at room temperature for 5 min followed by addition of 2.0 ml saturated Na_2CO_3 (75 g / L) and incubation for 90 min at 30°C. After incubation, absorption was measured at 765 nm using gallic acid as standard.

Silver Nanoparticle Synthesis Using VMEX

50 mL 0.5M $AgNO_3$ solution was mixed with 4850 mL VMEX. The solution is mixed properly and then 100 mL of 0.01M KOH solution was added. Immediately, red colloidal silver nanoparticle solution is observed and labelled as VMSNP. Other negative controls were prepared accordingly and composition is presented in Table 1.

The characterization of the CSNP was performed using UV-Vis wavelength scanning from 200-800 nm, transmission electron microscopy (TEM) JEOL JEM 2100, Japan. Fourier transform infrared spectroscopy (FTIR) of Nicolet Impact 410 spectrometer.

Antibacterial Analysis

Antimicrobial analysis was performed following the method described by Bharali et al. (2013) [15]. The test was performed against *Staphylococcus aureus* (MTCC 3160) and *Escherichia coli* (MTCC 40). A total of 6 wells were prepared in Muller Hilton Agar (Himedia, India) and CSNP, negative controls and streptomycin (STR) (50 mg / ml) solution of 100 ml is poured into respective wells. After antimicrobial activity evaluation VMSNP2 is excluded from further study as it has been confirmed that it is only a bigger sized slowly synthesized silver nanoparticle. Therefore, VMSNP1 is referred hereafter as VMSNP.

Antibacterial Activity

The Agar Well Diffusion method described by Bharali et al. [7] was used to determine the antibacterial assay. The media used for the assay was Mueller Hinton Agar (Himedia). A gram positive strain, *Bacillus subtilis* (MTCC 121) and a Gram negative bacteria, *Escherichia coli* (MTCC 40) were used for the antibacterial assay. The bacterial culture was adjusted to the McFarland standard No. 0.5 before the tests. Streptomycin (STR) (50 mg / ml, Sigma) was taken as the positive control. The test was performed in triplicates.

Chemotaxis Assay

The assay for chemotaxiswas performed following

the method described by Bharali et al. (2013) [15]. The bacteria used for analysis were *Staphylococcus aeurius* (MTCC 3160) and *Klepsiala pneunomonia* (MTCC 618).

- Cytotoxicity assay was performed on RAW 246.7 (murine macrophage) cell line and *Vigna radiata* Linn. and *Oryza sativa* (*variety Ranjit*) seeds.

Mouse macrophage cells were plated in 96 well plates ($1x10^4$ cells/well) and kept for 24 h using DMEM media supplemented with 10% FBS (fetal bovine serum) 200 U/mL penicillin, 100 μg/mL streptomycin, 0.3 g/mL L-glutamine and 2 mM $NaHCO^3$ in 5% CO_2 atmosphere at 37 °C so that cells can reach confluency. The cells were then treated with distilled water (control), VMSNP and $AgNO_3$(0.001M) solutions of volume 1, 5 and 10 mL and then incubated for 24h. After incubation 20 mL of 3(4, 5dimethyl-2-thiazolyl)2,5-diphenyl-2H-tetrazoliumbromide (MTT) is poured to each well and incubated. After incubation for 4 h the MTT solution was discarded without disturbing the formed formazone crystal and 100 mL of MTT solvent was added to each well to dissolve the complex. The optical density of the solution was measured at 580 nm using Multiskan Go equipment (Thermo Scientific, India).

The toxicity on plant cells were evaluated using germination process. In short, 2.50 g (dry weight) of *Vigna radiata* Linn.and *Oryza sativa* (*variety Ranjit*) seeds were surface sterilized and then soaked in 50 mL volume of distilled water, $AgNO_3$, PC, VMSNP1 and DIEX for 24h. After imbibitions the seeds were washed thoroughly under sterile condition and allowed to germinate in germination chamber under ambient temperature and light/dark phase using plant tissue culture facility. Number of seeds germinated was recorded after 7 days.

Results

Biochemical Characterization of VMEX

The biochemical characterization of VMEX with respect to protein, reducing sugar and polyphenol is presented in Figure 1. Reducing sugar content is found to be the highest followed by polyphenol and protein. With respect to soaking time period leaching of all content is found to be increasing.

Synthesis and Characterization of the Nanoparticle

VMSNP1 start turning into a red colour solution from a colourless one, after addition of 100 mL KOH. The red colour becomes deeper with respect to time. Figure 2 shows the UV-Vis absorption spectroscopic analysis of the VMSNP1 nanoparticle along with other negative and positive controls. In case of negative control VMSNP2 (without KOH) it has been seen that nanoparticle formation takes more time compared to VMSNP1 (with KOH). Nanoparticle formation is confirm from the absorption between 400-500 nm in PC, VMSNP1 and VMSNP2, which is not visualised in other negative controls including *V. mungo* exudates.

For further confirmation and to evaluate the nanoparticle shape and size TEM and FTIR analysis were performed and presented in Figure 3. As seen in the Figure 3 (a, b and c) the nanoparticle size of VMSNP2 is bigger compared to PC and VMSNP1. The fact is correspond with broad absorption spectra of VMSNP2 compared to VMSNP1 as presented in Figure 2(c).

Antibacterial and Chemotaxis Activity

The result obtained for antibacterial and chemotaxis property of the VMSNP1 is presented in Figure 4. Except VMEX all other samples are found to be antibacterial including VMSNP1 (Figure 4, a and b). *Staphylococcus aeurius* is found to be chemotactic and VMSNP is not a good chemo-attractant compared to glucose and PC. *Klepsiala pneunomonia* is found to have poor chemotactic property and slight movement towards glucose and PC has been detected.

Cytotoxicity Assay

Figure 4 (a) viability of the mouse macrophage cells with respect to treatment with different samples, represented in the form of optical density and figure 4 (b) shows the number of seeds germinated with respect to different treatment.

In case of mouse macrophage cell line, the toxicity corresponds directly to lower optical density as observed for $AgNO_3$ and VMSNP. VMSNP toxicity is found to be equivalent to $AgNO_3$, whereas VMEX does not show any toxic effect for the applied concentration as the absorbance of the solution was found to be slightly more than the controls treated with sterile distilled water (Figure 5, a).

Figure 5 (b) shows number of seeds germinated under different sample treatment. Total in the figure suggest the total number of seeds from each species. Numbers represented against all other sample represent the number of seeds germinated after soaking in the sample solution for 24h. Silver nitrate solution was found to be highly toxic as it is already known to inhibit germination by increasing

sensitivity to abscisic acid during germination through its inhibitory effects on ethylene activity [29]. VMEX have distilled water like activity. VMSNP was found to be highly toxic and reduce the number of germinating seed to more than five and three folds in case of *V. mungo* and *O. sativa* seeds respectively. The effect on *O. sativa* might be little less because of the hard seed coat compared to *V. mungo*. Germinating seeds in PC treatment was found to slightly more which is reported by many researchers as toxic for germination. As there are excess of $NaBH_4$ is present in PC (10 ml 0.001M $AgNO_3$+30 ml 0.002M $NaBH_4$) therefore, same concentration $NaBH_4$ is also evaluated for the germination assay. $NaBH_4$ is found to be non toxic rather it is a germinations stimulator at least in case of *V. mungo*.

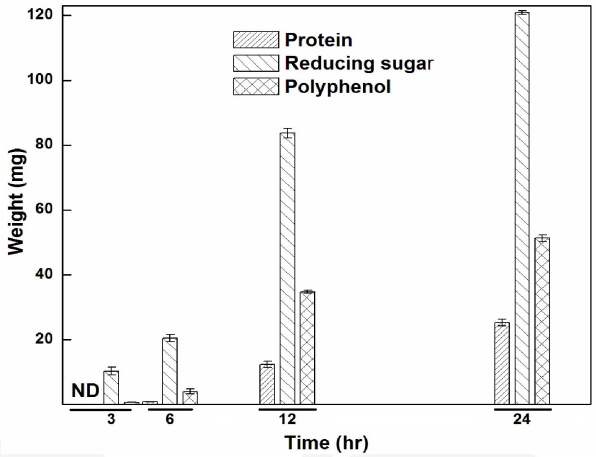

Fig. 1: Total protein, reducing sugar and polyphenol leached out of 50 gm*Vigna mungo*(dw) seed at different time intervals. ND= not detected.

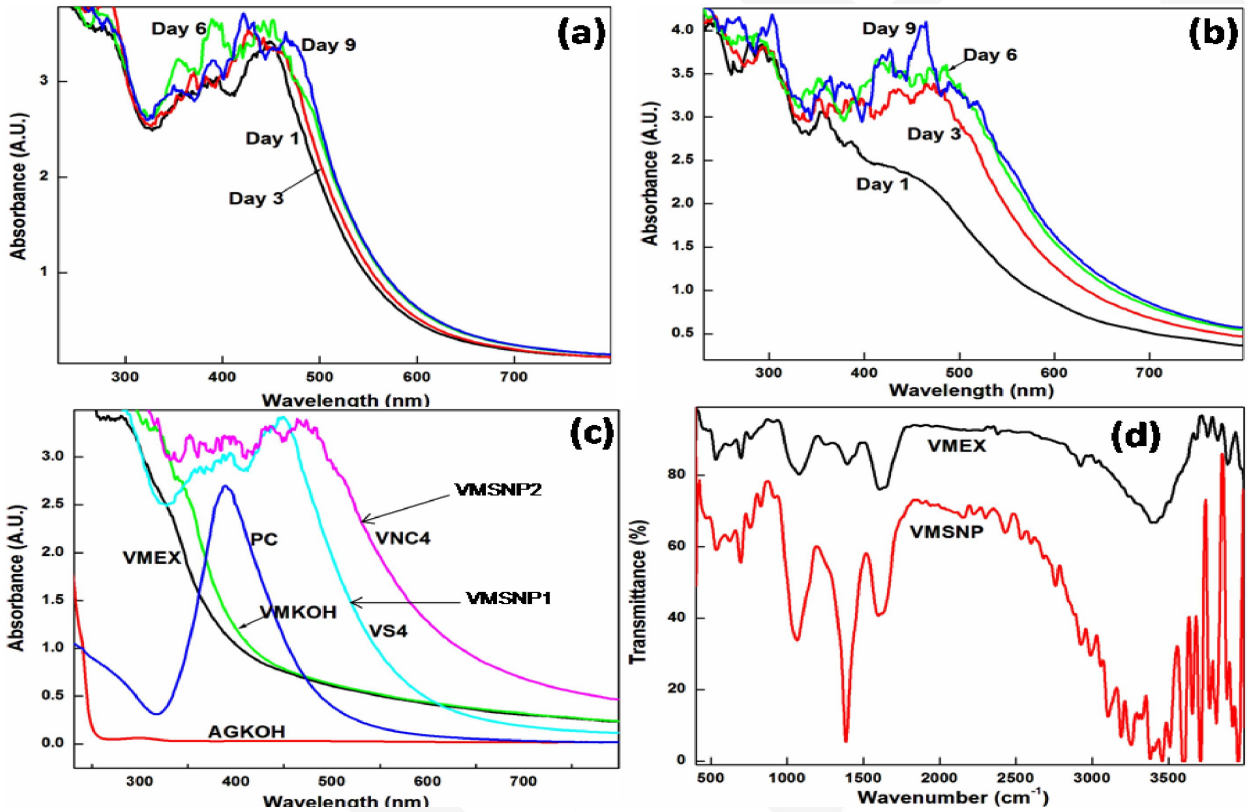

Fig. 2: UV-Vis absorption spectroscopy of *Vigna mungo* based silver nanoparticle. Day wise absorption spectra of VMSNP1 (a), VMSNP2 (b) and comparative absorption spectra of different samples on day 3 (c).FTIR spectra of VMEX and VMSNP1 (d).

Table: 1: Composition of VMSNP and negative controls

Name of the sample	$AgNO_3$ (0.5M) ml	VMEX ml	KOH (0.01) ml	Distilled water ml
VMSNP1	50	4850	100	0
VMSNP2	50	4850	0	100
VMEX	0	4850	0	150
AGKOH	50	0	100	4850
VMKOH	0	4850	100	50

NB: PC was prepared using 10 ml 0.001M $AgNO_3$ and 30 ml 0.002M $NaBH_4$.

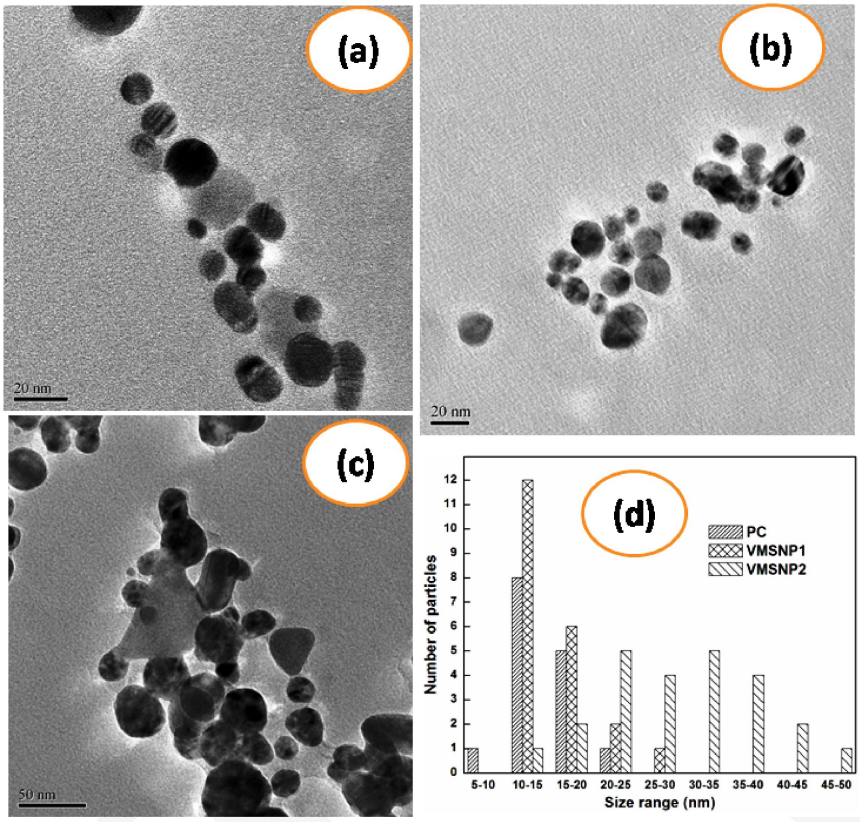

Fig. 3: TEM image of PC (a), VMSNP1 (b) and VMSNP2 (c). Number of particle in different range in presented from the TEM figures (d)

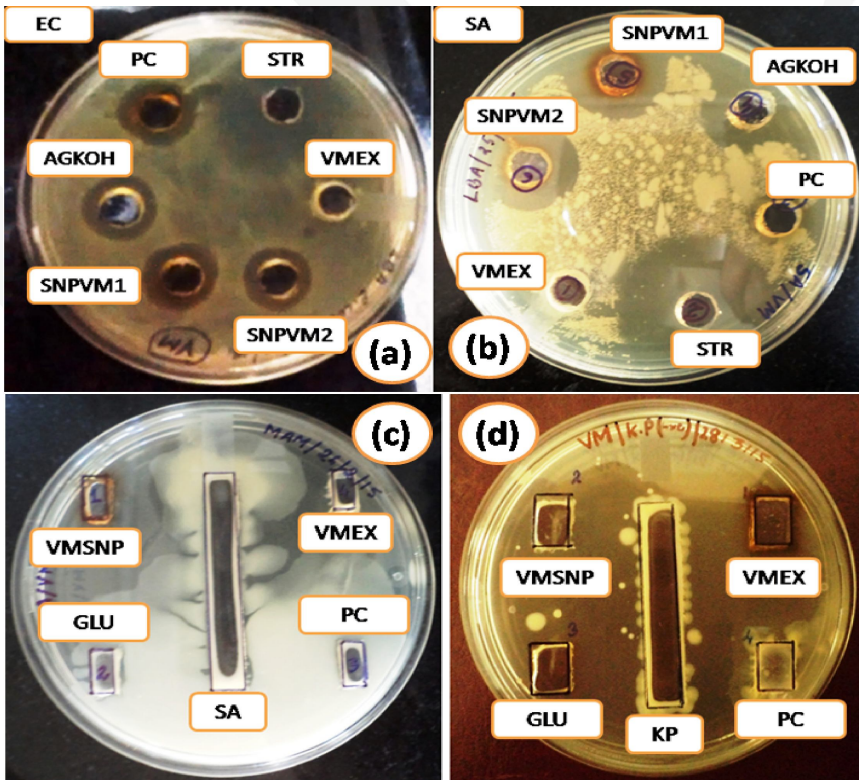

Fig. 4: Antibacterial (a and b) and chemotaxis (c and d) activity of SNPVM1. EC=*Escherichia coli* (MTCC 40), SA=*Staphylococcus aureus*(MTCC 3160), SA=*Staphylococcus aeurius* (MTCC 3160), KP=*Klepsiala pneunomonia* (MTCC 618), GLU=glucose solution, VMSNP=VMSNP1 and STR=streptomycin.

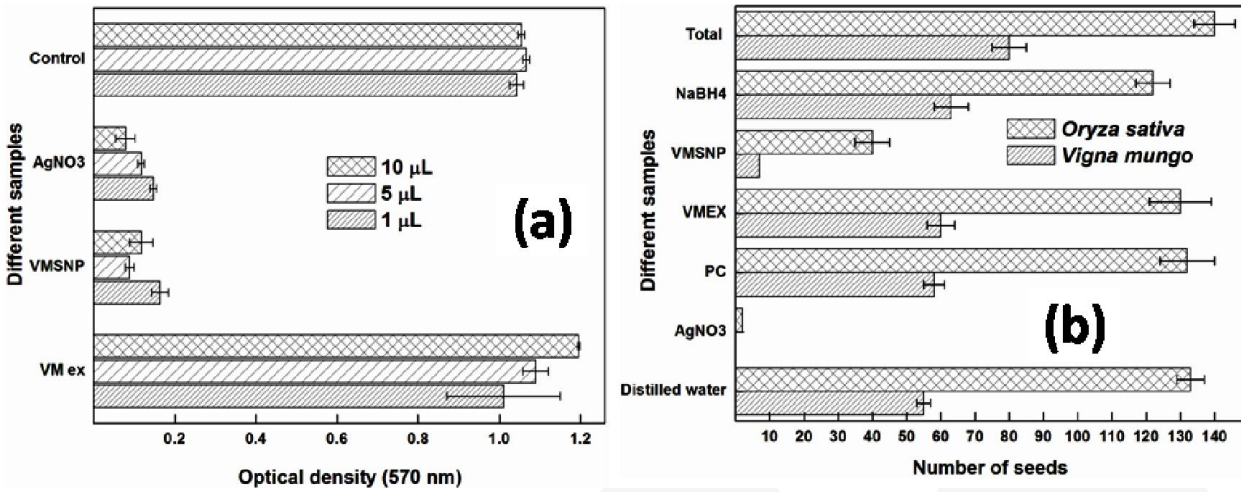

Fig. 5: Toxicity assay of silver nanoparticles in (A) RAW 246.7 (murine macrophage) cell line, (B) seeds of *V. radiata* and *O. sativa*.

Discussion

Ahmed et al. reviewed the green nanoparticle size reported for different plant extract showing 0.5 to 150 nm ranges using different plant extract [30]. In their review they did not referred any article using seed exudates for silver nanoparticle synthesis. As presented in Figure 3 (d) the maximum number of silver nanoparticles of sample PC and VMSNP1 are present the range of 10-15 nm and pattern of distribution of particle size range of PC and VMSNP1 are similar. Maximum numbers of VMSNP2 particles are found in the range of 20-35 nm range. The size distribution of PC and VMSNP1 is also narrow (PC 5-25 nm, VMSNP1 10-30 nm) compared to wide size distribution of VMSNP2 (10-50 nm). This suggest that for narrow size distribution and small sized nanoparticle the reduction method should be fast and in the present case KOH perform the same. Further, it can be observed in Figure 3 (a, b and c) the PC and VMSNP1 particle morphology is spherical and the same for VMSNP2 is irregular and triangular.

Antimicrobial activity of all the samples was found to be equal and no significant difference is found except streptomycin and VMEX [30]. Another noticeable event is that the exudates (VMEX) alone do not have the antimicrobial activity but on addition of $AgNO_3$, antimicrobial activity is incorporated tomicrobesusceptible exudates rich in reducing sugar, protein and polyphenol. From the chemotaxis assay it is clear that KP strain might not have taxis or movement. SA shows good chemotaxis and VMEX was found to be a chemo-attractant for SA but VMSNP is not. This suggests that VMSNP might be a good repellent for SA.

The toxicity assay suggests that he VMSNP is highly toxic to animal cell line like $AgNO_3$. Similar toxicity is found in germination test with two different plant seeds. These results suggest that green nanoparticles should not declare merely nontoxic rather their toxicity should be evaluated from case to case basis.

Conclusion

Silver nanoparticle synthesized using *V. mungo* seed exudates without destroying the seeds is a novel method. Green nanoparticle is found to be highly toxic to animal cell line and plant seeds. Nanoparticles are found to be repelling activity towards *Staphylococcus aeurius* suggesting possible application in bacteria repelling surface fabrication. $NaBH_4$ germination stimulating activity might be submerging the toxicity of PC type silver nanoparticle to some extent. Further investigation is needed to evaluate the mechanism of PC mediated germination stimulation of *V. mungo* seeds. The present approach of using food industry waste like seed exudates might provide more novel nanoparticle and will not harm the medicinal plants and biodiversity on extensive use.

Acknowledgement

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Original Article

Optimization of Nitrogen Source(s) for the Growth and Amylase Production from *Bacillus licheniformis* JAR-26 under Submerged Fermentation

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Abstract

The growth and enzyme production in microorganisms need optimum physical (temperature, pH, and aeration) and chemical (carbon, nitrogen and mineral ions) environment which have critical role in determining growth behaviour and biochemical production in cultured microorganisms. The present study aimed to investigate effect of different concentrations of organic nitrogen sources i.e. peptone, tryptone, soytone, beef extract, malt extract and yeast extract (0.5, 1.0, 1.5, 2.0, 2.5% w/V) and inorganic nitrogen sources i.e. $NaNO_3$, KNO_3, $(NH_4)_2SO_4$, NH_4Cl and $(NH_4)_2HPO_4$ (0.1, 0.25, 0.5, 1.0, 1.5% w/V) on growth and amylase (EC 3.2.1.1, 1, 4-á-D-glucanglucanohydrolase) enzyme production from *Bacillus licheniformis* JAR-26. Among the tested nitrogen sources, organic sources proved superior over inorganic sources and malt extract proved best for amylase production (maximum at 1.5%, 4.427 U/ml of medium) with growth/OD of 1.766 and bacteria could utilize 98.4% of the available sugar in the medium. Yeast extract was second suitable organic source for enzyme production (maximum enzyme production at 2%, 4.314 U/ml) and Growth/OD of 1.650, and bacteria could utilize 97.1% of total sugar in the medium. Soytone proved poorest organic nitrogen source for amylase production and growth of bacteria with maximum enzyme yield of 3.08 Uml^{-1} at 2.0% and growth/OD of 0.95 at 2%. Among the five inorganic nitrogen sources, none was found suitable for amylase production by *B. licheniformis* JAR-26.

Keywords: Amylase; Submerged Fermentation; *Bacillus licheniformis* JAR-26; Nitrogen Source.

Introduction

Amylases are one of the most utilized industrial enzymes for hydrolyzing starch molecules to diverse products like dextrins and progressively smaller polymers composed of glucose and/or maltose and glucose units. A significant increase in amylase production and utilization occurred in the early 1960s when *Bacillus subtilis* α-amylase and *Aspergillus niger* glucoamylase were used for the production of dextrose from starch as alternative to acid hydrolysis. Amylases hold maximum (about 30%) market share of enzyme sales with major industrial applications in starch processing, brewing and sugar production, food and paper production, textile, and detergent manufacturing [1]. Amylases are calcium metalloenzymes, divided into three groups according to their amylolytic specificity i.e. α-amylase; which cleaves the bond in interior of the substrate (endoamylase); β-amylase, which acts on the reducing end of the substrate (exoamylase); and amyloglucosidase, which liberate glucose units from the non-reducing end of substrate molecules [2]. Microbial amylases have completely replaced chemical hydrolysis in starch processing industry [3]. The production of microbial amylases from bacteria depends on the type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements (particularly carbon and nitrogen), incubation period, pH, temperature and metal ions [4]. Applications of bacterial amylases at industrial level have stimulated the interest to explore several microbes as bioresources for their amylolytic activity. The nature and concentration of nitrogen source in

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the culture medium affects bacterial growth and amylase production and acts as pH stabilizer. Many investigators have recorded that organic nitrogen sources support maximum amylase production in several bacteria [5]. Almost all *Bacillus* species synthesize α-amylase, thus this genus holds promise to dominate the enzyme production industries. *Bacillus licheniformis* JAR-26, isolated from spoiled tomatoes, is an acidophilic and thermostable extracellular α-amylase producing acidophilic bacteria reported in a previous study [6].

In the present study, an attempt has been made to optimize growth and amylase production from *Bacillus licheniformis* JAR-26 using different concentrations of various organic (peptone, tryptone, soytone, casamino acid, beef extract, malt extract and yeast extract) and inorganic (KNO_3, $NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl, and $(NH_4)_2HPO_4$) nitrogen sources under submerged fermentation.

Materials and Methods

Microorganism

Starch hydrolyzing *Bacillus licheniformis* JAR-26 was isolated from spoiled tomatoes and collected in sterilized stoppered glass vials.

Media and Chemicals

Starch,Yeast extract, Peptone, $MgSO_4.7H_2O$, KH_2PO_4, NaCl, $CaCl_2$, Agar, Distilled H_2O, Phosphate buffer, Iodine solution, 3, 5 dinitrosalicylic acid (DNS), $NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl, KNO_3, $(NH_4)_2HPO_4$, Malt extract, Beef extract, Tryptone, Soytone, Sephadex G-100, DEAE-Cellulose (DE-52), CM-Cellulose, Acrylamide, Bis-acrylamide, N,N,N'n'-tetramethylethane-1,2-diamine (TEMED), Sodium dodecyl sulphate (SDS) Ammonium persulphate.

Isolation of Microorganism

The thermostable, acidophilic starch hydrolyzing bacteria (*B. licheniformis* JAR 26) was screened for extracellular acidophilic amylase production by using starch medium containing (g/L): Starch (Merck, Germany), 10.0; yeast extract, 5.0; peptone, 2.0; $MgSO_4.7H_2O$,0.5; KH_2PO4, 0.5; NaCl, 1.5; $CaCl_2$, 0.1; Agar, 20.0. Initial pH was adjusted to 5.5. One gram of each sample was suspended in 9.0 ml of sterile water and 0.1 ml of suitably diluted suspension was spread on the agar plates. The plates were incubated at 45, 50, 55 and 60°C for 24 to 48 h. The isolated colonies were flooded with iodine solution and colonies bearing good colorless halos around them were picked and maintained on starch agar slants at 4 °C and further assessed for enzyme production in liquid medium. The characterization and identification of the isolate was made following Bergey's Manual of Systemic Bacteriology. The method of identification used was as given by Collee *et al*. [7].

Amylase Production

The basal fermentation medium for enzyme production contained(g/L): Starch, 10.0; Maltose, 20.0; yeast extract, 5.0; peptone, 5.0; KH_2PO4, 0.12; $CaC1_2.2H_20$, 0.12; $MgSO_4.7H_20$, 0.12; $MnSO_4.4H_20$, 0.02. Initial pH of the medium was adjusted at 5.5 and 50 ml of medium in 250 ml of Erlenmeyer flasks were inoculated with a cell suspension of optical density (OD) 0.5 (prepared from 24 h old culture). All the flasks were incubated for four days on a rotary shaker (Remi) at 170 rpm at 45°C. Samples were drawn after a time interval of 12 h, centrifuged at 8000 Xg for 10 minutes and cell free culture supernatant was used as enzyme source.

Assay of Enzyme

Culture filtrate (Supernatant) was used for assessing enzymatic activity by the method of Srivastava and Baruah [4]. One ml of 1% (w/V) starch (Merck, Germany) solution was taken in test tube and 0.2 ml of 0.2 M phosphate buffer (pH 5.5) and 0.2 ml of deionized water was added to it. The mixture was equilibrated at 70°C for 10 minutes in a water bath. 0.1 ml of supernatant was added and then reaction was stopped by adding 1 ml of 3,5-dinitrosalicylic acid (DNS). The mixture was heated and the color intensity was measured at 540 nm [8] using a spectrophotometer (Systronics Spectrophotometer 169). One unit of amylase activity was defined as the amount of amylase that liberates 1.0 mg of glucose per minute under assay conditions. In all the above experiments the enzyme activity was calculated as the average of 3 independent sets of experiments (the s.d. in all cases was found negligible).

Effect of Nitrogen Source

The nitrogen content [yeast extract (10 g/L) + peptone (2 g/L)] of basal fermentation medium was replaced with different concentrations of organic nitrogen sources i.e. Yeast extract (YE), Peptone, Malt extract (ME), Beef extract, Tryptone, Soytone (0.5, 1.0, 1.5, 2.0, 2.5% w/V) and inorganic nitrogen sources i.e. $NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl, KNO_3, $(NH_4)_2HPO_4$

(0.1, 0.25, 0.5, 1.0, 1.5% w/V) and its effect was recorded on bacterial growth, amylase production and sugar utilization by test bacteria.

Results

The data on growth, amylase production and sugar utilization by *B. licheniformis* JAR-26 at different concentrations of organic and inorganic nitrogen supplements are shown in Fig. 1 to 6.

Among organic nitrogen sources, malt extract proved best for amylase production (maximum at 1.5%, 4.427 U/ml of medium) where growth/OD was 1.766 and bacteria could utilize 98.4% of the available sugar in the medium. Yeast extract was second suitable organic source for enzyme production (maximum enzyme production at 2%, 4.314 U/ml) with Growth/OD of 1.650, and bacteria could utilize 97.1% of total sugar in the medium. Beef extract, peptone and tryptone were moderately suitable nitrogen sources for amylase synthesis with maximum amylase yield of 4.025, 3.372 and 3.683 Uml^{-1}, respectively, at 2.0% Beef extract, 1.5% peptone and 2.0% tryptone. Soytone proved poorest organic nitrogen source for amylase production and growth of bacteria with maximum enzyme yield of 3.08 Uml^{-1} at 2.0% and growth/OD of 0.95 at 2%. Comparison of various treatment combinations revealed maximum biomass production of *B. licheniformis* JAR-26 on 2% Beef extract whereas maximum amylase production on 1.5% of malt extract under submerged fermentation.

Among the five inorganic nitrogen sources i.e. $NaNO_3$, KNO_3, $(NH_4)_2SO_4$ NH_4Cl and $(NH_4)_2HPO_4$ (0.1, 0.25, 0.5, 1.0 and 1.5% w/V), none was found suitable for amylase production by *B. licheniformis*

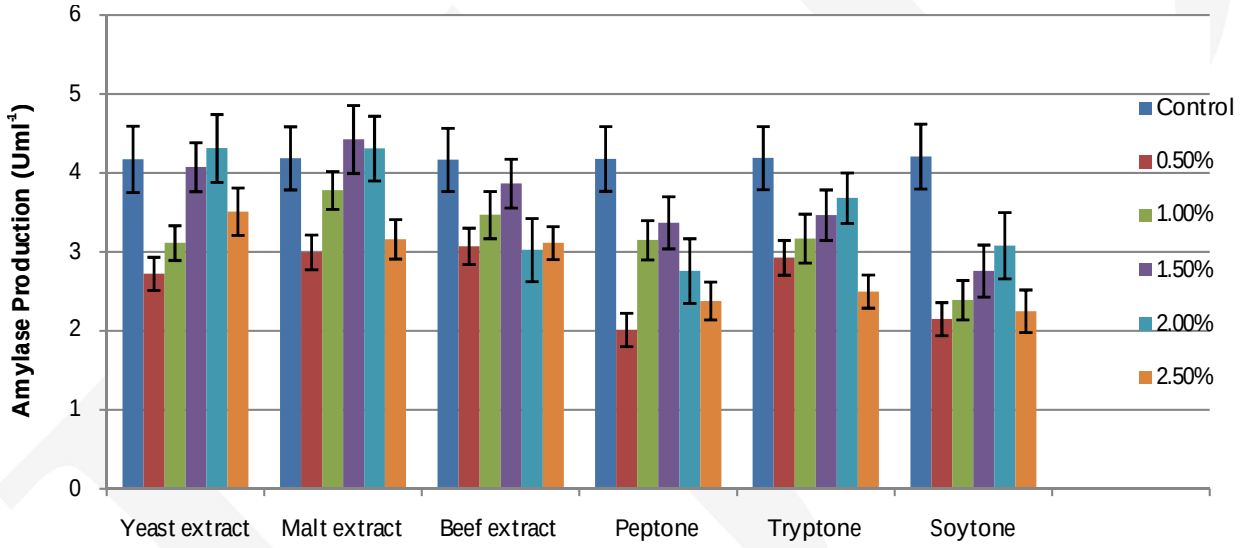

Fig. 1: Effect of different organic nitrogen source(s) on amylase production by *B. licheniformis* JAR-26

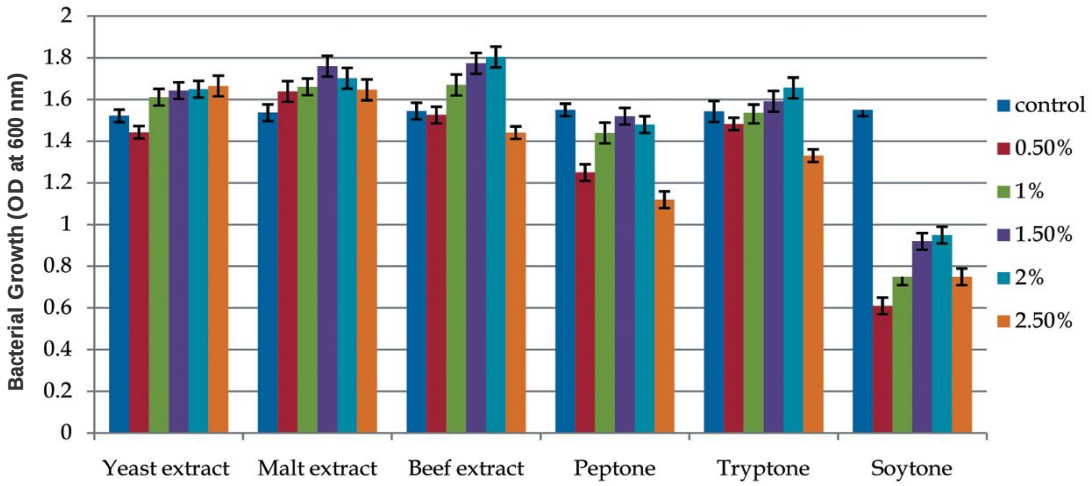

Fig. 2: Effect of different organic nitrogen source(s) on growth of *B. licheniformis* JAR-26

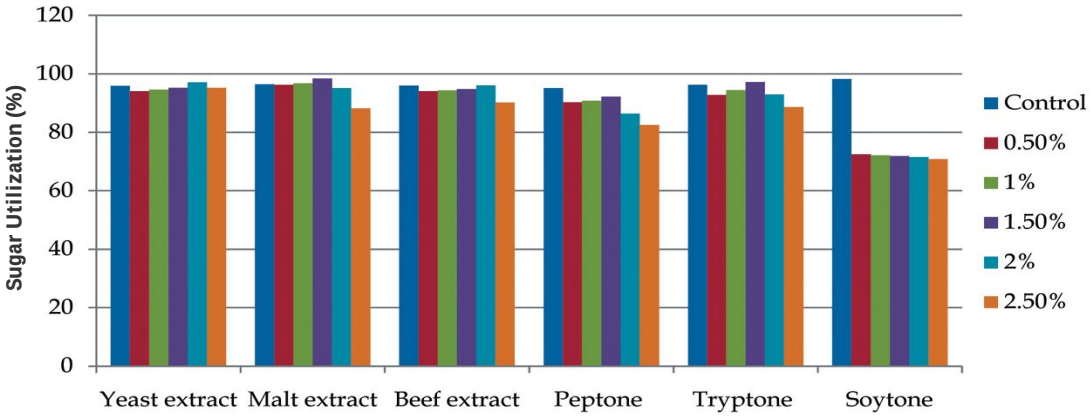

Fig. 3: Effect of different organic nitrogen source(s) on sugar utilization by *B. licheniformis* JAR-26

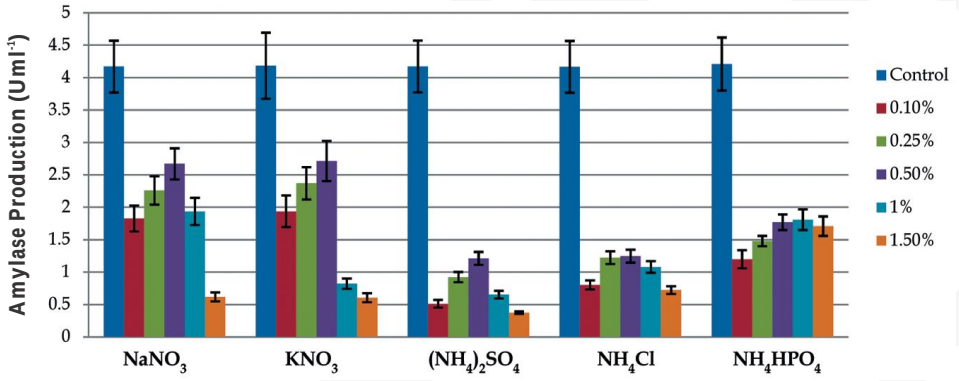

Fig. 4: Effect of different inorganic nitrogen source(s) on amylase production by *B. licheniformis* JAR-26

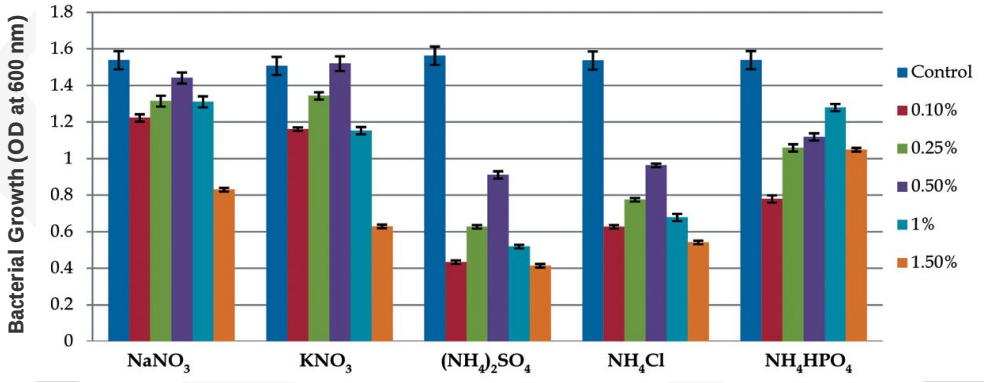

Fig. 5: Effect of different inorganic nitrogen source(s) on growth of *B. licheniformis* JAR-26

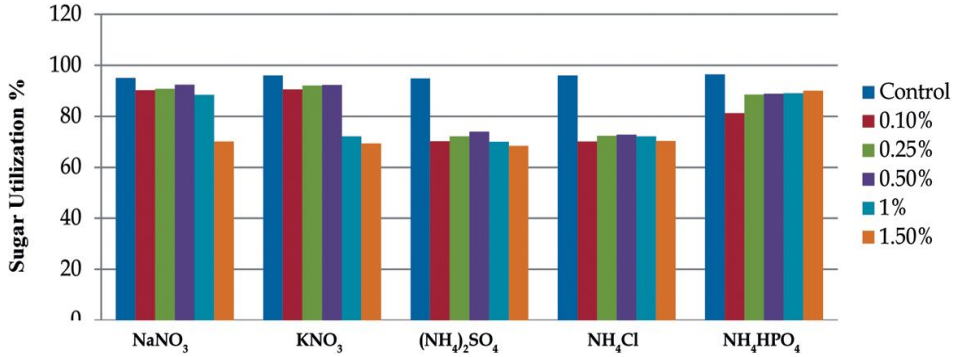

Fig. 6: Effect of different inorganic nitrogen source(s) on sugar utilization by *B. licheniformis* JAR-26

JAR-26. Both $NaNO_3$ and KNO_3 supported good growth of *B. licheniformis* JAR-26 when used as sole nitrogen source in the medium and the growth / OD obtained (1.442 and 1.521 OD at 600 nm, respectively) were more or less similar than those observed in case of control medium (1.539 and 1.521 OD at 600 nm). Among tested inorganic nitrogen sources KNO_3 shows maximum amylase production of 2.714 Uml^{-1} at 0.5% concentration. Inorganic nitrogen sources proved inferior in comparison to organic nitrogen sources with respect to amylase production as well as biomass production from *B. licheniformis* JAR-26.

Discussion

After Carbon, Nitrogen is another major nutrient that is required by the microorganisms in comparatively larger amounts. Nitrogen forms the essential part of proteins, enzymes, nucleotides and cofactors that play vital role in metabolism. The nature and relative concentration of nitrogen source in the medium affected growth and amylase production from *B. licheniformis* JAR- 26. Figures 1 to 6 indicate that the growth of *B. Licheniformis* and amylase production greatly depends upon the nitrogen source in the medium. Negi and Banerjee [9] have opined that not all nitrogen sources would act as enhancers for the production of amylases and the response differs from species to species. Among various organic nitrogen sources (peptone, tryptone, soytone, beef extract, malt extract and yeast extract) tested, malt extract was found to be the best source for amylase production (4.427 Uml^{-1}) as well as growth (1.766 OD at 600 nm) of organism in medium containing 1.5% malt extract. Very few previous studies included malt extract among their organic nitrogen sources tested for optimization of bacterial amylase production. However, similar results have been reported by Demirkan [10] on *Bacillus subtilis* wild type and mutant form (U2-6) and malt extract and tryptone were found best for amylase production from wild type and mutant form, respectively. Yeast extract was proved to be second most amylase producing nitrogen source with highest enzyme yield of 4.314 Uml^{-1} recorded at 2.0% in the present study. Similar results have been reported by Narang and Satyanarayan [11] in case of *Bacillus thermoolevorans* where Yeast extract individually and in combination with other nitrogen sources favored growth as well as amylase production.Yeast extract has also been reported better nitrogen substrate for amylase enzyme production in many previous studies of *Bacillus* species [12, 13]. In this study, beef extract, peptone and tryptone were moderately suitable nitrogen sources for amylase synthesis with maximum amylase yield of 4.025, 3.372 and 3.683 Uml^{-1} at 2.0% Beef extract, 1.5% peptone and 2.0% tryptone, respectively.

In contrast to present findings, various previous studies have reported peptone as a good organic nitrogen source for bacterial amylase enzyme production [14, 15, 16]. In case of *B. subtilis* KC-3, Vijaylakshmi *et al.* [15] proved peptone (24.64 Uml^{-1}) to be the most suitable organic nitrogen substrate followed by tryptone (21.66 Uml^{-1}). Soytone proved poorest organic nitrogen source for amylase production with maximum enzyme yield of 3.08 Uml^{-1} at 2.0%.

Among the five inorganic nitrogen sources tested, none was found suitable for amylase production by *B. licheniformis* JAR-26. Among inorganic nitrogen sources tested, KNO_3 showed maximum amylase production (2.714 Uml^{-1}) at 0.5% concentration and a slight increase in growth rate (1.521 OD at 600 nm) than control (1.508 OD at 600 nm). Avdiiuk and Varbanets [17] reported sodium nitrate (0.2%) as best inorganic nitrogen source for a-amylase production by *Bacillus subtilis* 147. Similarly, Zar *et al.* [18] reported slightly superior production of a-amylase using *Bacillus amyloliquefaciens* IIB-14 using NH_4NO_3.

Conclusion

The present findings reveal that organic nitrogen sources are better far amylase production by *B. licheniformis* than the inorganic sources. Malt extract was most suitable for amylase production at the 1.5% concentration. Yeast extract ranked second suitable source for amylase production with 4.314 Um^{-1} at 2.0%. Beef extract, peptone and tryptone were found to be the moderate and Peptone proved poorest organic nitrogen source for amylase production. Among the five inorganic nitrogen sources none was found suitable for amylase production and enzyme yields in all cases were poor than that of malt extract and yeast extract. However, comparatively good biomass growth was observed at 0.5% KNO_3 in comparison to control (OD-1.521).

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Original Article

Development and Evaluation of Polyherbal Hair Oil Fortified with Milk of Indegenous Cow

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Abstract

Hair, an accessory part of the body is important for overall appeal of the body. Ailments related with hair and scalp is chronic dermatological condition, for which several chemical based products are available in the market but with adverse side effects. In the present study a polyherbal hair oil fortified with milk of indigenous cow was developed, standardized using physico-chemical parameters such as Refractive index, weight per ml, iodine value, acid value, peroxide value, saponification value and HPTLC. Efficacy of Hair Oil was carried out by repeated open application test - (ROAT) on adult human volunteers for four weeks to determine if the Hair oil is efficient in controlling Dandruff and itching of scalp and thus improving overall hair quality in human subjects. Parameters used for efficacy with respect to Hair were dryness, roughness and texture of hair and with that of Scalp were dandruff and itching of scalp. At the end of the fourth week, more than 80% participants got Excellent results for quality of hair and scalp. Based on the results obtained from the Study, it can be concluded that use of polyherbal hair oil fortified with cow milk produces an evident clinical improvement in hair and scalp ailments. The product is suitable for micro industries.

Keywords: Hair; Scalp; Polyherbal; Oil**.**

Introduction

Hair is a sting of dead keratin cells [1]. It is an accessory part of the body derived from ectoderm of the skin. It is important for overall appeal of the body [2]. They also can be termed as a mirror of nutritional imbalance of the body. Ailments related with hair and scalp is chronic dermatological condition, its etiology is not properly understood but its unpredictability of the condition together with its visible nature can result in stressful and psychological inferiority for the patients [3]. Fashion pro Hair strengthening, highlighting, coloring, use of chemical based creams lotions result in hair loss, premature graying, Minoxidil, a common chemical based treatment for hair ailments show side effect after prolong use [4]. Herbal hair care oils are one of the most well recognized hair treatments. Ayurvedic hair oils not only moisturize scalp, reverse dry scalp and dry hair conditions, but provide numerous essential nutrients required to maintain normal functions of sebaceous glands and promote natural hair growth. Hair oils have been traditionally used to treat irritated stressed scalp, reduce effect of aging on hair shape and growth, combat seborrhoea and alopecia. Herbal hair care oils work through nutritional support of natural skin restorative and hair growth processes. Regular continuous application is the best way to achieve significant improvement. In various ailment of hair, preference is being given to herbal hair oils [5]. They not only support hair growth and improve elegance of hair but also prevent hair fall [6]. Hair oil also provides essential moisture to the scalp depicting in beautiful hair [7].

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Many herbs and oils like coconut oil, sesame oil show excellent effect on hair and scalp ailments. The present study aims at formulation, standardization, and efficacy study of polyherbal hair oil based on kshirpak vidhi of its preparation. Use of indigenous cows milk enhances the extraction of the herbs along with minimizing the rancidity of the oils. The use of herbs like *Amla, Hirda, Baheda, Bhrungraj, Jatamansi, Kapoorkachari* as an oil base extracts are found to be very effective. *Emblica officinalis, Gaertn* (Fruit), *Terminalia chebula Retz* (Fruit), *Terminalia bellirica (Gaertn) Roxb* (Fruit) belonging to family (Euphorbiaceae) contains tannins and minerals, providing nutrition to hair (8), Amla is rich in vitamin C, tannins and minerals such as phosphorus, iron and calcium which provides nutrition to hair and also causes darkening of hair (9), *Eclipta alba* Hassk (Whole plant) is well known in Ayueved for hair treatments. *Nardostachys jatamansi* (Dried rhizome), *Hedychium spicatum (Rizome)* are known for their soothing effect and mental peace.

The present study aims to standardize the product according to Ayurvedic Pharmacoepia of India, and study the efficacy of the product.

Materials and Methods

The dried rhizome of *Nardostachys jatamansi* DC., *Hedychium spicatium,* dried whole plant of *Eclipta alba* Hassk, dried fruits of *Emblica officinalis* Gaertn, *Terminalia chebula* Retz, *Terminalia bellirica* (Gaertn) Roxb, fresh fruits of *Citrus lemoni* and dried root and rootstock of Arnebia euchroma (Royle)Johnston var were purchased from local market and authenticated by Dr. S.K. Padoley, Head, Dept. of Botany, Porwal college of Science, Kamptee, R.T.M. Nagpur University, Nagpur. Sesame oil and Coconut oil were purchased from locally from *Ghani.* Milk of indigenous cow (Gir) was provided by *Goshala* of Go-Vigyan Anusandhan Kendra, Dewalapar, Nagpur.

Formulation of Hair Oil

The herbal materials used for the formulation were checked as per the Ayurvedic Pharmacopoeia of India. There are various methods available for the preparation of hair oils direct boiling method, paste method and cloth method (10). All the herbal materials except *Citrus lemoni* were dried in shade, crushed and passed through the sieve number 80. Measured quantity of Sesame oil and Coconut oil were taken in a pan and heated on a low flame. Pieces of *Citrus lemoni* were added to it. Measured quantity of milk of indigenous cow (Gir) was added in pan. Arnebia euchroma (Royle) Johnston var was added to it to get naturally red coloured hair oil. Rounded dough mentioned as *Kulk* in Ayurveda were prepared by adding *minimum* quantity of water in weighed quantity of *Eclipta alba* Hassk and *Triphala* powder prepared by mixing equal quantity of *Emblica officinalis Gaertn, Terminalia chebula Retz.* and *Terminalia bellirica (Gaertn) Roxb.* Dough were added in boiling oil. Completion of heating process was tested by deeping cotton weaks and holding it under the flame for smooth burning. After satisfactorily heating, the oil was filtered through a muslin cloth and allowed to cool. Weighed quantity of Nardostachys *jatamansi* DC. and *Hedychium spicatium* were mixed in a piece of cotton cloth which was tied, dipped in the oil and left for seven days. Finally the oil was filtered to get finished product. Composition of the formulated polyherbal hair oil is summarized in Table 1.

Table 1: Composition of polyherbal hair oil fortified with cow milk

S. No.	Botanical name/English name of ingredients	Hindi names	Quantity
1.	Sesame oil	Til Tail	200 g
2.	Coconut oil	Nariyal tail	1800 g
3.	Arnebia euchroma (Royle)Johnston var (Dried root and rootstock)	Ratanjot	25 g
4.	Nardostachys jatamansi DC. (Dried rhizome)	Jatamansi	25 g
5.	Hedychium spicatum (Rizome)	Kapoorkachari	25 g
6.	*Eclipta alba* Hassk (Whole plant)	Bhrungraj	25g
7.	Mixture of	Trifala	75 g
	1. Emblica officinalis Gaertn (Fruit)	Mixture of 3 herbs in equal	
	2. Terminalia chebula Retz (Fruit)	parts :	
	3. Terminalia bellirica (Gaertn) Roxb (Fruit)	1. Amla	
		2.Haritki	
		3.Baheda	
8.	Citrus lemoni (Fruit)	Nimboo	6
9.	Milk of indigenous cow	Godugdha	2 L

Ingredients of hair oil

Preparation of hair oil

Filtration of hair oil

Standardization of Developed Formulation (DF)

The formulated hair oil was prepared in three batches and tested for following physico-chemical parameters as per methods mentioned in Ayurvedic Pharmacopoeia of India published by Government of India.

Refractive Index

The refractive index was measured at 25° C by placing a drop of DF in the prism of Abbe's refractometer.

Weight /ml

Weight per ml of the oil was measured at 25° C using dry pycnometer.

Saponification Value

The saponification value is the number of mg of potassium hydroxide required to neutralize the fatty acids, resulting from the complete hydrolysis of 1 g of the oil or fat, when determined by the following method:

35 to 40 g of potassium hydroxide was dissolved in 20 ml water, sufficient alcohol was added to make 1,000 ml. and allowed to stand overnight. The clear liquor was poured off. 2 g of the DF was weighed in a tared 250 ml flask, 25 ml of the alcoholic solution of potassium hydroxide was added. The flask was attached to a reflux condenser and boiled on a water-bath for one hour, with frequently rotating the contents of the flask. It was cooled, 1 ml of solution of phenolphthalein was added and the excess of alkali was titrated with 0.5 N hydrochloric acid. The number of ml required (a) was noted. The experiment was repeated with the same quantities of the same reagents in the manner omitting the sample. The number of ml required (b) was noted. The saponification value was calculated using the following formula:—

$$\text{Saponification Value} = \frac{(b-a) \times 0.02805 \times 1.000}{W}$$

Where 'W' is the weight in g of the sample taken.

Iodine Value

It was determined by using Iodine Monochloride Method

Acid Value

The acid value is the number of mg of *potassium hydroxide* required to neutralize the free acids in 1 g of the substance, when determined by the following method:

10 g of the oil was weighed into a 250 ml flask , 50 ml of a mixture of equal volumes of alcohol and solvent ether were added to it, which was neutralized after the addition of 1 ml of solution of phenolphthalein. The flask was heated gently on a water-bath and titrated with 0.1 N potassium hydroxide, shaking constantly until a pink colour with persistance for fifteen seconds was obtained. The number of ml required was noted. The acid value was calculated from the following formula:

$$\text{Acid Value} = \frac{a \times 0.00561 \times 1000}{W}$$

Where 'a' is the number of ml of 0.1 N potassium hydroxide required and 'W' is the weight in g of the sample taken.

Rancidity Test

The rancidity test was carried out as per Kreis test.

Mineral Oil

The test for detection of mineral oil was carried out as per Holde's test.

High Performance thin Layer Chromatography

2 g of formulated Hair oil was taken in a flask and 20 mL methanol was added to it. The flask was placed in rotator shaker for 3 hours at 40^{0}C, then cooled and alcoholics layer was separated to get methanolic extract. It was concentrated to about 5 ml. 10 µl of the methanolic extract of hair oil along with same quantity of methanolic extracts of all the ingredients was applied on Silica gel "G" plate (F 254) and developed to a distance of 8 cm using Toluene: Methanol: Ethyl acetate (6: 0.5: 3) as mobile phase.

Evaluation of Efficacy

Efficacy of Hair Oil was carried out by repeated open application test - (ROAT) on adult human volunteers for four weeks to determine if the Hair oil is efficient in controlling Dandruff and itching of scalp and thus improving overall hair quality in human subjects.

Selection of Volunteers for Study

A total of 07 volunteers (2 male and 5 female) of age group 18-45 years satisfying following eligibility criteria were enrolled in the study.

Inclusion Criteria

Healthy male and female volunteers having at least one problem related hair / Scalp ailments such as dry hair, rough hair, damaged hair, dandruff & itching of scalp were selected and asked to refrain themselves from the use of any other means of Dandruff control or hair treatment other than the test product. Written Informed Consent was received from the volunteers after informing details of the treatment procedures, benefits and potential risks, if any.

Exclusion Criteria

Exclusion criteria were individuals under Hair / Scalp therapy at least one month prior to the study and using Specific anti dandruff products, individuals who were medically compromised, individuals who had undergone pharmacological treatments, individuals with past history of contact dermatitis or who were using any other branded Hair products.

Design of Efficacy Study

The volunteers were enrolled for the efficacy study after satisfying inclusion criteria. Case history of subjects with regard to Hair / Scalp related problems were obtained and their demographic medical histories were recorded. All the subjects were provided with the product and instructed to massage gently their Scalp and hairs with the allocated Hair oil for 10 minutes both in the morning and before going to bed in the night. Subjects were instructed to refrain from any other Hair / Scalp products. The volunteers were examined for hair quality and scalp quality at baseline and after hair wash on 2nd, 3rd, and 4th week of the study with the help of magnifying lens.

Assessment of Efficacy

Four point scale (i.e. bad, average, good & excellent) for hair & scalp related ailment was the basis for overall efficacy assessment of therapeutic effect. Volunteers underwent for follow up for four weeks on the basis of following criteria:

Hair: dryness, roughness and texture of hair

Scalp: Dandruff and itching of scalp

Results and Discussion

Standardization of developed formulation (DF)

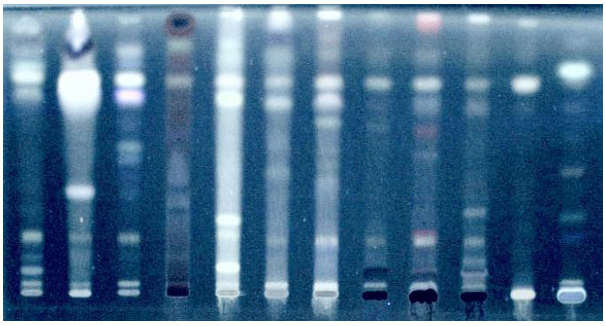
Based on data generated from analysis of three batches of hair oil, range of values for each parameter was obtained and summarized in Table 2.

HPTLC Studies

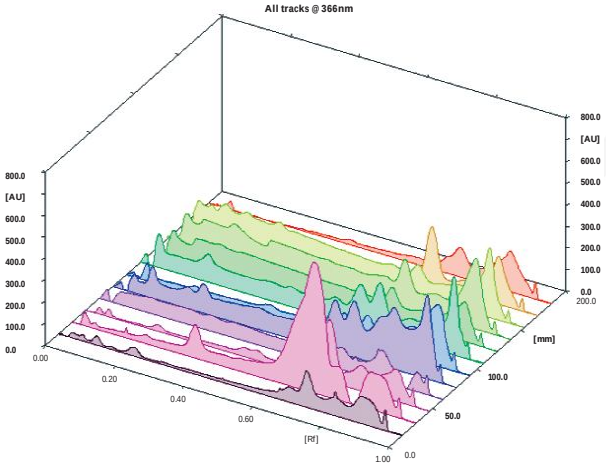
HPTLC of methanolic extract on Silica gel "G" Toluene:Methanol:Ethyl acetate (6:0.5:3) shows 3 major spots at 254nm at Rf.0.11, 1.84 and 1.93 (all black). On spraying with 5% Methanolic Sulphuric acid reagent and heating the plate at 105^{0}C for ten minutes; shows under U.V. 366 at Rf 0.04 (yellowish), 0.07(blue), 0.11(faint blue), 0.22(yellow), 0.67(faint blue), 0.72(pink), 0.81, 0.89, 0.95 .

Table 2: Standards Developed for DF

S.no.	Parameters	Obtained range
1.	Refractive index at 25 °	1.450 to 1.460
2.	Weight / mL at 25 °	0.90 to 0.92
3.	Saponification value	210 to 230
4.	Iodine value	10.00 to 12.00
5.	Acid value	Not more than 4
6.	Rancidity test	Negative
7.	Mineral oil	Absent



Chromatogram: Derivatized at 366 nm



Graph for Derivatized plate at 366 nm

Efficacy of DF

Results obtained for evaluation of hair quality and scalp quality are as summarized in table 3 and 4 respectively. Total of 07 Participants were enrolled in the present study out of which 02 were male and 05 were Female. Among 7 participants, 06 participants enrolled in the study were complaining about dry / rough or damaged hair and 05 were complaining for Dandruff and itching of scalp. At the end of the study there were total of 07 participant with no drop offs.

At the end of the fourth week, out of total 07 participants, 05 participants got Excellent results for Hair quality (Dryness, Roughness, texture of hair) and 02 participants got Good results for Hair quality.

At the end of the fourth week, out of total 07 participants, 04 participants got Excellent results for Scalp quality (Dandruff and itching of Scalp) and 03 participants got Good results for Scalp quality.

In all the participants, any kind of complications or adverse effects was neither reported by participants nor observed by the investigator. It is obvious that addition of herbs in cosmetics does not have any side effect on skin (11, 12, 13). Results obtained for evaluation of hair quality and scalp quality are as summarized in Table 3 and 4 respectively.

Table 3: Observation sheet,
(Assessment results for Hair quality)

Participant no.	Age/Sex of participant	Week of observation	Assessment results for Dryness, Roughness, texture of hair			
			Bad	Average	Good	Excellent
1	24 / F	0th	■			
		2nd			■	
		3rd				■
		4th				■
2	30 / F	0th	■			
		2nd		■		
		3rd				■
		4th				■
3	26 / M	0th		■		
		2nd		■		
		3rd			■	
		4th			■	
4	24 / M	0th		■		
		2nd			■	
		3rd			■	
		4th			■	
5	19 / F	0th	■			
		2nd			■	
		3rd				■
		4th				■
6	25 / F	0th	■			
		2nd			■	
		3rd			■	
		4th				■
7	42 / F	0th			■	
		2nd				■
		3rd				■
		4th				■

■ Indicates positive results

Table 3: Observation sheet
(Assessment results for Scalp quality)

Participant no.	Age/Sex of participant	Week of observation	Assessment results for Dandruff and itching of Scalp			
			Bad	Average	Good	Excellent
1	24/F	0th				
		2nd				
		3rd				
		4th				
2	30/F	0th				
		2nd				
		3rd				
		4th				
3	26/M	0th				
		2nd				
		3rd				
		4th				
4	24/M	0th				
		2nd				
		3rd				
		4th				
5	19/F	0th				
		2nd				
		3rd				
		4th				
6	25/F	0th				
		2nd				
		3rd				
		4th				
7	42/F	0th				
		2nd				
		3rd				
		4th				

Indicates positive results

Conclusion

Based on the results obtained from the Study, it can be concluded that use of polyherbal hair oil fortified with cow milk produces an evident clinical improvement in hair and scalp ailments. The demand for medicines that modify hair augmentation and look has led to a multibillion dollar industry [14]. The product is suitable for micro industries.

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Original Article

Isolation and Characterization of PGPR from Wheat (*Triticum aestivum*) Rhizosphere and Their Plant Growth Promoting Traits *in Vitro*

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Abstract

The PGPR have been divided into two groups based on their involvements in (i) nutrient cycling and phytostimulation, and (ii) the biocontrol of plant pathogens. A total of 72 rhizobacterial isolates belonging to genera *Bacillus, Pseudomonas* and *Rhizobium* were isolated from Wheat (*Triticum aestivum* L.) rhizospheric soils collected from various locations of Kanpur region, India. These rhizobacterial isolates were characterized biochemically and screened for their PGP (plant growth promoting) activities *in vitro.* Plant growth promoting traits screened with the test rhizobacteria included production of indole acetic acid (IAA), ammonia (NH_3), hydrogen cyanide (HCN), siderophore and catalase. All test isolates turned positive for catalase production. The rhizobacterial isolates of *Pseudomonas spp.* (100%), *Bacillus spp.* (100%) and *Rhizobium spp.* (67%) produced IAA. Production of ammonia (NH_3) was commonly detected in the rhizobacterial isolates of *Bacillus* (100%), Pseudomonas (85%) and *Rhizobium* (70%). *Bacillus Spp.* sample KNP-7 showed high level of tolerance to the multiple heavy metals tested whileas tolerance to heavy metals was observed less frequently in *Rhizobium spp*. sample KNP-36 and KNP-5. Rhizobacteria tolerant to multiple heavy metals and exhibiting a couple of PGP traits in the present study hold promise as effective PGPR with wheat and / or other compatible crops.

Keywords: Ammonia; HCN; Heavy Metal Tolerance; Indole Acetic Acid; Plant Growth-Promoting Rhizobacteria; Siderophore; *Triticum aestivum* L.

Introduction

Plant growth-promoting rhizobacteria (PGPR) form a highly diverse group of indispensable soil bacteria of plant rhizosphere that influence the plant growth through a range of mechanisms and have been classified into three broad categories i.e. bioprotectants: strains that suppress the pathogens and hence control plant diseases, biofertilizers: strains which improve the nutrient uptake of the plant and biostimulants. The rhizobacteria enhance the plant biomass and nutrient availability either by the solubilization of phosphate and other mineral complexes or by nitrogen fixation, production of siderophores for the acquisition of trace metals or by the release of phytohormones for better root growth and controlling the harmful effects of deleterious / pathogenic organisms [1-3]. Many studies have been conducted to evaluate the role of PGPR in phytoremediation efficiency action on metal contaminated soils [4]. These bacteria generate a stimulating effect on the growth of plants via giving to them a continuous supply of nutrients and hormones through their metabolic activities [5]. PGPR such as *Agrobacterium*, *Alcaligenes (Ralstonia), Arthrobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Serratia, Pseudomonas* and *Rhizobium* [6-12] are particularly interesting for metal extraction by plants since they increase both the rate of metals accumulated by plants and the plant biomass. Plant growth promoting rhizobacteria when applied to seeds or incorporated into soil reduce the toxicity of heavy metals and consequently enhance the growth and yield of plant. Further, the nodule bacteria can protect the plants against the toxic effects of nickel

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and zinc through adsorption or desorption mechanism [13]. In addition, the plant growth promoting rhizobacteria also synthesize plant growth promoting substances (siderophore, indole acetic acid, hydrogen cyanide and ammonia), which augment the crop productivity [14]. Thus, it may be said that there are plethora of mechanisms that may be explored for developing various PGPR strains as the successful eco-friendly tools to implement sustainable agricultural practices in all parts of the world. There is very little information regarding use of PGPR as bioinoculants / biofertilizers in wheat. Therefore, the present study was undertaken with the view to isolate and characterize PGPR strains from wheat growing fields in Kanpur agro-ecological region of Uttar Pradesh, India.

Materials and Methods

Collection of Sample

The rhizospheric soil samples were collected from sewage irrigated fields growing *Triticum aestivum* L. from rural areas of Kanpur region, India. The fields are being irrigated with domestic sewage last 7 to 8 years. Randomly chosen plants from different locations were uprooted carefully and the excess of soil was removed by gentle shaking and the soil adhering to roots formed composite samples. The collected samples were placed in plastic bags and kept at 4°C in the laboratory until processed further.

Isolation of Rhizobacteria

Soil samples were serially diluted up to 10^{-5} to 10^{-7} in sterile phosphate-buffered saline (Hi-Media, pH-7.2) and plated on the appropriate culture medium for isolating different rhizobacteria. All bacterial strains were isolated on their respective media; *Rhizobium* was isolated on yeast extract mannitol agar [15]. *Pseudomonas* and *Bacillus* were isolated on King's B agar selective medium [16] and nutrient agar, respectively. Isolated colonies of rhizobacterial strains were randomly selected and further purified by streaking. Pure isolates were maintained as glycerol stocks at -80°C for further use.

Identification and Biochemical Characterization of Rhizobacteria

Isolated rhizobacterial strains were characterized and tentatively identified on the basis of their morphological, biochemical and / or physiological characteristics according to Bergey's manual of determinative bacteriology. Selected isolates of *Bacillus* (55), *Pseudomonas* (45) and *Rhizobium* (45) were biochemically characterized for Gram's reaction, carbohydrate fermentation, oxidase test, O-F test, H_2S production, IMVIC tests, NO_2 reduction, and starch and gelatin hydrolysis as per the standard methods [17].

Characterization of Rhizobacteria for PGP Traits

Production of Indole Acetic Acid

Indole acetic acid (IAA) production was detected as described by Brick *et al.* [18]. *Pseudomonas, Bacillus* and *Rhizobium* cultures were grown separately on their respective media with 100 and 200 μg / ml of L-tryptophan at 30°C for 48 hours. Fully grown cultures were centrifuged at 8000 rpm for 10 min. The supernatant (2 ml) was mixed with two drops of ortho-phosphoric acid and 4 ml of the Salkowski reagent (concentrated H_2SO_4:150 ml, 0.5M $FeCl_3 \cdot 6H_2O$:7.5 ml, distilled water: 250 ml). Development of pink colour indicates IAA production.

Production of Ammonia

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48-72 hours at 36±2°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production [17].

Siderophore Production

Siderophore production was detected by the method of Schwyn and Neilands [19] using blue agar plates containing the dye chrom azurol S (CAS). Orange halos around the colonies on blue were indicative for siderophore production.

Phosphate Solubilization Activity

All isolates were first screened on Pikovskaya's agar plates for solubilization of insoluble inorganic phosphate as described by Gaur [20]. Bacterial cultures were inoculated on centre of agar plate through inoculation loop under aseptic condition. Inoculated plates were incubated for 3 days at 30°C. Presence of clear zone (halozone) around the colony was recorded on Pikovskaya's agar plates. Formation of halozone showed positive phosphate solubilization ability.

Catalase Production

Bacterial cultures were grown in nutrient agar medium for 18-24 hours. The cultures were mixed with appropriate amount of H_2O_2 on a glass slide to observe the evolution of oxygen.

HCN Production

Hydrogen cyanide (HCN) production from glycine was tested growing the bacteria in 10% trypic soy agar (TSA) supplemented with glycine (4.4 g l^{-1}) and cyanogenesis was revealed using picric acid and Na_2CO_3 (0.5 and 2%, respectively) using the method of Donate-Correa *et al.* [21]. Impregnated filter paper fixed to the underside of the Petridis lids. Results were read after five days of culture at 28°C. A change in filter paper colour from yellow to orange-brown indicated production of HCN as indicated below:

Yellow (1) - limited cyanide production, orange (2) - moderate cyanide production, light brown (3) - relatively high cyanide production and brown (4) - high cyanide production.

Heavy Metal Tolerance

The selected bacterial strains were tested for their resistance to heavy metals by agar dilution method. Freshly prepared agar plates were amended with various soluble heavy metal salts namely Cr, Pb, Hg, Cd, Zn, and Cu, at various concentrations ranging from 25 to 200 μg ml^{-1} were inoculated with overnight grown cultures. Heavy metal tolerance was determined by the appearance of bacterial growth after incubating the plates at room temperature for 24-48 hours.

Results and Discussion

In the present study, the rhizobacterial isolates were identified based on morphological and biochemical characteristics and were tested for their beneficial traits like ability to production of indole acetic acid ammonia and production of other plant growth promoting substances. Efficient rhizobacterial isolates selected based on the above characters were examined for their *in vitro* screening methods.

Isolation and Identification of Rhizobacteria

On the basis of cultural, morphological and biochemical characteristics (Table 1), a total of *Bacillus* (55), *Pseudomonas* (45) and *Rhizobium* (45) were identified from domestic sewage irrigated rhizospheric soil samples as described in Bergey's Manual of Determinative Bacteriology. *Bacillus* represents the predominant bacterial genera of the tested wheat rhizosphere. This observation is in conformity with Rawat *et al.* [22] and may be attributed to the ability of *Bacillus* to form endospores and produce antimicrobial substances that inhibit other competitors in the rhizosphere. Among the 145 isolates, 72 isolates (*Bacillus*-35, *Pseudomonas*-20 and *Rhizobium*-17) were selected for further studies based on the efficiency of multiple plant growth promoting activities *in vitro*.

Plant Growth Promoting Characteristics of Test Isolates

Screening results for PGP traits of selected isolates are presented in Table 2. IAA production was shown in most of the isolates of *Bacillus* (100%), *Pseudomonas* (100%) and *Rhizobium* (67%) thus showing positive PGP activities in relation to IAA. IAA is the most important auxin (phytohormone) produced by plants and many soil bacteria. It has a crucial role to play in a variety of plant activities, including embryo development; root initiation and development; apical dominance; leaf formation and fruit development. IAA is derived mainly from tryptophan through multiple enzymatic pathways by many different genera of PGPR like *Rhizobium*, *Bacillus*, *Pseudomonas*, *Azotobacter*, *Enterobacter*, *Bradyrhizobium*, *Xanthomonas* and *Alcaligenes* [23]. All rhizobacterial test isolates turned positive for catalase production. Catalase activity in the bacterial strains may be potentially very advantageous and bacterial strains showing catalase activity must be highly resistant to environmental, mechanical and chemical stress. Selected rhizobacterial isolates did not exhibit significant phosphate solubilisation activity in the present study. Siderophore production was detected in *Pseudomonas* (70%), *Bacillus* (33%) and *Rhizobium* (0%). Siderophore ability was detected significantly higher among isolates of *Pseudomonas* spp. Siderophores are low-molecular-weight molecules that are secreted by many microorganisms and act as solubilising agent for iron from minerals under iron shortage condition. In addition, siderophores form stable complexes with heavy metals including U, Np, Al, Cu, Cd, In, Ga, Zn and Pb and increases the soluble metal concentration [24], thus, it helps to alleviate the stresses imposed on plants by heavy metals in soil. Siddiqui *et al.* [25] reported that AY197010 isolate of *Pseudomonas* and AY197006 and AY197009 isolates of *Flavobacterium* could manufacture siderophore. HCN production was detected higher in *Pseudomonas spp.* as compared to the *Bacillus spp.* and *Rhizobium spp.* isolates. Higher HCN production by *Pseudomonas fluorescens*, *P.*

aeruginosa and *Chromobacterium violaceum* has also been reported by other researchers [11, 26]. Ammonia production was detected in *Bacillus* (100%), *Pseudomonas* (85%) and *Rhizobium* (70%) of test isolates which is an important attribute of PGPR that influences plant growth indirectly [27]. Hydrogen cyanide was detected in *Bacillus* (70%), *Pseudomonas* (100%) and *Rhizobium* (50%) among the test isolates. HCN production by soil bacteria is reported to play a role in disease suppression, as in the case of tobacco

Table 1: Morphological, cultural and biochemical characteristics of rhizobacteria associated with rhizosphere of *T. aestivum* L

Morphological and Biochemical Characterization	*Bacillus* (35)	*Pseudomonas* (20)	*Rhizobium* (17)
Gram's reaction	G +ve	G -ve	G -ve
Shape	Rods	rods	rods
Pigments	-	+	+/-
Dextrose	+	+	-
Sucrose	+	+	-
Mannitol	+	-	+
Oxidase	-	+	+
OF test	-	+	-
H_2S production	-	+	+
Indole	-	-	+
Methyl red	-	-	+
Vogues Proskauer	+	-	+
Citrate utilization	+	+	-
Starch hydrolysis	+	+	+
Gelatin hydrolysis	+	-	-

Table 2: Plant Growth Promoting Characteristics of rhizobacteria associated with rhizosphere of *T. aestivum* L.

Organism	Sample No.	IAA	Catalase	HCN	Ammonia	Siderophore
Bacillus spp.	KNP-4	+	+	+	+	+
Bacillus spp.	KNP-7	+	+	-	+	-
Bacillus spp.	KNP-13	+	+	+	+	-
Pseudomonas spp.	KNP-19	+	+	+	+	+
Pseudomonas spp.	KNP-11	+	+	+	+	+
Pseudomonas spp.	KNP-27	+	+	+	+/-	+/-
Rhizobium spp.	KNP-36	-	+	-	+	-
Rhizobium spp.	KNP-5	+	+	-	+/-	-
Rhizobium spp.	KNP-22	+	+	+	+	-

Table 3: Heavy metal tolerance of selected of rhizobacterial isolates associated with rhizosphere of *T. aestivum* L.

		Heavy Metal Tolerance (µg ml^{-1})					
Organism	Sample No.	CR	PB	HG	CD	ZN	CU
Bacillus spp.	KNP-4	200	100	50	100	100	100
Bacillus spp.	KNP-7	200	200	100	100	200	100
Bacillus spp.	KNP-13	200	200	50	100	100	100
Pseudomonas spp.	KNP-19	200	100	100	100	50	100
Pseudomonas spp.	KNP-11	150	100	100	100	100	100
Pseudomonas spp.	KNP-27	200	200	100	100	200	100
Rhizobium spp.	KNP-36	100	100	100	100	50	100
Rhizobium spp.	KNP-5	100	100	50	50	100	50
Rhizobium spp.	KNP-22	200	100	200	100	100	200

where *Pseudomonas fluorescens* helped suppression of black root rot disease [28].

Heavy Metal Tolerance of Test Isolates associated with Rhizosphere of T. aestivum.

Thirty-five (35) *Bacillus*, 17 *Rhizobium* and 20 *Pseudomonas* rhizobacterial isolates was checked against different heavy metals Cr, Pb, Hg, Cd, Zn and Cu and data on few selected isolates is presented in Table 3. This study observed rhizobacteria particularly *Bacillus* and *Pseudomonas* isolates tolerant to multiple heavy metals and exhibiting a couple of PGP activities (Table 2 and 3). The metal-microbe interaction in natural environment is influenced by pH and organic matter content. In the present study, selected strains showed heavy metal tolerance up to 200 µg / ml. *Bacillus sp.* (KNP-7) tolerated Pb and Cr (200 µg / ml), Hg (50 µg / ml) and tolerance exhibited

towards Zn, Cu and Cd was 100 µg / ml, respectively. *Pseudomonas sp.* (KNP-27) proved more heavy metal tolerant as compared to other isolates, tolerating up to 200 µg / ml of Cr, Zn and Pb, and 100 µg / ml of Hg, Cd and Cu. A varying level of resistance to heavy metals among the PGPR (*Bacillus* and *Pseudomonas*) have also been reported [29] as seen in present findings. *Bacillus* and *Pseudomonas* induced larger inhibition zones showing their high heavy metal tolerance activity and exhibiting high metal tolerance activities against heavy metals compared to the other *Rhizobium* isolates. Tolerance to heavy metals was observed less frequently in *Rhizobium spp.* isolates. The present study shows the significance of rhizobacteria under *in vitro* conditions for multiple PGPR traits and their evaluation under controlled conditions for selection of effective PGPR isolates of *Bacillus*, *Pseudomonas* and *Rhizobium.* Their multiple plant growth promoting activities are highly effective in improving the plant growth parameters. Several studies have also established a correlation between bacterial antibiotic resistance and metal tolerance [27, 30].

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Original Article

Diversity and Community Structure of Aquatic Insects in a Fresh Water Lentic System of Purba Medinipur District, W.B., India

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Abstract

29 species of aquatic insects have been recorded from a weed infested man made wetland near Tamluk Station. Hemiptera was numerically the most abundant group comprising 39% of the total aquatic insects followed by Coleoptera (36%) and Odonata 25%. Hemiptera and Odonata were represented by 10 species each while Coleopteran was represented by 9 species. Of these only one coleopteran species, *Canthydrus latitabilis* was found to be dominant. The water body under investigation was considered moderately polluted. On the basis of Diversity index, Evenness value and Dominance value indicated the equitability and heterogeneity of the aquatic system. While Coleoptera and Odonata exhibited a peak in July and May respectively but no distinct peak could be seen for Hemiptera. Correlation between the abiotic factors and insect species revealed that abiotic factors had some regulatory effects on aquatic insect population.

Key words: Aquatic Insects; Aquatic Ecosystem; Biodiversity.

Introduction

Among the fresh water organisms aquatic entomofauna may comprised more than 95% of all the species of macro-invertebrates (Ward, 1992) in some lentic water bodies. There are about 45000 species of insects known to inhabit diverse fresh water ecosystem (Balaram, 2005) and about 5000 species of aquatic insects are estimated to inhabit inland wetlands of India (Subramaniam and Shivaramakrishnan 2007). Aquatic insects are involved in nutrient recycling and form an integral part of natural food web in aquatic ecosystem. These constitute a dominating group of littoral, benthic and limnetic biodiversity of the freshwater ecosystem because of their high abundance, high birth rates, short generation time, large biomass, high turnover rates and rapid colonization to habitats (Roy *et al.*, 1988). These are also considered as model organism in analysing ecological characteristics of inland water bodies and thus serve as a reliable bioindicator of aquatic ecosystem. Both larvae and adult of aquatic insects prey on various kinds of aquatic organisms and also offered themselves as food for carnivores fishes. As such, these are of immense value form the point of aquaculture and public health. Some recent works on aquatic entomofauna of India are those by Bhattacharyya (2000), Pal *et al*. (2000), Khan and Ghosh (2001), Anbalagan *et al*. (2004), Saha *et al*. (2007), Das and Gupta (2010), Hazarika and Goswami (2010), Sharma and Agarwal (2012), Barman and Baruah (2013), Jenila and Nair (2013), Abhijna *et al.* (2013), Gupta and Narzary (2013), Samweel and Nazir (2014), Vasantkumar and Roopa (2014), Choudhury and Gupta (2015) and Susheela and Radha (2015). Although Pahari *et al*. (1997, 1999) and Jana *et al*. (2009) have studied some taxonomic and ecological aspects of aquatic insects in West Midnapore District. So, far no comprehensive work has been done on the quantitative ecology of the aquatic insects of Purba Medinipur District.

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Materials and Methods

The present study was conducted in a man-made perennial pond (Tamluk Station Pond, 22° 17' 52.56'' N, 87° 55'16.72'' E). The area of ponds is about 2.3 acre with an average depth of about 3.6 meter. This water body is infested with many aquatic weeds like *Nelumbo nucifera* Gaertner*, Alterhennthera sessilis* Linn., *Eclipta alba* Hassk., *Monochoria hastate* Solms., *Scirpus articulatus* (Linn.), *Cyanotis axillaries* Roem & Sch., *Aeschynomene ampera* Linn., *Hygrorryza aristata* Nees., *Hydrocotyla asiatica* Nees., *Hydrophylla difformis* L.f., *Utricularia stellaris* L.f., *Jussiaca repens* Linn., *Nymphaea nouchali* Burm. f., *Marsilea minta* Linn., *Nymphoides indica* (Linn.), *Eichhornia crassipes* (Mart.) Solms*, Commelina bengalensis* Linn., *Hydrilla vercillata* Casp.*, Vallisneria spiralis* Linn.*, Chara sp., Nitella sp., Salvinia sp., Learsia sp.*.

Insects were collected at monthly interval from Jan 2015 to December 2015 between 8.00 am to 10.00 am. The collections were made by hauling of a dip net with a mesh sizeof 245 gm Nylobolt PA, (Dukay Nilobolt Industries Pvt. Ltd., Mumbai, India). The area of the circular net was 4208 cm^2. Samples were taken from four sites at four corners. Collected insects were preserved in 70 % ethyl alcohol in specimen bottles and identified upto the species level. Water quality parameters *viz.* pH, temperature, conductivity, dissolved oxygen and carbon-di-oxide were analysed following APHA (2005).Community analysis represence to abundance, relative abundance, general diversity index (Shannon-Wiener, 1963) and evenness index (Pielou, 1966), Dominance Diversity index (Mc-Naughton, 1968)were determined using the package Ecological Methodology version 6.1 (Krebs, 2002) & Multivariate Statistical Package (MVSP) version 3.13n

Result and Discussions

During this investigation 29 species of aquatic insects were recorded (Table 1), belonging to Hemiptera, Coleoptera, and Odonata. Among these Hemiptera was numerically the most abundant comprising 39% of the total insect fauna (Figure 1). This order was represented by 04 families *viz.* Belostomatidae (41%), Corixidae (27%), Nepidae (23%) and Notonectidae (9%) (Figure 2). Coleopter-aconstituted of 36% of the total insect population with 2 families *Viz.* Dytiscidae (90%) and Hydrophilidae (10%)(Figure 3). Odonatawas 25% of the insects collected was represented by 4 families *viz.* Coenagrionoidae (47%), Libellulidae (25%), Aeshnidae (21%) and Ptatycnemididae (7%)(Figure 4).Hemiptera, Odonataand Coleoptera were represented by 10, 10 and 09 species respectively. As in present study preponderance of Hemiptera in freshwater lentic system has also been reported in earlier studies by Bhattacharya (1998) from West Bengal, Hazarika and Goswami (2010), Das and Gupta (2010), Gupta and Narzary (2013), Choudhury and Gupta (2015) and Barman & Baruah (2013) in Assam and Abhijna *et al*. (2013) in Vellayani lake in Kerala. Numerical abundance of Hemiptera over Coleoptera has also been observed by Khan and Ghosh (2001) in West Bengal and Johri *et al.* (2010) in Uttar Pradesh. Family Dytiscidae was taxonomically more diverse (7 species) (Table 1) and numerically more abundant (Figure 3) than Hydrophilidae among Coleoptera.

The member of the family Dytiscidae prefer weed infested freshwater bodies as they inhabit leaf of the submerged macrophytes. The naid of Odonata prefer macrophyte infested wetland for their better survival. Hydrophilidae on the contrary is water scavenger beetles generally occur in shallower regions of the wetlands and feed mainly on detritus (Khan and Ghosh, 2001). Findings pertaining relative abundance (Table1) revealed that out of 29 species only one species *Canthydrus laetubilis* was dominant (11.9%). Tis species appears to be the good exploiters of resources in weed infested aquatic ecosystem as compared to others. Of the remaining species 11 were subdominant (RA 5% -10%) and 17 were recedent species (RA 3.2% - 10%).

The diversity index indicated a seasonal trend. It was lowest in January and increased till June. Thereafter it progressively decreased till December. According to Wilhm & Dorris (1966) diversity index between 01 to 03 indicates a moderately perturbed condition of the water body. Since the diversity index in the present study ranged between 1.131 to 1.332, the water body under investigation may be considered as moderately polluted. Smith (1997) suggested that high species diversity indicated that such community has their resources more finely distributed among individuals of many species. Iwaski (1999), however opined that environmental stability rather than heterogeneity has greater influence on it. The value of evenness index was considerably high and ranged from 0.855 to 0.955, indicating the heterogeneity of the community. In the present study dominance index was quite low and varied from month to month without any trend. Dominance index increases with the increase in the harshness of environment and decreases with the vegetational development (McNaughton and Wolf,

1970). This finding suggests that the waterbody exhibited a relatively equitable environment. While Coleoptera and Odonata exhibited a unimodal pattern of temporal variation with a peak in July and May respectively no such trend could be seen for Hemiptera (Figure 5).

Correlation between aquatic insect population are shown in Table 3. In the present study *Laccophilus anticatus* (Coleoptera) and *Diplonychus rusticus* (Hemiptera) had a significant positive correlation with pH.Jenila and Nair (2013) also observed a similar relationship of pH with *Diplonychus indicus* and *Ranatra filiformis*. Water temperature had a significant positive correlation with *Ischnura verticalis, Ranatra varipes* and *Urothemis signata*. Jenila and Nair (2013) found that change in water temperature had a profound influence on the population of aquatic insect. In the present study two odonate species *Anax imperator* and *Aeshna fabricius* and ahemipteran species *Ranatra varipes* showed significant negative correlation with D.O..Thirumalai and Raghunathan (1988) however, opined that D.O. had no impact on aquatic insect population. *Anisops bouvieri* showed a negative correlation with conductivity while *Aeshna fabricius* had a positive correlation with it. *Hydrovatus bonvoluri, Sternolophus rufipes and Brachydiplax chalybea* exhibited positive correlation with salinity where as *Hydrocoptus subvittulus, Laccophilus anticatus, Helochares anchoralis* and *Diplonychus rusticus* showed negative correlation with salinity. Thus it is seen that influence of abiotic factors varies from species to species.

Table 1: Relative Abundance and dominance status of insect species

Order- Coleoptera	Abundance	Relative abundance (RA)%	Dominance status
Family – Dytiscidae			
***Canthydrus laetabilis* (Walker, 1858)**	114	11.92	Dominant
***Canthydru sluctuosus* (Aube, 1838)**	65	6.80	Sub Dominant
***Hydrocoptus subvittulus* (Motschulsky, 1859)**	46	4.81	Sub Dominant
Laccophilu spurvulus (*Aube, 1838*)	34	3.56	Sub Dominant
***Laccophilus anticatus* (Sharp, 1890)**	13	1.36	Recedent
***Hydrovatus bonvoluri* (Sharp)**	22	2.30	Recedent
***Cybester tripunctatus* (Sharp, 1882)**	11	1.15	Recedent
Family –Hydrophilidae			
***Helochares anchoralis* (Sharp, 1890)**	21	2.20	Recedent
***Sternolophus rufipes* (Fabricius ,1792)**	13	1.36	Recedent
Order- Odonata			
Family -Coenagrionoidae			
***Ischnura verticalis* (Say, 1839)**	29	3.03	Sub Dominant
***Pseudogrion rubriceps* (Selys, 1876)**	37	3.87	Sub Dominant
***Enallagma parvum* (Selys, 1876)**	18	1.88	Recedent
***Pseudogrion microcephalum* (Rambur, 1842)**	28	2.93	Recedent
Family -Ptatycnemididae			
***Coperam arginipes* (Rambur, 1842)**	18	1.88	Recedent
Family –Aeshnidae			
***Anax imperator* (Leach, 1815)**	25	2.62	Recedent
***Aeshna fabricius* (Syst, 1775)**	25	2.62	Recedent
Family –Libellulidae			
***Brachydiplax chalybea* (Brauer, 1868)**	24	2.51	Recedent
***Urothemis signata* (Rambur, 1842)**	20	2.09	Recedent
***crocothemis servilia* (Drury, 1773)**	17	1.78	Recedent
Order - Hemiptera			
Family - Nepidae			
***Laccotrephes ruber* (Linnaeus, 1764)**	24	2.51	Recedent
***Laccotrephes maculates* (Fabricius, 1775)**	16	1.67	Recedent
***Ranatra filiformis* (Fabricius, 1790)**	32	3.35	Sub Dominant
***Ranatra varipes* (Stal, 1861)**	13	1.36	Recedent
Family - Belostomatidae			
***Diplonychus annulatam* (Fabricius, 1803)**	88	9.21	Sub Dominant
***Diplonychus rusticus* (Fabricius, 1794)**	43	4.50	Sub Dominant
***ethocerus indicus* (Lepeletier and Serville, 1825)**	22	2.30	Recedent
Family - Notonectidae			
***Anisops bouvieri* (Kirkaldy)**	35	3.66	Sub Dominant
Family-Corixidae			
***Micronectascutellaris* (Dist)**	36	3.77	Sub Dominant
***Plea liturata* (Fieber)**	67	7.01	Sub Dominant

R.A. <1 = Subrecedent, 1.1-3.1 = Recedent, 3.2-10% Subdominant, 10.1-31.6 = Dominant & >31.7% = Eudominant (Engelmann, 1973)

Table 2: SpeciesDiversity, Evenness and Dominance Index of the insect community

Months	Shannon-Weiner Diversity Index (H')	Evenness index (e)	Dominance Index (d)
Jan	1.131	0.855	35.4
Feb	1.154	0.887	34.5
Mar	1.234	0.906	29.5
Apr	1.260	0.901	27.4
May	1.303	0.957	15.7
Jun	1.332	0.953	15.2
July	1.235	0.895	27.4
Aug	1.237	0.908	23.1
Sep	1.255	0.935	17.9
Oct	1.274	0.952	17.1
Nov	1.2	0.894	24.7
Dec	1.173	0.917	25.4

Table 3: Correlation coefficient between insect species and abiotic factors

	pH	Temp (°c)	D.O(ppm)	Cond (ms)	Sal (ppt)
Hydrocoptus subvittulus	0.25	0.18	-0.27	0.16	-0.63*
Laccophilus anticatus	0.60*	0.29	-0.27	0.34	-0.75**
Hydrovatus bonvoluri	0.00	-0.03	0.12	0.03	0.60*
Holochares anchoralis	0.13	0.10	0.04	0.44	-0.60*
Sternolophus rufipes	0.08	0.09	-0.14	-0.03	0.63*
Ischnura verticalis	0.28	0.80**	-0.21	-0.34	-0.07
Anax imperator	-0.03	0.29	-0.74**	0.47	0.10
Aeshna fabricius	0.02	0.17	-0.73**	0.59*	0.06
Brachydiplax chalybea	-0.40	-0.17	0.08	0.22	0.60*
Urothemis signata	0.51	0.68*	-0.55	0.09	-0.31
Ranatra varipes	0.26	0.63*	-0.63*	0.22	-0.17
Diplonychus rusticus	0.81**	0.05	-0.05	0.23	-0.68*
Anisops bouvieri	0.33	0.55	-0.31	-0.59*	0.17

* = p≤0.05, ** p≤0.01

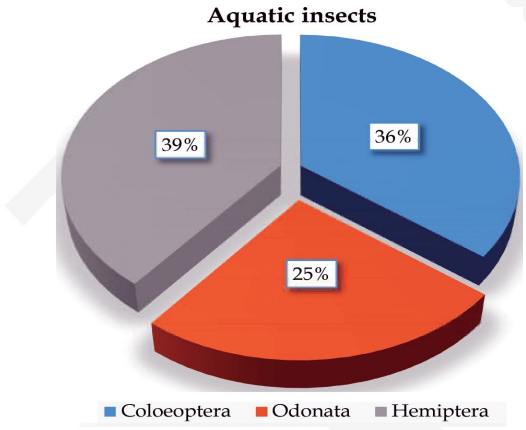

Fig. 1: Relative abundance of orders of insect fauna

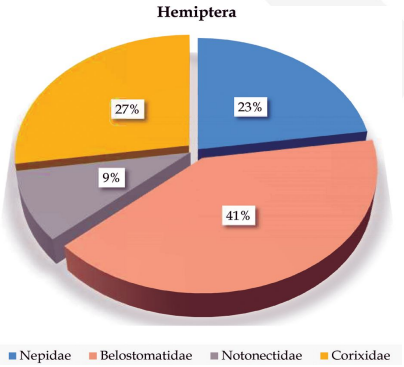

Fig. 2: Relative abundance of families of order Hemiptera

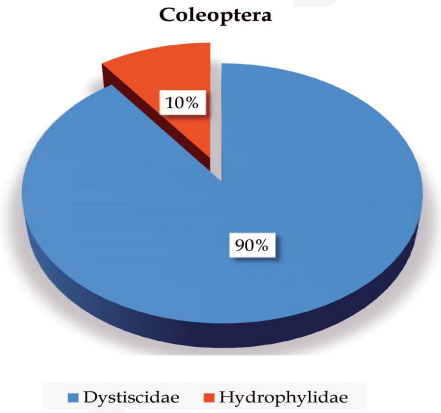

Fig. 3: Relative abundance of families of order Coleoptera

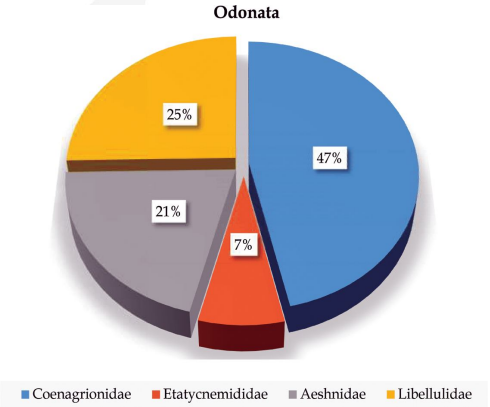

Fig. 4: Relative abundance of families of order Odonata

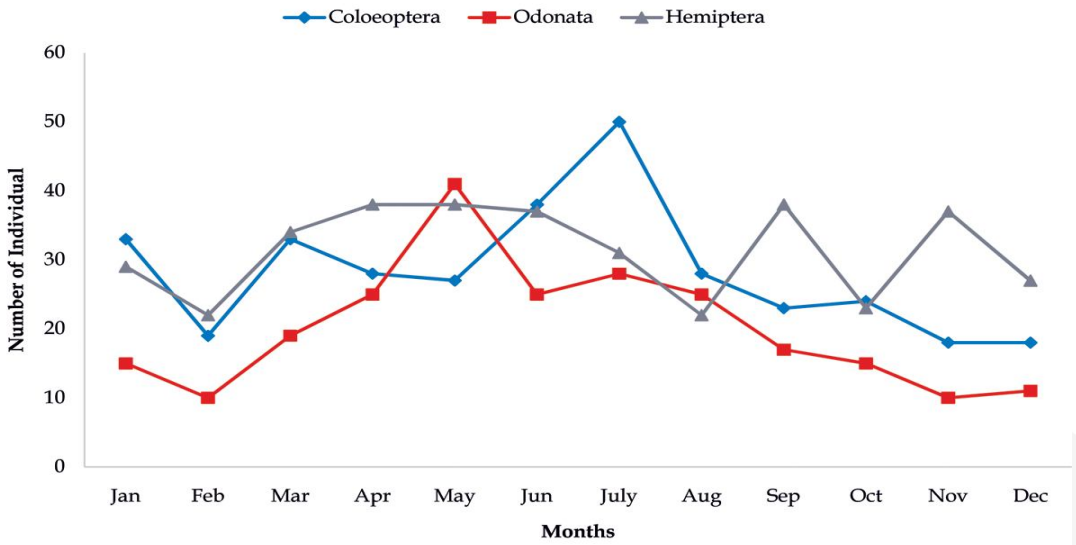

Fig. 5: Temporal variation in number ofinsects orders

Acknowledgement

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Original Article

Haematology of Grey Heron (*Ardea cinerea*) and Black Crowned Night Heron (*Nycticorax nycticorax*)of Chilika Wetland

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Abstract

A study of the haematological profile of two common resident heron species of Chilika wetland(Asia), namely black crowned night heron and grey heron was undertaken during their wintering period to record the baseline data on haematology of these two species in the region. Significant differences were noted in blood parameters like haemoglobin concentration, total WBC count, PCV, thrombocyte count and MCHC between the two heron groups. However, no significant variation was noticed in parameters like total RBC, MCV, MCH and differential leucocyte count. It was observed that the night heron showed higher values for most of the parameters in comparison to grey heron.

Keywords: Ardeidae; Haemogram; Leucogram; Wading Birds.

Introduction

Herons belong to the family Ardeidae along with other members like egrets.They have an extensive range of distribution throughout the temperate regions especially the wetland habitat where they enjoy the status of 'Least Concerned' as declared by the International Union of Conservation of Nature and Natural Resources (IUCN). Hunted down extensively in the 19th century either in the lieu of being a prized delicacy for human consumption or for their ornamental plumes to be used as fashionable human adornments, they suffered mass extinction in many parts of Europe and this led to laws for their conservation in those regions. The members are mostly residents or semi-migratory in nature and are one of the widely distributed populous avian species with top feeding positions to be found in most of the wetland ecosystems.

In 1960s black crowned night heron population showed decline around Michigan(USA), reportedly due to presence of excessive amount of DDT in water [2]. The same species has shown decline in 1970s in northeastern US estuaries [14] and again in 1990s due to contaminants and habitat destruction [3]. The status of the population of herons and egrets has been proved to be an important factor for the assessment of overall environmental wellbeing. Thus, these birds occupy a sensitive yet strategic position in their ecosystem. Along with other assessment methods like habitat and population studies, non-invasive and effective faster health assessment tools like haematological analysis are also important for wildlife studies. However, being categorised as least concerned, the members of this family perhaps fail to draw attention in this respect and thus, very few literature is available on haematology of herons and egrets [5,8,12] . Most of the studies conducted are on other popular species having either economic importance or having a threatened existence [15,18]. Moreover, no proper haematological data are on record for grey herons except a few [10,13]. A recent study on grey heron has shown that blood is the fourth tissue after feather,liver and kidney to show a higher concentration of mercury contamination[10] thus, emphasizing the importance of studying base line values of blood parameters , as an important tool for overall assessment of the health of a

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population and its environment.This study has been undertaken on two common yet important resident wading birds of Chilika, namely black crowned night heron and grey heron with an assumption that the blood value of these avian species may serve as an indicator of the wellbeing of the wetland.

Materials and Methods

The site of this study was Chilika lagoon which is located between the coordinates of 19º 28' and 19º 54'N and 85º 05'and 85º 38' E and is a part of Odisha state in the eastern coasts of India.It enjoys the status of being world's second largest brackish water lagoon and the 1st Ramsar site of India.It hasbeen declared to be an wetland of international importance by the Ramsar convention in 1981.It is a favourite wintering destination for many migratory and semi-migratory birds as well as a host to many breeding resident bird species [1]. Out of the 71 reported resident avian species, family Ardeidae contributes 14 species of herons and egrets [6].

The grey heron is a large predatory wader,having long yellowish brown legs, an 's' shaped long neck with black-brown stripes, grey feathers covering the upper part of the body, while under part being white and characteristic pinkish yellow beak (Figure 1). It is a diurnal feeder showing solitary foraging habit mostly depending upon aquatic insects,fish, amphibians, small mammals as their food. These birds are active mainly during day time and are found to be aggressive defenders of their nesting and feeding territories. In contrast to other herons, black crowned night heron is a small size bird with shorter legs,neck and a stocky hunched posture.It is a nocturnal species having characteristic large and widely separated red eyes for night vision (Figure 2). They have been known to fly with faster beating wings in comparison to other herons. They are solitary foragers like grey herons, maintaining exclusive feeding territories and showing similar diet with an additional propensity to eat young birds, which is unusual among herons.

Blood samples were collected from 10 non-breeding , adult, black crowned night herons and 10 grey herons during the month of February and March in the year 2015. The blood samples were collected between 6.00 a.m. and 9.00 a.m. in the morning in all cases to avoid diurnal variation. Clinically sound adult birds were captured using nets from their nests and blood samples were collected by venepuncture of the ulnar vein by trained veterinary professionals.The blood collected using 2.5ml disposable syringes was immediately stored in EDTA vials in ice box. Few drops of fresh blood was used to prepare the bloodfilms on the site. The anti-coagulated samples were taken to the laboratory at adistance of three hours, for further study within 24 hours of sample collection following standard procedure[16]. Haemoglobin concentration was estimated using Sahli's Haemoglobinometer and PCV was estimated by microhaematocrit method running the sample at 2500 rpm in centrifuge for 15 minutes.Total RBC, WBC and thrombocyte [4,17] counting were done with the help of Neubaurer's haemocytometer.The mean corpuscular volume (MCV), mean haemoglobin concentration (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated using standard formulae [3]. For the DLC, blood smears were stained in Romanovsky stain (Leishman's Stain). WBCs were counted and classified according to their staining and morphologic properties [16]. The data was analysed in MS Office Excel 2010 and were presented as Mean ±SE(standard error).Further, Student's t-test was performed assuming equal variance and the differences were considered significant at $p < 0.05$,$p < 0.01$ and $p < 0.001$.

Results

The haemogram and leukogram of avian species vary in response to factors like age, species, hormonal, physiological, pathological and environmental influence apart from handling and blood collection procedures [17].

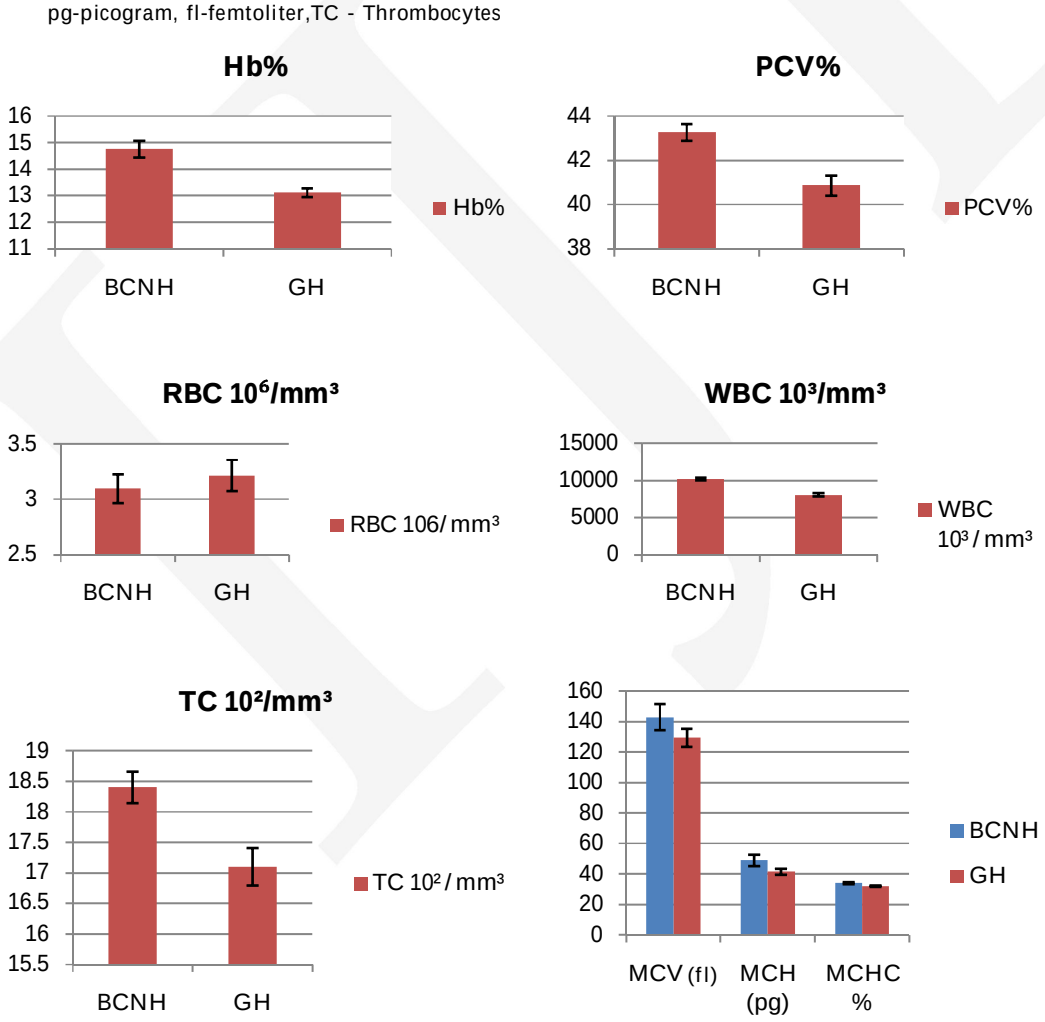
Significant difference was found between the two heron species for some of the parameters like PCV, Hb, WBC, thrombocyte count and MCHC. While, in others like RBC, MCV, MCH and differential leucocyte count the difference was non-significant. The parameters in case of black crowned night herons were visibly higher than those of grey herons. Hob concentration for night heron was 14.77± 0.31 but was 13.12 ± 0.31 for grey heron, the difference was found to be highly significant. Similarly, PCV was recorded as 43.26 ±0.38 for black crowned night heron while it was 40.87 ±0.46 for grey heron. The total thrombocyte per mm^3 blood was also higher in black crowned night heron having a value of 18.4±0.26 in comparison to that of grey heron which is 17.1±0.31.This difference was significant. Black crowned night heron also showed higher number of WBC, 10190±183.75 in comparison to the total WBC for grey heron, 8071±201.8.Heterophils were found to be predominant leucocytes followed by the lymphocytes in both the birds.The difference between

the percentage of the five types of leucocytes was recorded to be no significant. The MCV and MCH value of black crowned night heron which are 142.96±8.5 and 49.06±3.6 respectively were higher than those of grey heron. However, the difference was nonsignificant. The MCHC of night heron (34.12±0.54) showed significant difference with that of grey heron (32.1±0.49) (Table 2 and Fig. 3).

Table 1: Haematological parameters in black crowned night heron and grey heron

S. No	Parameters	Black Crowned Night Heron(10)	Grey Heron(10)	p- value
1	Hb(g/dL)	14.77 ± 0.31	13.12 ± 0.15	0.0005***
2	PCV(%)	43.26 ± 0.38	40.87 ± 0.46	0.003***
3	RBC(10^6/mm^3)	3.09 ± 0.13	3.21 ± 0.14	0.94 NS
4	WBC(10^3/mm^3)	10.190 ± 0.18	8.071 ± 0.2	3.3E-06
5	TC(10^3/mm^3)	18.4 ± 0.26	17.1 ± 0.31	0.01**
6	MCV(fl)	142.96 ± 8.5	129.49 ± 5.9	0.54 NS
7	MCH(pg)	49.06 ± 3.6	41.56 ± 1.8	0.1 NS
8	MCHC(%)	34.12 ± 0.54	32.1 ± 0.49	0.05*
9	Heterophil(%)	61.8 ± 1.12	61 ± 1.8	0.5 NS
10	Lymphocyte(%)	33.6 ± 1.2	33.8 ± 1.9	0.7 NS
11	Eosinophil(%)	2.7±0.21	2.8±1.6	0.8 NS
12	Monocyte(%)	1.6±0.37	1.6±0.22	1.0 NS
13	Basophil(%)	0.3±0.15	0.4±0.16	0.3 NS

Note: Values given as Mean± Standard Error (SE). Parameters showing significant difference * at p<0.05 ,**at p<0.01 ,***at p<0.001. NS-non significant.



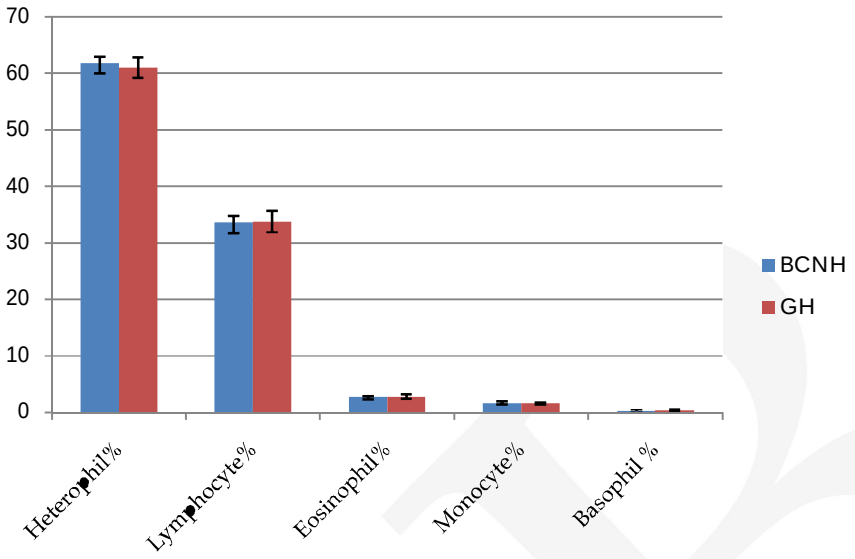


Fig. 3: Comparative account of different haematological parameters between black crowned night heron (BCNH) and grey heron(GH) shown by standard error bars

Fig. 1: Grey heron (*Ardea cinerea*)

Fig. 2: Black crowned night heron (*Nycticorax nycticorax*)

Discussion

The present study shows results similar to those reported by other studies [5,8,13]. This study indicates a slightly lower PCV, MCV, heterophil and lymphocyte value but higher value of RBC, WBC and MCHC for black crowned night heron in comparison to that reported by Celdran et al. [5]. The difference noted may be due to difference in the season of study or reproductive and physiological status of the birds or environmental conditions like temperature, availability of food etc.[7,9,11]. The difference may also be due to the fact that earlier reports were on captive herons[4] whereas the present study projects the blood values of wild free living herons.

The difference in value of Hb,PCV,WBC ,thrombocyte count and MCHC between the two species may be due to several factors like metabolic status, nocturnal or diurnal habit[17], feeding habit, age and sex [9]apart from their taxonomic position and genotype [7]. For instance, black crowned night heron is nocturnal in nature and with a higher metabolic rate at night which may be reflected in their higher haematological value in samples collected early morning in comparison to grey herons which are diurnal and supposedly show a higher metabolism during day time.

Hb concentration is an index of the quantity of haemoglobin per unit volume of blood and is always proportionate to haematocrit.In this study, the Hb value was found to be 14.77 and 13.12 for the two herons which falls within the normal range of avian Hb concentration 11-16 mg/dL.PCV is the quickest

method for evaluating the red cell mass and normal range for avian species has been calculated to be 35%-55%. PCV value less than this range is assumed to be the indictor ofan anaemic condition where as a higher value outside this range may indicate dehydration or polycythaemia [17]. These results (43.26% and 40.87%) indicate that the birds taken for study were neither anaemic nor dehydrated. This is also supported by the MCV value (142.96 and 129.49) which falls within the normal range of 90-200fl.A similar conclusion may be drawn about these results with respect to MCH and MCHC values as they are in normal range [17]. Thrombocyte concentration of most avian species [17] studied ranges between 20 to30 (10^3 / mm^3). In the present study, it was recorded to be 18.4 and 17.1 for the two herons.No other study was found to have reported on thrombocyte count of herons.The leucogram in birds covers a broad range as it often varies widely even between normal birds of the same species.Though H:L ratio has been conventionally used as a parameter of stress in birds, many birds including the herons and egrets show a higher percentage of heterophils than lymphocytes. Moreover, birds that normally have greater number of heterophils, show less drastic change in stressful conditions [17]. This fact also is in support of the differential leucocyte count found in the present study which comes within the ranges reported normal in other studies [5]on herons.

Conclusion

Herons are an important group of birds in any wetland ecosystem.Being one of the major avian groups at the tertiary trophic level in an ecosystem as well as having a noticeable wide spread population by virtue of their breeding success, health of their heronries may easily reflect any alteration in biotic or abiotic components of the ecosystem , whether natural or induced by human activities.Thus, they may play a key role in studying the wellbeing of their environment.This study is an effort to record haematological data of two common wading species of the Chilika wetland which may be useful for future reference in ecological,wildlife as well as veterinary purposes.

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Original Article

The Effect of the Seasonal Variation and Sexual Dimorphism on the Erythrocytic Indices of Black Bengal Goats (*Capra aegagrus hircus)* in Nadia, West Bengal

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Abstract

Goat is a multipurpose animal and it is capable of producing meat, milk and hide. Nadia has a good number of Black Bengal goat populations, which has a vital role in the lives of local goat rearers in Nadia. The intention of the present study is to find out the effects of altering seasons on blood elements in Black Bengal goat (*Capra aegagrus hircus*) in Nadia district of West Bengal. The highest mean value of temperature (ºC) has been reported during the month of April and May in the pre-monsoon. The lowest mean value of temperature (ºC) has been reported during the month of December and January in the post-monsoon season. The parameter studied here are concentration of Hb, RBC count, PCV, MCV, MCH and MCHC. Data has been analyzed for the effect of seasonal variation among both the bucks and does. The present findings imply that seasonal variation plays the major role to influence the erythrocytic indices.

Keywords: Black Bengal Goats; Erythrocytes; Pre-Monsoon; Post-Monsoon; Nadia.

Introduction

Goat is a multipurpose animal; it can produce meat, milk and leather. According to All India Livestock census, the total goat population of the Nadia district is about 952143 [1]. Black Bengal goats or *Capra aegagrus hircus* [2] are among the best meat producer of India and are reputed as good meat producer of West Bengal and Bangladesh. Although goats are known to be adapted to harsh environments but their productivity is affected adversely by extreme climatic conditions. Lowering of food intake and decreasing in meat as well as milk production are commonly observed in heat stressed goat. Proper understanding of how the way climate plays a significant role in the physiological response of the goats gives us a proper idea for improving the husbandry and health status of goats [3].

Blood sample composition studies are essential to assess of health status and predict a number of diseases. The monitoring of blood constituents in a regular interval can predict the unnatural physiological condition and respective necessary action may prevent sudden mass destruction in goat husbandry from any sort of physiological changes due to pathogens or climatic factors. The present study was carried out to study seasonal variation in erythrocytic indices of Black Bengal goat in Nadia. The data is needed for physiological characterization of Black Bengal goat and helps to interpret the influence of ambient temperature on physiological condition of the Black Bengal goat [4].

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Materials and Methods

Animals: The animals used in this study were clinically healthy and physically normal looking Black Bengal goat (both Bucks and does) of 1 – 3 years of age and has an average body weight of 15 kg. The animals were taken from the Gayeshpur farm (KVK) ($22^0$57'19''N, $88^0$28'45'' E) and Mohanpur farm (WBUAFS research and extensions) ($22^0$56' N, $88^0$31' E) in Nadia district of West Bengal and there are no

feed restrictions to the goats [5].

Study Area

Nadia, fall under Lower Gangetic Plains region of India [6]. About 68% of the land is cultivated. The soil of this sub-zone is deltaic alluvial and the climate is per humid to humid. Annual rainfall ranging between 1200 mm to 1700 mm. The zone has a tropical climate with a short spell of winter season. The hot season lasts from mid-March to mid-June, with the day temperature ranging from 38°C to 45°C in different parts of this region. The monsoon arrives by the month of middle June. Winter extends about three months; the average minimum temperature not goes down below 10°C. Average rainfall of this area is 1,435.8 mm [6].

Climatological Measurement

The last five year data on temperature of the study area has been collected from the state meteorological department and the mean of the five years with standard deviation was calculated (Table 1) using MS-Excel 2013 and shown here in a tabular form.

Table 1: Mean temperature of last three years of Nadia district

Temperature (°C)	Pre-Monsoon			
	March	**April**	**May**	**June**
Max (ºC)	37 ± 2.45	38 ± 1	39.2 ± 1.5	36 ± 4.7
Min (ºC)	16 ± 3.9	19.4 ± 3.3	23.4 ± 1.5	23.6 ± 1.3
	Post-Monsoon			
	November	**December**	**January**	**February**
Max (ºC)	31.6 ± 1.2	28.75 ± 0.5	28.6 ± 1.5	32.2 ± 3.6
Min (ºC)	14.4 ± 2.8	11.5 ± 1.3	10.2 ± 1.6	12.6 ± 3

Blood Collection and Clinical Analysis

Data on blood parameters have been collected on apparently healthy goats using purposive sampling technique [7] for the year and categorized into two seasons. The season includes pre-monsoon and post-monsoon. About 6 ml of blood was collected by jugular venipuncture from each goat between 12 o'clock to 2 pm under the intense sun using standard method. The collected blood has been dispensed into di-potassium ethylene diamine tetra acetic acid (K_2EDTA) vials and labeled accordingly. The anticoagulants mixed blood then used to analyze for the packed cell volume (PCV), red blood cell (RBC), haemoglobin (Hb). Total erythrocyte count (TEC) or the RBC count has been done with the help of improved Neubauer counting chamber. The total hemoglobin (calculated in 10^6/µl) concentration in blood has been determined by the methods adapted from Jain et al. [8]. Determination of Hematocrit value or PCV (in %) has been done by hematocrit tube method and *mean corpuscular volume* or *mean cell volume or* MCV *(in* femtoliter per cell or fl), *mean corpuscular hemoglobin or* MCH *(*in picogram per cell or pg) and *mean corpuscular hemoglobin concentration or* MCHC *(in %)* have been calculated from the values of PCV, RBC and Hb [9].

Statistical Analysis

The statistical analysis of the data was performed using SPSS 21.01 [10]. Analysis of variance (ANOVA) test was used to determine the effects of season on the parameters studied here [11]. Mean separation was performed using MS-excel 2013.

Results

Effect of the Sexual Dimorphism

In the season of pre-monsoon there are no significant difference in the studying parameters between the bucks and does, this is happened may be due to the severe heat stress where both the sexes are respond similarly to the environment, so the parameters studying here has not been showing significant difference (Table 2). On the other hand in the post monsoon season where the mean temperature is lower, there are significant differences in the studying parameters between two sexes (Table 3) has been observed. Hb concentration has been significantly higher in bucks ($p < 0.05$). RBC count not significantly ($p > 0.05$) but higher in does. PCV, MCV and MCH have been significantly higher in bucks ($p < 0.05$) whereas MCHC has not been differing significantly.

Effect of the Seasonal Variations

There are significant differences between two

seasons (Table 4) in the bucks. Hb concentration is count significantly higher ($p < 0.01$) in pre-monsoon than in post-monsoon on the other hand RBC count is much lower ($p < 0.01$) in pre-monsoon than post-monsoon. PCV and MCV is significantly higher ($p < 0.01$) in pre-monsoon than the other season. MCH is significantly higher ($p < 0.01$) in pre-monsoon whereas MCHC has not been significantly ($p > 0.05$) differs between the two seasons.

In the case of does (Table 5) besides MCHC ($p > 0.05$), all the parameters are showing significant difference between the season of pre and post-monsoon. Hb concentration, PCV and MCV are significantly measured low during post monsoon than pre monsoon ($p < 0.01$) while on the other hand total erythrocyte count is higher in post monsoon ($p < 0.01$).

When we compile all the above findings in a single table (Table 6), we have been found that there was a significant difference between the parameters of pre-monsoon and post-monsoon. Hb concentration shows a significant difference ($p < 0.01$) between two seasons. RBC count was significantly low ($p < 0.01$). in pre-monsoon than post-monsoon. PCV and MCV has been significantly higher ($p < 0.01$) in pre-monsoon than post-monsoon. MCH count has been significantly goes down in post-monsoon ($p < 0.01$) and MCHC count has not been significantly differs ($p > 0.05$) between two seasons. Although in pre-monsoon MCHC value has been higher than post-monsoon like other above findings.

Table 2: The one-way ANOVA showing the effects of **sexual variations** on the erythrocytic indices of Black Bengal goats during in the season of pre-monsoon in Nadia

Parameters (unit)	Buck	Doe	Over all	*P-value*
Hb (g/dl)	12.64 ± 0.064	12.72 ± 0.25	12.7 ± 0.63	0.341^{NS}
RBC (millions/ mm^3)	7.22 ± 0.42	7.08 ± 0.51	7.15 ± 0.98	0.222^{NS}
PCV (%)	37.12 ± 0.22	36.98 ± 1.8	37.05 ± 1.86	0.311^{NS}
MCV (fl)	52.43 ± 3.54	52.54 ± 5.1	52.48 ± 6.02	0.968^{NS}
MCH (pg/ cell)	18.06 ± 1.3	17.93 ± 1.58	18 ± 2.02	0.49^{NS}
MCHC (%)	33.28 ± 0.58	33.54 ± 0.46	33.91 ± 0.61	0.455^{NS}

NS: not significant

Table 3: The one-way ANOVA showing the effects of **sexual variations** on the erythrocytic indices of Black Bengal goats during in the season of post-monsoon in Nadia

Parameters (unit)	Buck	Doe	Over all	*P-value*
Hb (g/dl)	9.04 ± 1.51	8.06 ± 0.62	8.45 ± 1.5	0.034*
RBC (millions/ mm^3)	8.35 ± 2.2	10.03 ± 1.9	9.19 ± 2.79	0.231^{NS}
PCV (%)	27.08 ± 3.46	25.06 ± 2.29	26.07 ± 4.02	0.045*
MCV (fl)	31.44 ± 4.84	28.36 ± 4.26	29.9 ± 6.78	0.039*
MCH (pg/ cell)	10.4 ± 1.38	9.34 ± 1.37	9.87 ±2.16	0.01*
MCHC (%)	33.38 ± 0.54	33.14 ± 0.6	33.26 ± 0.82	0.481^{NS}

*: $P < 0.05$; **: $P < 0.01$; NS: not significant

Table 4: The one-way ANOVA showing the effects of **seasonal variations** on the erythrocytic indices of Black Bengal bucks in Nadia

Parameters (unit)	Pre-monsoon	Post-monsoon	Over all	*P-value*
Hb (g/dl)	12.64 ± 0.064	9.04 ± 1.51	10.84 ± 2.12	0.0001**
RBC (millions/ mm^3)	7.22 ± 0.42	8.35 ± 2.2	7.79 ± 1.61	0.29^{NS}
PCV (%)	37.12 ± 0.22	27.08 ± 3.46	32.05 ± 5.68	0.0000014**
MCV (fl)	52.43 ± 3.54	31.44 ± 4.84	41.94 ± 11.34	0.000000004**
MCH (pg/ cell)	18.06 ± 1.3	10.4 ± 1.38	14.22 ± 4.04	0.0000000000064**
MCHC (%)	33.28 ± 0.58	33.38 ± 0.54	33.33 ± 0.45	0.74^{NS}

**: $P < 0.01$; NS: not significant

Table 5: The one-way ANOVA showing the effects of **seasonal variations** on the erythrocytic indices of Black Bengal does in Nadia

Parameters (unit)	Pre-monsoon	Post-monsoon	Over all	*P-value*
Hb (g/dl)	12.72 ± 0.25	8.06 ± 0.62	10.28 ± 2.52	0.0000000003**
RBC (millions/ mm^3)	7.08 ± 0.51	10.03 ± 1.9	8.555 ± 2.03	0.009968999**
PCV (%)	36.98 ± 1.8	25.06 ± 2.29	31.02 ± 6.25	0.000000000060**
MCV (fl)	52.54 ± 5.1	28.36 ± 4.26	40.45 ± 13.02	0.00000000014**
MCH (pg/ cell)	17.93 ± 1.58	9.34 ± 1.37	11.64 ± 4.57	0.0000000000033**
MCHC (%)	33.54 ± 0.46	33.14 ± 0.6	33.32 ± 0.52	0.25^{NS}

**: $P < 0.01$; NS: not significant

Table 6: The one-way ANOVA showing the effects of seasonal variations on the erythrocytic indices of Black Bengal goats in Nadia

Parameters (unit)	Pre-monsoon	Post-monsoon	Over all	*P-value*
Hb (g/dl)	12.7 ± 0.63	8.45 ± 1.5	10.58 ± 2.45	0.000000045**
RBC (millions/ mm^3)	7.15 ± 0.98	9.188 ± 2.79	8.17 ± 2.28	0.14^{NS}
PCV (%)	37.05 ± 1.86	26.07 ± 4.02	31.56 ± 6.4	0.00000017**
MCV (fl)	52.48 ± 6.02	29.9 ± 6.78	41.23 ± 13.11	0.00000069**
MCH (pg/ cell)	18 ± 2.02	9.87 ±2.16	13.84 ± 4.73	0.00000012**
MCHC (%)	33.91 ± 0.61	33.26 ± 0.82	33.59 ± 0.78	0.061^{NS}

**: P < 0.01; NS: not significant

Discussion

During the period of experiment the animals were gone through a marked seasonal variation of ambient temperature. The goat body temperature is rises along with the rising of ambient temperature, which secondarily increase the uptake of the water. The enormous drinking of water reduces the feed intake in extreme heat condition in pre-monsoon. In the tropical condition of India water intake by goat is relatively high in pre-monsoon than any other season [12]. Such variations in food habit influence the composition of blood in goat. Moreover, increased ambient temperature, and increased heart rate are linked with expansion of blood volume [13]. Feed intake of goat is lowers with the increase of ambient temperature [14]. Hemodilution can observe both well fed and non fed goats [15].

A high mean value of RBC count during pre-monsoon is obtained in present study. Holman and Dew [16] has been reported higher values of RBC count as well as PCV and Hb during summer compared to winter is the concord with the result of the present work. Another work by Pospisil et al. [17] reported lower values of RBC, PCV and Hb in winter than summer for Cameroon goats kept in temperate environment. The current findings indicate that MCV and MCH were significantly low. The low MCV value obtained could be related to the negative correlation between size and number of RBC that has been suggested by Holman and Dew [18]. The values obtained in the present study for MCV and MCH during post-monsoon and post-monsoon are in general concord with the findings of previous work by Gutierrez-De La *et al*. [19].

The virtual constancy of MCHC level in the present investigation may be recognized to associated increase as well as decrease of Hb concentration [20 - 21].

So we can conclude that, it is seasonal variation not the sexual one which influenced the erythrocytic indices of Black Bengal goat.

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Conflict of the Interest Statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Erratum

Article Titled **"Changing Climatic Scenarios: Role of Crop Growth Simulation Models"** Published in **Indian Journal of Biology, Volume 3 Number 1, January - June 2016, page 81-84.**

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Keywords in said article has been replaced by the author and now to be read as

"Keywords: Dynamic models; Climate change; Agricultural Crops."

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Review Article

Variation of Thermal Environment and its Effect on Performance of Wheat (*Triticum aestivum* L.) Under Future Global Warming

Ravi Kiran

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Abstract

High temperature during post-anthesis period has been found to decrease the yield and the yield attributing characters of Wheat (*Triticum aestivum* L.). While, High yield is correlated with low soil temperature between shoot elongation and beginning of heading. The increased carbon dioxide concentration is helpful in simulating the development of tiller buds. Carbon dioxide enriched wheat produced about twice the dry matter of control plants. Tillers and earhead numbers is also increased by carbon dioxide enrichment irrespective of N supply. Agroforestry can provide suitable microclimatic conditions for growth and development of wheat and also help to mitigate the hot weather and dry wind effects which are injurious to wheat plant at grain filling. Grain filling is not greatly affected by short shading period but increasing the length of period of shading brought about an accelerating yield reduction. The crop is most sensitive to shading at the time of rapid ear growth.

Keyworde: Wheat (*Triticum aestivum* L.); Modified Thermal Environment; Agroforestry; Climate Change.

Introduction

Global circulation climate models predict an increase in mean ambient temperatures between 1.8 and 5.8^{0}C by the end of this century (IPCC, 2007). Future climates will also be affected by greater variability in temperature and increased frequency of heat waves (Pittock, 2003). In addition to the general warming, a predicted increase in the occurrence of heat waves is likely to result in further yield losses (Long and Ort 2010).Exposure to excessive temperatures during development reduce the yield of many in the tropics. Increasing global temperatures and increasingly frequent heat waves are likely to have similarly negative effects on natural systems. The heat stress is mainly encountered in combination with water deficit and excess load of radiation , it can be difficult to separate the effects of the three factors. The effect of heat stress on staple crops like wheat can be severe depending on the developmental stage of the plants. High temperatures shorten the duration of various phenophase, accelerating their development and thereby limiting the ability of the plant to accumulate the carbohydrate necessary for grain growth. Wheat is exposed to high temperatute in many arid countries mean day temperatures beyond threshold during much of the growing season and so heat stress can significantly reduce crop yield by accelerating plant senescence, diminishing seed number and seed weight. Therefore, it is very much needed to acess the impace of changing climatic condition on overall growth and yield of wheat.

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Effect of Shading on Wheat

The effect of light on growth inhibitor in wheat root was studied. It was found that a growth inhibitor was present in the acid fraction and its concentration was much higher in root tips grown in light than in those grown in darkness. Masuda (1962). Friend *et al.*

(1962) reported that the length of lemina increased with each increase in temperature, but the breadth and thickness decreased. The greatest area of individual leaves was formed at 20°C. The greatest leaf area was also formed at 1000- 1750 f.c. Change in leaf shape under different environmental conditions were not directly related to change in leaf dry weight. Hsia and Wang (1964) studied the effect of light intensity on growth and development of wheat. In shaded plants (to 10 per cent of sunlight intensity) from sexual cell formation to flowering filled grain number / spike was markedly decreased, but weight / grain was increased. On shading from flowering to ripening number / spike was somewhat decreased and both weight / spike and weight / grain increased. There was a similar difference between the effects of shading from the first 10 days after flowering and for the following 10 days.

Pendleton and Weible (1965) conducted the shading studies (30, 60 and 90 per cent of shading) on winter wheat. It was concluded that all degrees and periods of shading adversely affected grain yield, which were reduced by 37, 70 and 99 per cent respectively, by the 3 degree of shading. Light was a critical factor during the heading stage, even slight restriction for short periods reduced yield. Wardlaw (1970) concluded that low yield which resulted from high temperature during the early period was caused by a reduction in seed set, which was partially compensated by increased grain size. In contrast high temperature during the later stages reduced the weight of individual grain. Low light at both stages of development significantly reduced grain weight per ear at maturity.

Effect of temperature change on wheat

Halsa and Weir (1974) studied the effect of temperature on spikelet number of wheat and found that temperature affected spikelet number. On providing increased total amount of illumination spikelet numbers were increased. Warrington *et al.* (1977) found the most important temperature effects during the ear development phase. Plants grown at low temperature at this time had long culms large flag leaves and more potentially fertile florets in each spikelet. The number of florets which produced harvestable grains and the weight of these grains at maturity, were affected by temperature during the grain growth stage. Closed relation was found between wheat and barley crop growth rates and radiation absorbed. It was reported that 2.2 g dry matter was produced per Maga Joule of radiant energy absorbed which was equivalent to a growth efficiency of approximately 3.9 percent. Gallagher and Biscoe (1978) The increasing temperature from 21 / 16°C to 30 / 25°C, during the period of development from anthesis to maturity found to substantially reduce grain dry weight in wheat. Altering either the demand for photosynthate by grain removal or the supply of photosynthates by a defoliation and shading treatment, did not prevent the reduction in grain dry weight due to high temperature and this was a further indication that the temperature effect occured mainly within, or close to the grain itself and did not result from an effect on availability of photosynthate (Wardlaw et al. ,1980). Manogaran (1982) proved that factors most significantly associated with wheat yield were monthly and seasonal values of potential evapotranspiration, soil moisture deficit and precipitation. Climatic variables accounted for > 30 per cent of yield variance. Bhuller and Jenner (1983) observed that brief warming of ears of wheat cv. Sonora reduced total grain weight due to reduction in grain number, individual grain weight and water content of the grain. Amores-Vergara and Cartwright (1984) showed that when wheat cv. Sonoro 64 was exposed to high temperature during 6 developmental stages and compared with growth of controls at continuous high (27°C) and continuous low (17°C) temperatures, the time for sowing to maturity was reduced more by later exposure to high temperature, especially from anthesis to maturity.Timefeenko and Urusova (1984) determined the correlation between grain yield and different meteorological factors using several regression equations and showed that heat sum, photosynthetically activated radiation, number of sunshine hour and R.H. were not factors limiting yield of winter wheat in dry zone of North-Caucasus. Iizuka *et al.* (1987) studied the influence of air temperature on growth of wheat cv. Norin - 61 from 1972 until 1986. Photoperiod affected maturity date more than air temperature with little variance between years. Morrison (1988) studied about the profound effect of increased atmospheric CO_2 concentration on plant growth and discussed the interactions of increased CO_2 concentration with low light intensities, restricted water supply, low temperature and atmospheric pollutants in wheat, *Vicia faba,* peas, *Liquidambar styraciflua,* soybean and okra. Caldiz and Sarandon (1988) grew wheat variety cv. Klein Toledo and Lapaz INTA that were and were not shaded with plastic grey mosquito mesh from seedling emergence until maximum spikelet number (M.S.N.), from MSN until maximum floret number stage (M.F.N.), from M.F.N. until anthesis, and from anthesis until grain maturity. Shading reduced final yield from 4.5 t / ha to a minimum of 2.65 t / ha with shading during development stages. Shading had no effect on 1000-grain weight or dry matter distribution between

leaves, stems and ears. Shading did not decrease maximum spikelet number in either cv. but spikelet number / ear decreased 15 per cent in Lapaz INTA and was not affected in Klein Toledo during period between terminal spikelet and maximum floret number stages of development.

Tashiro and Wardlaw (1989) reported that grain weight at maturity of wheat cultivars Banks was reduced by about 5 per cent for each 1°C rise in daily mean-post anthesis temperature in the range from 17.7 to 32.7°C using grain weight at 17.7°C as the base. In both wheat and rice there was a reduction in the duration of grain growth with increasing temperature up to a mean of about 26.7°C. In this range wheat did not show compensating increase in rate of dry matter accumulation. Above 26.7°C, the rate of dry matter accumulation feel down and the duration of grain growth continued to decrease.

Dawson and Wardlaw (1989) confirmed that exposure to high temperature only during anthesis generally had a small effect on grain set, where as other showed a decrease. Al-Khatib and Paulsen (1990) grew wheat of ten genotypes from major world wheat producing regions under moderate (22 / 17°C day / night) and high (32 / 27°C day / night) temperatures for 2 weeks as seedlings or from anthesis to maturity and showed that high temperature reduced mean kernel weight 20 per cent and mean grain yield 23 per cent relative to the moderate temperature. Relative grain yield was strongly influenced by decreased duration of photosynthetic activity which ranged widely in 10 genotypes. Genotypes that were most tolerant of high temperature had stable rates and / or long duration of photosynthetic activity high kernel weights, and high harvest indices.

Tashiro and Wardlaw (1990) reported that grain sterility was increased by high temperature 2-3 day prior to anthesis in wheat cv. Bank and this response was enhanced by high humidity. Parthenocarpic grains were induced by high temperature between anthesis and 3 days after anthesis. High temperature, 6-10 d after anthesis resulted in notched, split and opaque grains.

In an experiment Hunt et al. (1990) grew winter habit cultivars in doors under 16-h photo period and at a day / night temperature 20 / 15°C and spring habit cultivars at temperature ranging from 15 / 15°C to 30 / 25°C. Grain filling duration of spring wheat ranged from 56.4 to 47.0 d at 15 / 15°C and from 23.8 to 18.1 d at 30 / 25°C. Grain number per spike decreased from 15 / 15°C to 30 / 25°C. Hocking and Meyer (1991) studied the effect of CO_2 enrichment and nitrogen stress on growth in wheat and maize and concluded that CO_2 enriched wheat produced about twice the dry matter of control plants at all levels of N supply. There was no effect of CO_2 enrichment on specific leaf weight. CO_2 enriched wheat plant accumulated more N than the control but the proportional increase in N content was not great as than in dry matter. CO_2 enrichment increased N-use efficiency by wheat.

Savin and Slafer (1991) studied the effects of shading on the yield of Argentinian wheat crop. It was found that an applied shading treatment of 50 per cent of incident radiation reduced biological yields, above ground dry matter and grain yield of field grown wheat. Mehra *et al.* (1991) reported that in wheat cv. Kalyan sona and HD 2428, high temperature during the Ist week after anthesis decreased grain number / spike but increased 1000 grain weight, while high temperature in the 2nd and 3rd weeks after anthesis decreased 1000 grain weight particularly in HD 2428.

Rawson and Zajac (1993) reported fewer spikelets production per leaf at short photopheriod. High temperature delayed spike emergence in wheat both in controlled environments and in field. Lombardo et al. (1993) conducted field trial on four wheat cv. and showed that effective filling period duration decreased and grain filling rate increased with increasing air temperature. Both were positively correlated with number of seed per spike and grain yield. Sun (1994) reported that seedling growth and dry weight and were higher in different day / night temperatures than in constant temperatures. The shoot : root ratio was lower with diurnal temperature variation. Lower night temperatures were advantageous to root growth.

Scheeren et al. (1995) reported that shading significantly reduced the yield components and increased number of sterile spikelets per spike. The reduction caused by excess soil water were less significant, mainly as a result of environmental conditions during the growing season. Wardlaw and Moncur (1995) reported that high temperature resulted in a considerable drop in kernel dry weight at maturity. The importance of rate of kernel filling in determining varietal responses to high temperature illustrated the need to isolate the effect of temperature on process in kernel during the linear phase of growth. Chaurasia *et al.* (1995) showed in a field trial that grain filling duration depended on temperature and rainfall during February and March, but rate of grain growth depended on PAR interception and canopy temperature.

Delayed planting of wheat reduced the plant height, days to heading, days to maturity and grain

filling duration and ultimately showed the reduction in yield and yield components (Din & Singh, 2005).

To sustain wheat productivity under late planting, development of heat tolerant genotypes has been suggested. There is an average yield loss 1.7% per day, when sown beyond optimum time (Mohammadi, 2002).Grain filling duration contribute to the final yield of a plant that is a product of rate of grain filling and du-ration of the grain filling period. High temperature during grain-filling period may be with a degree of plant heat escape due to shortening of the grain filling duration by 0.4 day for each 1 °C increase in mean temperature from optimum temperature. (Tahir & Nakata, 2005).

Hays et al. (2007) reported that stress occurring after anthesis mostly has detrimental effects on wheat grain yield by hastening maturity, trigger premature senescence, shortening grain filling duration and reducing net assimilates and 1000 kernels weight. Heat stress during grain filling is responsible for shortening of grain growth period and improper grain filling affects over-all yield of wheat crop (Rane et al. 2007).

Conclusion

Excessive heat load affect the wheat crop during reproductive phase Under Mediterranean climate after anthesis. Modification of microclimatic conditions on field basis is not so easy. Introducing trees in the wheat field offers much scope for useful management of micro-climatic conditions for favourable purpose and thereby may also increase the yield as compared to monoculture. Therefore further research is needed to utilize the trees to optimize microclimatic conditions under global warming.

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29	Journal of Animal Feed Science and Technology	2	4100	3600	410	360
30	Journal of Cardiovascular Medicine and Surgery	2	10000	8600	910	860
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