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International Journal of Forensic Sciences	Semiannual	10000	9500	781	742
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Journal of Gerontology and Geriatric Nursing	Semiannual	5500	5000	430	391
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Indian Journal of Law and Human Behavior	Semiannual	6000	5500	469	430
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International Journal of Food, Nutrition & Dietetics	Triannual	5500	5000	430	391
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Original Article

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Exploration and Documentation of Flora and Fauna of Sacred Groves of Murshidabad, West Bengal, India

Biplab Bandyopadhyay[1], Malay Mandal[2], Ankush Pal[3], Santi Ranjan Dey[4], Mitu De[5]

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Abstract

Sacred groves are a common phenomenon in Murshidabad district. Specific areas of vegetation are conserved by the local people because of their religious beliefs. Usually vegetation that surrounds a temple, mosque, church, graveyard are regarded as sacred. It is usually a taboo to destroy flora and fauna in these areas. An extensive field work with the survey/exploration and documentation of flora and fauna within sacred groves of Murshidabad district was carried out over a period of two years. A total of 153 (One hundred and fifty three) sacred groves were recorded in Murshidabad with high floral diversity along with faunal diversity. This paper is the documentation of the flora and fauna within the sacred groves of Murshidabad following the survey work.

Keywords: Sacred groves; Documentation; Biodiversity; Repositories.

Introduction

Murshidabad district of West Bengal is located on the left bank of the mighty Ganga River. The Bhagirathi River flows through this district. This region had a rich history. Ancient mosques, temples, churches, cemeteries, monuments are quite common in Murshidabad district. Areas of lands around these structures are considered to be sacred among the people of different religious beliefs who reside here. It is usually a taboo to destroy flora and fauna in these areas.

Conservation of biodiversity due to religious beliefs helped flora and fauna to thrive within these tracts of vegetation, now popularly known as sacred groves. Often within the sacred groves local deities are found which are worshipped by the tribal communities even in recent times. Some other sacred groves become a religious place example Takib Shah Pirtala, which is about 200+ years old. It was observed by Deb and Malhotra in 1997 that often the sacred grove was used for religious purposes like fairs during the celebration of certain festivals. Some workers felt that the sacred Groves were examples of the love humans have towards animals and plants which is just an expression a of the love and respect of nature (Wilson 1988; Deb and Malhotra 2001).

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Both ancient and recent sacred groves around temples are found in Murshidabad, some examples Pataleswar Shiv Mondir (250+ years), Kiriteswari Temple (from time immemorial), Domdoma Kali Mondir (40+ years), Ramnagarghat Radhagobinda Mondir (20 years). In Murshidabad district there are several cemeteries with lush vegetation around them. They are usually known as 'Koborsthan' in the local language meaning place of burial of the dead. Some sacred groves with cemeteries in Murshidabad are Talbagan Kaborsthan (100+ years), Elahiganj Cemetry (150+ years), Baro Bigha Kaborsthan (100+ years) etc. There are graveyards too example Residency Cemetery of Babulbona (European Cemetery about 200+ years).

A total of 153 (One hundred and fifty three) sacred groves were recorded in Murshidabad with high floral diversity along with faunal diversity. An extensive field work with the survey/exploration and documentation of flora and fauna within sacred groves of Murshidabad district was carried out over a period of two years. This paper is the documentation of the flora and fauna within the sacred groves of Murshidabad following the survey work.

Material and Methods

An extensive field work with the survey/ exploration and documentation of flora and fauna within sacred groves was carried out during 2014 to 2016. The plants within the Sacred groves were identified with the help of local Floras. The animals within the sacred groves were listed. However the insects and invertebrates are not recorded during this investigation. Among the vertebrates Amphibia, Reptilia and Mammalia were listed during this study.

Study area

Location & Geographical Area

Murshidabad district lies between latitude $23^0 43'30''$ & $24^0 50'20'''30''$ North and longitude $87^0 49'17''$ & $88^0 46'00''$ East.

Results

The entire Murshidabad district was surveyed in search of Sacred Groves. It is found that sacred Groves were of two types in this district. In the first type the sacred groves surrounded temples of Hindu deities. In the second type a Muslim graveyard or "Muslim Kabarsthan." Around the Hindu temples there were several plants which considered as religious trees like *Cocos nucifera* (coconut), *Ficus religiosa* (sacred fig), *Aegle marmelos* (wood apple) etc. Around these trees which have a religious importance there are several herbaceous plant associations. Similarly many small patches of vegetation were found in the "Kabarsthan" where many trees were considered as "Sacred Trees". In this investigation only the places having high diversity of vegetation, protected by walls, maintained by a statutory authority and age old were listed as sacred groves in this study. A total of 153 (One hundred and fifty three) sacred groves were recorded with high floral diversity along with faunal diversity. A list of major flora of sacred groves in Murshidabad is given in Table 1. A list of major fauna of sacred groves in Murshidabad is given in Table 2.

Table 1: Major Flora of Sacred Groves of Murshidabad

Type	Name of the plants
Sacred Tree and associated vegetation	*Aegle marmelos, Alocasia fornicata, Annona reticulate, Areca catechu, Bombax ceiba, Cassia fistula, Cocos nucifera, Comellina benghalensis, Costus speciosa, Ecbolium viridae, Ficus hispida, Ficus recemosa, Ficus religiosa, Hemidesmus indicus, Litsea glutinosa, Moringa oleifera, Polyalthea subarosa, Tinospora cordifolia.*
Major plants in Sacred Groves with Temples and Kabarsthan (graveyard)	*Aegle marmelos, Annona reticulate, Azadiracta indica, Bombax ceiba, Calotropis gigantean, Cassia fistula, Cassia sophera, Chrozophora rottleri , Ceratophylum sp. Clerodendrum viscosum, Croton bonplandianus, Cynodon dactylon, Cyperus rotendus, Eichornia crassipes, Ficus religiosa, Glycosmis pentaphylla, Hydrilla verticillata. Lantana camara, Moringa oleifera, Scirpus articulates, Trewia nudiflora, Typha domingensis, Zizipus mauritiana.*
Wall flora in old walls of Ancient monuments	*Murshidabad district is famous for historical places and there are a widespread of monuments, temples, mosques like Katra Mosque, Hazarduari, Khosbagh, Siva-temple of Rani Bhabani, Moti Jhil. Temple of Jagat Seth, Palace of Kashimbazar, Lalkuthi and many others which have recognizable wall floral association of Boerhaavia diffusa, Euphorbia hirta, Euphorbia prostrate, Ficus benghalensis, Ficus religiosa, Lindenbergia indica, Peperomia pellucida, Pilea microphylla, Tridex procumbens, Vernonia cinerea.*
Parasitic plants	*Cuscuta reflexa, Macrosolen cochinensis.*
Common Epiphytes	*Vanda tesselata, Rhyncostylis retusa.*

Table 2: List of Amphibia, Reptilia and Mammalia in the Sacred Groves of Murshidabad.

Type	Names of Animals	Common Name in Bengali
Amphibia	*Haplobatracus tigrina*	Sonabeng
	Duttaphrynus melanostictus	Kunobeng
	Rhacophorus sp	Gechobeng
	Euphlyctis hexadactyla (Lesson)	Sonabeng
	Limnonectes limnocharis	Jhijhibeng
	Pedostibes tuberculosus Gunther	Gechobeng
Reptilia	*Trionyx gangeticus*	Kochop
	Chrysemys picta	Kocchop
	Varanus monitor	Gosap
	Hemidactylus flaviviridis	Tiktiki
	Mabuya mabuya	Sapermasi
	Calotes versicolor	Raktochosa
	Ptyas mucosus	Hele
	Naja naja	Keute
	Vipera russelli	Sakhamuti/chiti
	Gavialis gangeticus	Gharial [only near Jangipur and in Motijheel(reported)]
Mammalia	*Platamista gangetica*	Sushuk
	Funambulus sp	
	Mus musculus	Idur
	Rottus rottus	Dhere idur
	Pteropus sp	Badur
	Rhinolophus sp	Chamchiki
	Talpa Sp	
	Bandicota bengalensis	Dhere idur
	Herpestes javanicus	Beji
	Presbytis entellus	
	Felis domesticus	Biral
	Canis familaris	Sheal
	Canis aureus	Kukur
	Vulpes bengalensis	Sheal

Discussion

Sacred groves are found all over India. It is believed that the sacred groves represent a variety of ecosystems, social and ethnic identities, management regimes, legal tenures, and cultural traditions (Ray *et al.* 2014). Nowadays these sacred groves are a subject of great interest to biologists, social scientists, anthropologists and policy makers. These sacred groves provide are believed to be the strong link between our past and present in terms of biodiversity and ethnic heritage (Khan *et al,* 2008).

Urbanization has been cited as a major cause of biodiversity loss in many countries. In the 2016 publication on an overview on sacred groves it was stressed that the pressures of growing urbanization and industrialization, the need for roads and housing and other infrastructure had eaten into the area of the groves (Amirthalingam, 2016). Sacred groves are those patches of vegetation that escaped the destruction as the place was considered religious by the local people. Ethnic heritage seems to have played a significant role in the conservation of flora within the sacred grove over many years. Many of the sacred groves in Murshidabad are more than 100 years old, a few over 200 years. These sacred groves are samples of the climax vegetation that once covered the area. Within the plant community various faunal groups are still thriving. Had it not been for the sacred groves many of the Reptilia, Amphibia and Mamamlia members would have been killed out of fear by humans.

Conclusion

In the present investigation it has been found that flora and fauna are conserved within the sacred groves of Murshidabad to a great extent till now. Sacred groves are the repositories of the local flora and fauna of Murshidabad. It is of paramount importance to conserve the sacred groves itself for overall biodiversity conservation.

Conflicts of Interest The authors declare that there are no conflicts of interest regarding the publication of this work.

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Original Article

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Green Synthesis, Characterization, and Antibacterial Activity of Silver Nanoparticles using *Ocimum sanctum* (Tulsi) Leaf Extract

Gitanjali Mishra[1], Sagarika Satapathy[2], B. Rabiprasad[3], Diptikanta Acharya[4]

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Gitanjali Mishra, Sagarika Satapathy, B. Rabiprasad Diptikanta Acharya. Green Synthesis, Characterization, and Antibacterial Activity of Silver Nanoparticles using *Ocimum sanctum* (Tulsi) Leaf Extract. Indian J Biol. 2019;6(1):9-14.

Abstract

Green nanotechnology has now developed as a new distinct field of research in modern science and technology. The green nanotechnology, which can be also termed as "bionanotechnology" is based upon the nanoparticles synthesis from the natural sources. Among the various availability of natural sources the treatment of plant extract makes the process of synthesis of nanoparticles more easier, cost effective and eco-friendly. The leaves extract of *Ocimum sanctum* were used as a reducing and stabilizing agent for the synthesis of silver nanoparticles (AgNPs). The biosynthesized nanoparticles were characterized by the help of UV–VIS spectrophotometer, FTIR and SEM analysis and their antimicrobial activity was screened against *Micrcoccus luteus, Escherichia coli* and *Pseudomonas aeruginosa.*

Keywords: Biosynthesis; Plant extract; *Ocimum sanctum*– Silver nanoparticles; Antimicrobial activity.

Introduction

Nanotechnology is said as the field of engineering which deals with the fine tuning of matter at the atomic, molecular and sub molecular level. Nanoparticles are implicit to be equally natural and synthetic designed particles lesser than 100 nm. Green nanotechnology or nanobiotechnology has become apparent as a distinguish part of nanotechnology in which the nanoparticles are produces from the natural sources. Various natural sources including plants, algae and microbes have been widely used for the manufacture of nanoparticles. Nanoparticles manifest completely latest along with enhanced characteristics based on specific features as morphology, size and distribution, if compared with larger particles of the bulk material they are made of. These properties widely differ as such as mechanical, electrical conductivity, catalytic activity, melting point, thermal and, optical absorption. From the earlier period to till date, Indian greeneries have been widely explored for their medicinal properties. Recently, as the rapid development occur in the field of research, many such plants have been acquiring importance due to their unique constituents and their versatile application [1]. *Ocimum sanctum*, holy basil, or *tulasi* (also spelled *thulasi*), its an aromatic plant belongs to the family Lamiaceae which is native to the Indian subcontinent and worldwide as a cultivated plant all through the Southeast

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Asian tropics [2]. In a large scale polygeographical investigation of this species governed by utilizing chloroplast genome sequences, the plant originated from North Central India which have been propounded by a group of researchers from Central University of Punjab, Bathinda [2].

Basil is considered to be sacred, medicinal and has extensive application in the indigenous medicinal system in the Asian countries and also it having the well known medicinal properties such as antibacterial, antifungal, antiseptic, antipyretic, anticancer, and antioxidant [3]. Oleanolic acid, ursolic acid, rosmarinic acid, eugenol, carvacrol, linalool, β-caryophyllene (about 8%), β-elemene (11%) and germacrene D (about 2%) are some of the main chemical constituents present in tulsi.[4] *Ocimum sanctum* was generally used for the making of silver nanoparticles. Because of their large ratio of surface-to-bulk silver atoms silver nanoparticles are the kind of nanoparticles of silver composed of large amount of silver oxides. silver nanoparticles are even found to be better than the nanoparticles of other metals on various parameters. Both silver nanoparticles and gold nanoparticles are commonly used in the application of optical detection for their surface plasmon resonance effect [5]. The efficiency of Plasmon excitation of silver nanoparticles is known to be more distinct than that of gold nano particles, which has shown in their stronger, sharper plasmon resonance peaks at the same particle concentration. Thus silver nanoparticles can be provide better idea for certain applications like localized plasmon resonance or surface enhanced Raman scattering detection. When being used along with the fluorescence emission detection silver nanoparticles turn more advantageous than gold nanoparticles. The emission range of fluorophores at a wavelength above 500 nm but the absorbance range of Plasmon resonance of gold nanoparticles is primarily of 500-600 nm, and therefore satishfy the detectable fluorescence upto certain range, when the fluorescent dyes are in close proximity to the particle surface.By this concern the fluorescence quenching is minimized for silver nanoparticles, as their plasmon resonance absorbance is mostly underneath 500 nm, which is little convergence with the emission wavelength of mostly fluorescent dyes [6]. Conventionally, various types of physical as well as chemical methods were used for the manufacturing of the nanoparticles. The physical vapour condensation (PVC), arc-discharge method, laser ablation, evaporation-condensation, sputtering, and mechanical milling among which evaporation-condensation and laser ablation are the kind of common physical methods mostly used [7]. Physical methods for the synthesis process never involving toxic chemicals and becomes fast. To prevent against agglomeration these methods necessiatate the usage of stabilizers to secure the Ag nanoparticles so that pure silver nanoparticles can be produced. The chemical method includes variety of methods like pyrolysis (spray and aerosol), chemical etching, sputtering and sol-gel process amongst which chemical reduction using various reducing agents is most widely used [8]. For reduction of silver ions (Ag+) in aqueous or non-aqueous solutions different kinds of organic and inorganic reducing agents, such as sodium borohydride (NaBH4), sodium citrate, ascorbate, elemental hydrogen, Tollen's reagent, N,N-dimethyl formamide (DMF) and poly (ethylene glycol) block copolymers are commonly used [9]. The conventional methods have been used for a very long time and are still in use. But these methods are expensive, laborious and toxic. Hence, the alternative method such as biological method used for the synthesis of nanoparticles from natural source has been put forward that tend to be eco-friendly. The natural sources may be microbes, plants or algae [10]. But the plant extract involvement eliminates elaborate processes of maintaining microbial cultures. From the experimental Studies it have been manifested that *Alfaalfa* roots can bitterly absorb Ag (0) from agar medium and are also able to transport it to the plant shoot in the same state of oxidation. Various plants including M. *balbisiana*, *A. indica*, *Crataegus douglasi, and Acalypha indica* have been used for the manufacturing of silver nanoparticles [11]. Here we have been designed a rapid, convenient and eco-friendly green methodology i.e. for the synthesis of silver nanoparticles from silver nitrate by using leaf extracts of *Ocimum sanctum* (green tulsi). The plant mediated synthesized Ag NPs were properly characterized and studied in details with all of their properties which is most significant to current science and prevailing technologies as per the current research is concern.

The characterization was made to understand the potentiality of nanoparticles which can be done by having a understanding knowledge of their synthesis along with applications. The characterization techniques included UV-VIS spectroscopy, further, anti-microbial testing was performed using the three bacteria namely, *Escherichia coli*, *Micrcoccus luteus* and *Pseudomonas aeroginosa*. In response to the antimicrobial activity of the plant extract the zone of inhibition was obtained which was evaluated by disc diffusion method. From the obtained results from analysis

of the antimicrobial property of the plant extracts make sure that they are safe to be released into the environment and hence suitable to be concerned for pollution remediation.

Materials and Methods

Collection of Plant materials

O. sanctum leaves were taken from the botanical garden of Department of Biotechnology, GIET University Gunupur, odisha, India. The reagents such as Nutrient Agar was supplied by (Cat. No.: M001, Himedia, Mumbai and Silver nitrate (Cat.No.: 209139, Sigma Aldrich, India). Bacterial cultures (*Micrcoccus luteus* MCC 2408; *Pseudomonas aeruginosa*. MCC 2511; *Escherichia coli* MCC 2155) were procured.

Preparation of O. sanctum leaf extract and 1 mM $AgNO_3$

Fresh leaves of O. sanctum (100 g) were transferred to sterile 250 mL conical flask and diced into fine pieces. The leaves were washed with tap water and distilled water. The leaves were incubated in oven for four hours at 800C and then grinded into fine powder. 100 mL leaf powder solution was prepared and heated at 60 0C for one hour to prepare the aqueous extract. Then the extract was filtered using Whatman No. 1 filter paper and the filtrate was stored at 4^0C for further use. Silver nitrate ($AgNO_3$, Sigma Aldrich, USA), 0.0421 gm was added to 100 mL of double distilled water and dissolved thoroughly. To prevent the auto oxidation of silver, the solution was preserved in amber coloured bottle.

Determination and synthesis of silver nanoparticles

The aqueous leaf extract of O. *sanctum* and 1 mM $AgNO_3$ were mixed in the ratio of 1:10 and was kept in water bath shaker at 60^0C for 30 min until change in color was observed. The generation of silver nanoparticles in leaf extract solution is confirmed by changes in color.

UV–visible spectrometric analysis.

Samples (1mL) of the AgNPs solution were collected sporadically to observe the completion of bioreduction of Ag+ in solution, which is directed by dilution of the samples with 2 ml of de ionized water and consequent scan in UV-visible (vis) spectra, of wave length 450 nm in a UV-vis spectrometer (UV-1800 spectrophotometer, Shimadzu, Japan), having a resolution of 1 nm. UV-VIS spectra were recorded at intervals of 0 min, 30 min and 24 h.

Fourier transform infrared (FTIR) analysis of silver nanoparticles.

The uncapping ligands of 200 ml residual solution of AgNPs can be done by centrifuging at 10,000 rpm for 30 min and the precipitate was kept in 10 ml ethanol and distilled water and the process was repeated 3-4 times. The powder of purified AgNPs was prepared by drying in oven and then analyzed by Fourier Transform Infrared (PerkinElmer, MA, USA).

Scanning electron microscopy of silver nanoparticles.

The AgNPs pellet was dehydrated in an oven and thin films of dried samples (10 mg/mL) were arranged on carbon coated copper grid and analyzed for size determination. The SEM analysis was done to determine the particle size and texture of nanoparticles and the presence and formation of silver nanoparticles.

Minimum inhibitor concentration (MIC) and minimum bacteriocidal concentration (MBC) studies

The MIC and MBC studies were done to find out the concentration of biosynthesized silver nanoparticles showing growth inhibition of bacterial strains.

Results and Discussion

AgNP characterization

UV-vis analysis

As a result of surface Plasmon vibration Silver nanoparticles (AgNPs) appear yellowish brown in color in aqueous medium [12]. By the addition of leaf extracts to aqueous solution of silver nitrate, the color of the solution converted from faint light to yellowish brown to reddish brown and finally becames colloidal brown which indicating formation of AgNP. Similar changes in color also have been detected in previous studies [13] and therefore confirmed that completion of reaction occured between leaf extract and $AgNO_3$. After some time intervals of 15 min, 30 min, 45 min, 60 min and 24 h from the initiation of reaction are shown in Figure 1, the UV-VIS spectra recorded. Due to the surface Plasmon resonance of AgNPs, formation of absorption spectra of AgNPs occured in the reaction media with the absorption maxima

in the range of 450 nm. From the UV-VIS spectra, it has been indicated that most rapid bioreduction denoted by broadening of them peak which implied the formation of poly dispersion of large nanoparticles due to slow reduction rates [14]. And also the UV-VIS spectra also exhibited that formation of AgNPs occurred more rapidly within the first 15 mins and the AgNPs remained stabilized in the solution even after 24 h of completion of reaction.

FTIR analysis

From the FTIR analysis it has been characterized that the AgNPs acquired from tulsi plant extract (Curve A) which has shown in Figure 2. Prominent bands of absorbance were observed at around cm^{-1} in all the AgNPs solution. The stretched bands, vibrational bands responsible for the existence of compounds like flavonoids and terpenoids [15] and also may be responsible for stabilization of obtained AgNPs and efficient capping.

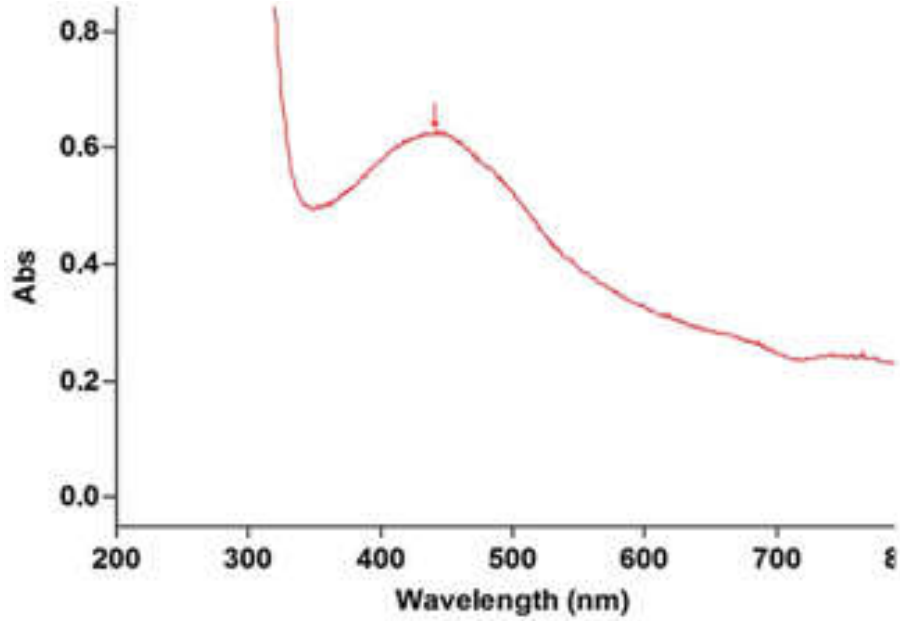

Fig. 1: UV-VIS absorption maxima of silver nano particles. From the data it has been estimated that based upon the presence of absorbance peak of AgNPs solution at the wavelength range of 300-800 nm the absorption maxima were found to be 451 nm.

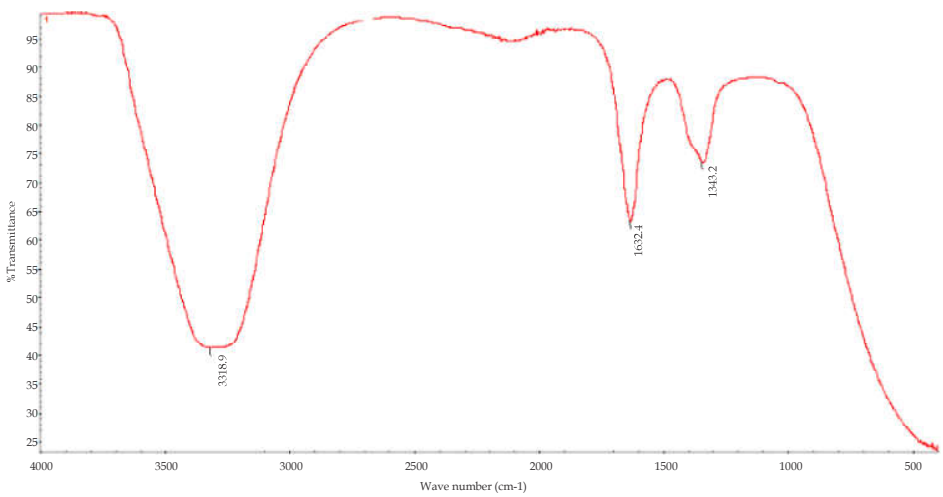

Fig. 2: FTIR graph of synthesised Silver Nano particle. The peaks are obtained at 1343.2,1523.4 and 3343.9.

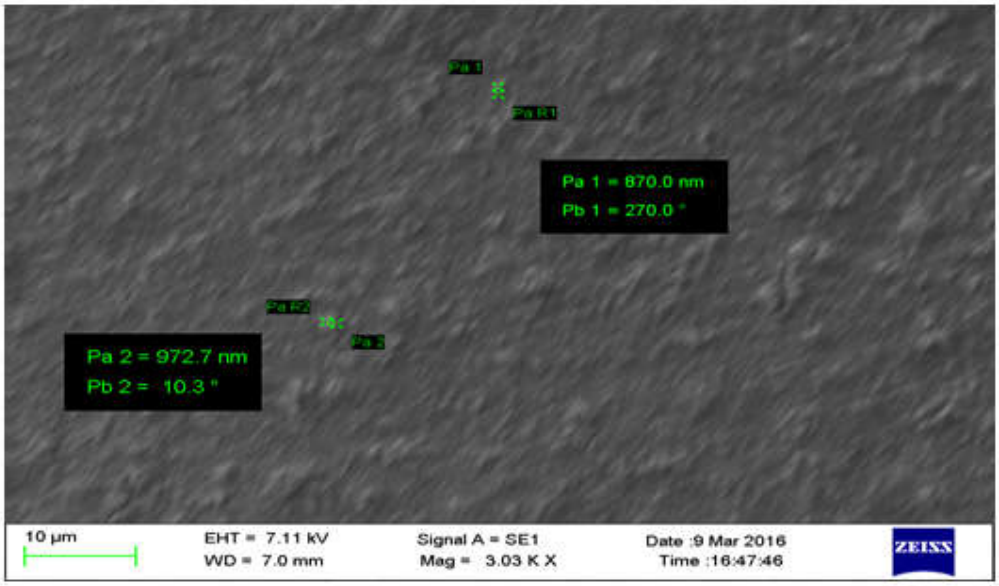

Fig. 3: SEM Analysis

Table 1: Antibacterial Properties

Extracts	Bacteria	Zone of inhibition in (mm)				
		By Leaf extract	By AgN	By AgNPs 1:4	By AgNPs 1:10	By AgNPs 1:20
Tulsi (leaf)	M. luteus	7 ± 0.03	6 ± 0.04	8 ± 0.004	13 ± 0.043	14 ± 0.002
	P. aeruginosa	7 ± 0.04	6 ± 0.002	7 ± 0.03	14 ± 0.008	17 ± 0.6
	E. coli	7 ± 0.15	6 ± 0.025	4 ± 0.006	3 ± 0.01	4 ± 0.022
Mehndi (leaf)	M. luteus	5 ± 0.07	6 ± 0.008	19 ± 0.1	13 ± 0.3	13 ± 0.04
	P. aeruginosa	5 ± 0.004	6 ± 0.05	11 ± 0.04	6 ± 0.009	15 ± 0.013
	E. coli	5 ± 0.023	6 ± 0.016	7 ± 0.021	10 ± 0.019	8 ± 0.009
Bramhi (leaf)	M. luteus	9 ± 0.004	6 ± 0.04	24 ± 0.02	19 ± 0.06	16 ± 0.002
	P. aeruginosa	9 ± 0.06	6 ± 0.015	7 ± 0.005	7 ± 0.003	10 ± 0.011
	E. coli	9 ± 0.05	6 ± 0.027	12 ± 0.4	11 ± 0.05	9 ± 0.035

SEM analysis

The AgNPs, SEM images has been shown in Figure 3. It has been estimated that AgNPs obtained in case of Tulsi leaf extracts appear to be cuboidal in shape and can be utilized as reducing as well as capping agents, availability of different quantity and nature of capping agents sustained in the leaf extracts. The shifts and difference in areas of the peaks obtained through FTIR analysis.

Antibacterial property analysis

By the supplement of AgNPs on nutrient agar culture media, its antimicrobial property investigated against *Micrcoccus luteus*, *Escherichia coli* and *Pseudomonas aeruginosa*. A control culture plate was separately maintained for the microorganisms from water. Results achieved has been shown in Table 1. The obtained zone of inhibition indicates that maximum antibacterial activity occurred in the prepared test sample. Results achieved in previous studies [16] also bear the antibacterial potential of AgNPs. In contrast to $AgNO_3$ and AgNPs,there is no such prominent antimicrobial activity found when crude form of plant extracts used and in control no zone of inhibition was acquired.

Conclusion

The green synthesis and characterization of AgNPs was done and confirmed by UV–VIS spectrophotometer. The nanoparticles appeared to be in shape with. The growth inhibitory value against bacterial species exhibited by MIC and MBC of the AgNPs. In summary, the extract of *O. sanctum* intervene the efficient synthesis of silver nanoparticles and provides additional property such as bacteriocidal efficiency and might act as long searched substitute and could be the response to antibiotic resistance.

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Prevalence of Hypertension, and Dietary Correlation with Blood Pressure in the Islamic Community of the District Murshidabad and Adjoining Areas

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Abstract

The present study was undertaken to evaluate the current trend in hypertension and its prevalence and awareness within the people of Murshidabad district of West Bengal. Multistage cross-sectional survey was carried out in adults aged between 18 to 67 living in the district. Structured interview schedule was used to interview 2114 study subjects. The study showed mean systolic and diastolic BP were 129.99 (± 15.95) mmHg and 81.50 (± 9.47) mmHg, respectively. The overall prevalence of hypertension was 33.54% and the sex-specific prevalence was 36.25% and 26.38% for males and females respectively. Higher age group subjects specifically males were at high risk. Only 192 (27.08%) of 709 hypertensive subjects were aware of hypertension and was taking medicine and out of which only 79 (41.15%) subjects had BP under control. So from the study it can be concluded that prevalence of prehypertension was very high in the study subjects (50.78%) and around one-third of the subjects were hypertensive. The treatment and control of high blood pressure were also very low.

Keywords: Hypertension; Prevalence of HTN; Murshidabad; BMI.

Introduction

Hypertension or high blood pressure is the major risk factor for public health because of its prevalence worldwide [1,8,16,19]. Around 7.5 million deaths or 12.8% of the total of all annual deaths worldwide occur due to high blood pressure [1,5]. Hypertension is a major risk factor for chronic heart disease, stroke, and coronary heart disease. Elevated Blood pressure is also associated with heart failure, peripheral vascular disease, renal impairment, retinal haemorrhage, and visual impairment.

The prevalence of Hypertension is increasing at an alarming rate throughout the globe [8] and particularity the countries with low and middle income, like India, are at higher risk [1]. Several studies in India suggest an increasing trend in its prevalence [11,13,14,21]. However, the data on trends of blood pressure distribution, hypertension prevalence, and awareness is scarce, fragmentary and heterogeneous. Particularly the data from the eastern region is poor [28]. These data are important in formulating key public health policies by the government, and non-government agencies. For Murshidabad condition is even worse the only data which is available on hypertension (HTN) is

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the National Family Health Survey – 4, Ministry of Health and Family Welfare, Government of India [4]. The data is showing an overall reduction in the prevalence of hypertension from 2012-13 to 2015-16 from a total of 23.8% (age 18 years and above) to 5.2% (age 15-49 years) [3,4]. The recent trend of rapid urbanization, sedentary life, dietary changes, and change in sleeping habit is acting to increase the risk of HTN throughout India and the Globe. To assess the actual situation regarding prevalence and awareness of HTN prevailing in this area we conducted multistage crosssectional surveys from 2013 to 2018. These surveys enabled us to estimate the true nature of blood pressure prevalence and its management in this population over this time period.

Materials and Methods

Study Area

Murshidabad is a district in the state of West Bengal, India. According to the 2011 Census it has a total population of 7,103,807. Out of this total population 3,627,564 is male and 3,476,243 is female and most of them (5,703,115) lives in rural area only 1,400,692 live in urban area [2].

Study Design and sample size

A multistage cross-sectional survey was carried out in the age group between 18 to 67 living in the selected area using a simple random sampling method. The minimum sample size calculated was 385 at 95% confidence level with permissible error as 5% and taking the most probable prevalence of hypertension as 50%. Considering the multistage nature of the study and to improve the accuracy 2114 individuals were surveyed.

Sampling methodology

Multistage sampling was used, at first out of 26 blocks of the district 5 blocks were selected using simple random sampling. Camps were set at each block and eligible candidates were selected randomly for the study. Individuals were then interviewed with prior consent using a structured interview schedule.

Selection of study subjects

Individuals of age group 18-67 were selected for the study, individuals suffering from serious physical or mental illness, pregnant women were excluded from the study.

Tools used in the study

Pretested structured interview schedule, OMRON digital body weight scale and KRUPS mechanical weighing scale for weight measurement, portable stature meter for height measurement, anthropometric tapes and for B.P. measurement OMRON digital (model no HEM 4030 two in number) and Dr. Morepen Aneroid blood pressure monitors were used.

Blood Pressure and Anthropometric Measurements

For Blood Pressure and Anthropometric Measurements, established methods were used.

Statistical Analysis

The data collected through the survey was initially entered into Microsoft Excel and analysed using IBM SPSS version 20.

Definitions Used

For Hypertension, Prehypertension, Isolated systolic/diastolic hypertension the classification of 7th report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC-7) was followed. And for BMI and WHR, classification of WHO was followed.

Hypertension is defined as systolic BP level of ≥ 140 mmHg and/or diastolic BP level of ≥ 90 mmHg or being previously diagnosed as hypertensive by any health professional.

Prehypertension is defined as 120-139 mmHg of systolic and/or 80-89 mmHg of diastolic BP.

The condition of systolic BP ≥ 140 mmHg and diastolic BP <90 mmHg is defined as Isolated systolic hypertension and systolic blood pressure < 140 mmHg and diastolic blood pressure ≥ 90 mmHg as Isolated diastolic hypertension.

Awareness of Hypertension was defined as history of diagnosis of hypertension by a healthcare provider. And BP under control was defined as blood pressure <140 and <90 mmHg in subjects who were taking medications.

BMI <18.5 was classified as "underweight"; 18.50–24.99 as "normal"; ≥ 25.00 as "overweight"; 25.0–29.99 as "pre-obese" and ≥30.00, as "obese".

Abdominal Obesity was defined as WHR >0.90 in case of male and WHR >0.85 in case of female.

Results

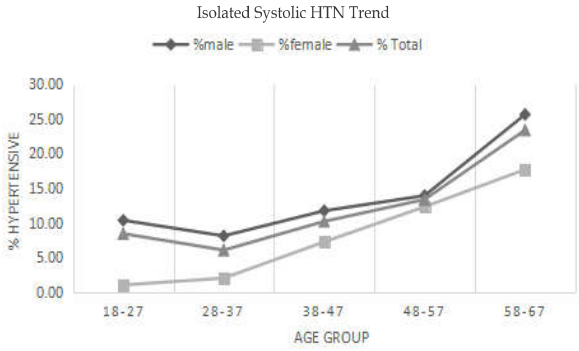
Out of 2114 persons interviewed during the survey, 1534 (72.56%) were male and 580 (27.44%) were female. The number of female participants was low because most refused to undergo the anthropometric measurements. The overall (including both male and female) median age (± *SD*) of the study subjects was 29 (± 12.57), for males it was 27 (± 12.78) and for females, it was 33 (± 11.77). More than half (55.82%) of the study subjects were married. On the obesity parameter (BMI), only 3.74% were obese 25.73% were overweight, a significant proportion (10.22%) were underweight, rest were normal. The mean BMI for male and female were respectively 22.79 (± 3.37) and 24.13 (± 4.10). According to abdominal obesity as measured by the waist-hip ratio (WHR) 48.06% (34.53%, 13.53% for male and female respectively) were at risk (Table 1).

Table 1: Background Characteristics of study subjects variables N (2114) Proportions (%).

Age group		
18-27	975	46.12
28-37	460	21.76
38-47	329	15.56
48-57	248	11.73
58-67	102	04.82
Sex		
Male	1534	72.56
Female	580	27.44
Other	0	00.00
Marital Status		
Married	1180	55.82
Unmarried	930	43.99
Other (divorced widow widower etc)	4	00.19
BMI		
Underweight	216	10.22
Normal	1275	60.31
Overweight	544	25.73
Obese	79	03.74
Abdominal Obesity (WHR)		
Male (>0.9.)	730	34.53
Female (>0.85)	286	13.53
Total	1016	48.06

The mean (± *SD*) overall systolic and diastolic BP of the study subjects were 129.99 (± 15.95) mmHg and 81.50 (± 9.47) mmHg, respectively. Male study subjects had higher mean BP than the females in both systolic and diastolic catagory (131.71 ± 15.02 vs 125.43 ± 17.41 in case of systolic BP and 81.98 ± 9.21 vs 80.22 ± 10.01). In case of diastolic BP across the age class, BP shows an increase in trend with the increase in age in both male and female. In case of men, highest mean BP (both systolic and diastolic) were among the eldest group (57-67) while in females the highest mean systolic BP was among eldest two groups and highest diastolic BP was in age group 48-57 (Table 2).

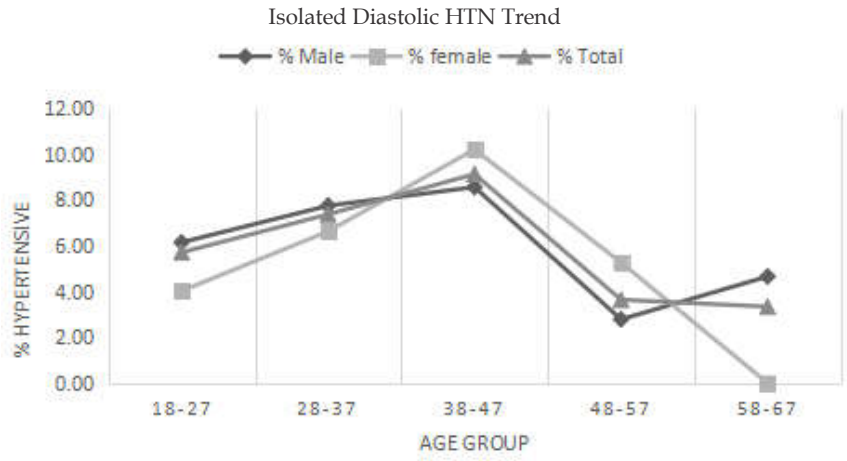
Table 3 depicts the prevalence of isolated systolic and isolated diastolic HTN across age and gender. The prevalence of overall isolated systolic HTN was found to be 9.00% and isolated diastolic HTN 6.35%. Like BP the prevalence of isolated HTN wad higher in males than in females. One interesting point about isolated HTN is that highest



Grpah 1: Trend in Isolated Systolic HTN

Table 2: Mean Systolic and Diastolic BP (mmHg) by age and gender

		Systolic BP (mean ± SD)			Diastolic BP (mean ± SD)		
Age groups (years)	**N=2115**	**Male**	**Female**	**Total**	**Male**	**Female**	**Total**
18-27	975	128.97 ± 13.05	117.73 ± 11.06	126.66 ± 13.45	80.30 ± 08.90	76.80 ± 08.96	79.58 ± 09.02
28-37	460	130.47 ± 12.96	121.68 ± 14.86	127.47 ± 14.25	82.73 ± 08.72	79.96 ± 09.41	81.78 ± 09.05
38-47	329	132.62 ± 14.80	130.88 ± 17.44	131.98 ± 15.81	83.87 ± 08.92	83.74 ± 10.91	83.82 ± 09.68
48-57	248	137.27 ± 17.88	140.27 ± 19.58	138.23 ± 18.45	84.39 ± 09.95	84.23 ± 09.24	84.34 ± 09.71
57-67	102	149.31 ± 19.08	140.79 ± 26.66	147.30 ± 21.27	85.40 ± 09.92	80.46 ± 11.33	84.24 ± 10.44
Total	2114	131.71 ± 15.02	125.54 ± 17.72	130.02 ± 16.04	81.98 ± 9.21	80.25 ± 10.07	81.50 ± 09.48
		F = 44.230	F = 41.407	F = 68.403	F = 15.276	F = 13.723	23.675
Significance		*df* = 4	*df* = 4	*df* = 4	*df* = 4	*df* = 4	*df* = 4
		p< 0.001	p< 0.001	p< 0.001	p< 0.001	p< 0.001	p< 0.001



Graph 2: Trend in Isolated Diastolic Blood Pressure

prevalence of isolated systolic HTN was found at age class 58-67 whereas this class had one of the lowest prevalence of diastolic HTN. The diastolic HTN was most frequent at age class 38-47 (Graph 1 and 2). The overall prevalence of HTN was 33.54% that is one-third of the population was under the grip of hypertension. The sex-specific prevalence was 36.25% for males and 26.38% for females. The overall prevalence of prehypertension was 52.39%. prevalence of prehypertension in male was 54.36% while in females it was 47.13% (Table 4).

Table 3: Prevalence of isolated systolic hypertensive and isolated diastolic hypertensive by age and gender

		Isolated systolic HTN (N = 1922)			solated diastolic HTN (N = 1922)		
Age groups (years)	**N=2114**	**Male**	**Female**	**Total**	**Male**	**Female**	**Total**
18-27	964	79	02	81	47	08	55
28-37	448	24	03	27	23	10	33
38-47	285	22	07	29	16	10	26
48-57	165	15	07	22	03	03	06
58-67	60	11	03	14	02	00	02
Total	1922	151	22	173	91	31	122

Out of 2114 study subjects interviewed, 192 (27.08%) were aware of hypertension and taking medicine. out of these 192 subjects taking medicine only 79 (41.15%) had their blood pressure under control (Table 5).

Table 4: Prevalence of hypertension and prehypertension by gender and age groups among the study subjects (N= 1922); excluding known Hypertensives (those taking medicine).

		Age groups (years)				
Category	**n**	**18-27**	**28-37**	**38-47**	**48-57**	**58-67**
Men	1401	765	297	188	108	43
Normal	217	137	40	27	13	00
Prehypertension	762	431	169	96	50	16
HTN stage 1	338	170	67	53	32	16
HTN stage 2	84	27	21	12	13	11
Women	521	199	151	97	57	17
Normal	181	93	56	24	05	03
Prehypertension	246	90	72	45	31	08
HTN stage 1	67	12	18	23	10	04
HTN stage 2	27	04	05	05	11	02

Table 5: awareness of hypertension.

Category	n (individuals with HTN)	No. of individuals tacking medicine	% aware of HTN and taking medicine	No. having BP under control after taking medicine	% having BP under control after taking medicine
Male	556	133	23.92	55	41.35
Female	153	59	38.56	24	40.68
Total	709	192	27.08	79	41.15

Discussion

India, like all other developing countries, is going through a rapid demographic and epidemiological transition and like all other developing countries the prevalence of hypertension is increasing [8, 27]. This increase in prevalence is particularly true in recent years after the introduction of mobile phones and internet [6]. And as to be expected Murshidabad is not an exception, in this study the prevalence of both prehypertension and hypertension was found to be very high which was 52.39% and 33.54% respectively.

The prevalence of hypertension in the present study (33.54%) was in parity with many of the other studies in India [6,10,13, 8,25]. However this prevalence of Hypertension 33.54% (male- 36.25% and female 26.38%) was much higher than it was reported in the National Family Health Survey - 4 Ministry of Health and Family Welfare Government of India (NFHS-4 2016)[4]. NFHS-4 2016 has used JNC 6 criteria and prevalence for stage -1, stage - 2 and stage -3 hypertension was 7.4, 0.0, 0.0 respectively for men and 5.0, 1.6, 0.3 respectively for women. The current study followed the JNC-7 criteria, it has only two stages and the prevalence of stage -1 and stage-2 was 24.13% and 6.00% respectively for men and 12.86% and 5.18% respectively for women. This huge difference was may be due inclusion of younger subjects and exclusion of elderly in the NFHS -4 2016 study (age 15-49 years).

For all stages of hypertension, the sex-specific prevalence was much higher in male than in females. Higher prevalence of hypertension in male was also reported by many of the similar studies [9,22,12]. This may be due to biological difference between two sex or due to behavioural risk factor difference, such as smoking, alcohol consumption, or difference if stress or physical activity.

Hypertension was found to be positively co-related with age irrespective of sex. Change in vascular system with age might be one of many underlying causes of this co-relation [20,26]. A number of studies demonstrated similar findings of an increase in the prevalence of hypertension with the advance in age [7,20,23].

On BMI parameter this study found 25.73% study subjects were overweight and 3.74% were obese. A very high proportion of study subjects (48.06%) had abdominal obesity based on waist hip ratio cut-off 0.9 for male and 0.8 for females. The higher prevalence of Hypertension was may be due to higher prevalence of overweight and obese study subjects as reported by many of the similar studies [24,15,17].

Conclusion and Recommendation

The systemic study carried out in the district Murshidabad shows a very high prevalence of both prehypertension and hypertension in this district and thus makes the people of the district vulnerable to hypertension associated chronic diseases. Older age group people were found to at more risk than the younger generation and specifically, males are at higher risk of being hypertensive than females. Initiatives are needed to improve the surveillance systems for the popper assessment of the disease burden and to develop key public health policies. For early detection of hypertension, community-based screening campaigns are also recommended.

Limitations of the Study

Among many others, the cross-sectional design was the most important limitation of this study, because it restricts association between cause and effect.

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Comparative Analysis of Oil Degrading Bacteria and Fungal Species to Manufacture Biosurfactant Using Neem Oil

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Abstract

Biosurfactant are attractive attention in recent year because they offer several advantages over chemical surfactants. Such as low toxicity, good biodegradability and ecological friendliness. In this respect, selected two oil degrading bacteria and two oil degrading fungal species. The selected microbes play major role in oil recovery, environmental bioremediation, food processing and pharmaceuticals owing to their unique properties such as higher biodegradability. The ability of oil degrading bacteria's such as *Pseudomonas putida, Pseudomonas aeruginosa* and fungal *species Aspergillus oryzae, Penicillium chrysogenum* to utilize Neem oil to produce biomass. The energy sources of neem contain different biologically active compounds undergoes into simpler compounds by the microbial activity. This will be helpful for environmental safe agricultural development. Bacterial species such as *Pseudomonas putida and Pseudomonas aeruginosa* could grow well in stone medium using water and hexane soluble fractions of neem oil and the presence of $(NH_4)_2 HPO_4$ seemed to be important for better production of biomass and Biosurfactant under the laboratory and neutral PH conditions than the fungal species. The composition of water and hexane soluble fractions of neem oil, non-degraded and degraded neem oil by microbes were analysed by using gas chromatography. The result suggested most of the components in hexane soluble extract were degraded by *Pseudomonas putida*. Hence, the present study aims to find out, oil degrading microbes for maximizing Biosurfactant productivity using Neem oil as an energy source.

Keywords: Biomass; Biosurfactant; Oil degrading; Neem oil; Hydrocarbon-degrading.

Introduction

Biosurfactant have advantage over synthetic surfactants, it can only replace the synthetic if the cost of the raw material and the process is minimal. So far, several renewable substrates form various sources; especially from industrial wastes have been intensively studied for microorganism's cultivation and surfactant production at an experimental scale. A variety of cheap raw materials, including plant-derived oils, oil wastes, starchy substances have been reported to support biosurfactant production. Researchers have used variety of vegetable oils from canola, corn, sunflower, safflower, olive, rapeseed, grape seed, palm, coconut, fish and soybean oil.

Azadirachta indica AJuss.; The neem tree is consider a good purifier of air due to its large leaf area. Parts of the plants are used medicinally. Neem oil contain several terpenoids, steroids, alkaloids, flavonoids, glycosides, etc., The isolated constituents are margosic acid, Nimbin, nimbidin, kaemperol,

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azadirone, quercursertan, b-sitosterol, praisine, vanilic acid, nimbicetin, meliacins, etc., Garg and Bhakuni, 1984 reported salanolide (a meliacin) as one of the bitter principles in neem seed oil. Furthermore, (Raman and santhanagopalan, 1979) reported tignic acid (5-methyl-2-butanoic acid as parted of the seed constituents. This compound is believed to be responsible for the distinctive odour of the neem oil. The limonoids are freely soluble in organic solvents such as hydrocarbon, alcohol and ketones, but are sparingly soluble in water. The tetranotriterpenoid compound azadirachtin exposed to neem compounds (Isman, 1990, Mordue and Black well, 1993, Verkerk and Wright, 1993). Different biologically active compounds have been isolated from neem seed (Lee et al., 1991). All biologically active neem components are suspected to be derived from one parent component which is tetra cyclic triterpenoid tirucallol (Ascher, 1993).

The complex biological structure of neem undergoes a break down into simpler compounds resulting in microbial succession favouring heterotrophic nitrogen fixers. These slow and gradual changes result in the formation of nitrogen pool which is available for plant growth and development (Barbara W. Ellis and Fern Marshall Bradley, 1996). Neem tree has identified as one of the most suitable candidate for environmentally safe agricultural development (Dhillon and Khajuria, 1996).

The oil degrading bacteria of *P.putida*, called "multi-plasmid hydrocarbon-degrading *Pseudomonas*," is the first patented organism in the world. It demonstrates a very diverse metabolism, including the ability to degrade organic solvents such as toluene. This ability has been put to use in bioremediation, or the use of microorganisms to biodegrade oil. Use of *P.putida* is preferable to some other *Pseudomonas* species capable of such degradation, as it is a safe species of bacteria, unlike *P. aeruginosa*, for example, which is an opportunistic human pathogen. This research uses the neem tree components like neem oil as a natural resource and the act of *P.putida* and *P.aeruginosa* on it in different environmental conditions.

Materials and Methods

Inoculum and Media

The bacterial species *Pseudomonas putida, Pseudomonas aeruginosa* and the fungal species *Aspergillus oryzae, Penicillium chrysogenum* were obtained from laboratory. The strain was cultured in 50 ml of stone medium, 10 ml of distilled water, and acetone and hexane extracts of Neem oil was amended in the medium as energy source. For further study analysis, Neem oil is used as substrate.

Control and Growth Conditions

Un Inoculated medium with the carbon sources (extract of neem oil) maintained at room temperature with neutral PH served as control. Another control was maintained with inoculated stone medium with carbon sources. The culture conditions are as follows PH4 - PH9, temperature 25c, 35c, 40c in BOD incubator, and 20mg of Nitrogen sources viz., $(NH_4)_2HPO_4$ (di-ammonium hydrogen orthophosphate), (NH4) 2SO4 (Ammonium sulphate), NH_4Cl (Ammonium chloride) and KNO_3 (Potassium nitrate) per 50 ml of stone medium with neutral PH was amended medium.

Biomass and Biosurfactant Production by Bacteria and fungi Using Neem Oil

Distilled water, acetone and hexane extracts of 5 ml of oil was obtained using 10 ml of extractives. The extract was centrifuged and the supernant was taken in conical flask. This extract served as carbon source. To each of these extracts 50 ml of stone medium was added and inoculated with bacterial species, Pseudomonas putida and Pseudomonas aeruginosa and fungal species *Aspergillus oryzae, Penicillium chrysogenum* are incubated for 6 days.

Fig. 1:

Fig. 2:

Isolation of Biosurfactant (Swaranjit Camorra, 1995)

After separating the biomass, the culture filtrate was centrifuged at 10,000 rpm for 30 minutes to remove any debris. The clear supernant was then treated with 3 volumes of ice cold acetone. The precipitate formed is collected by centrifugation at 5,000 rpm for 30 minutes.

Gas Chromatography Analysis

Working standard of 2 µl were prepared from the stock solution by dilution and used in finding of the retention times and qualification of the compounds in GC-ECD.

End Analysis

Oil was estimated by gas chromatography model varian Cp 3800 equipped with electron capture detector (ECD) fitted with capillary column. The following were the operating parameters.

Detector	ECD
Temperature C	Column – 25^{0}C Injector – 25^{0}C Detected – 25^{0}C
Column	1/8 inch 55 Packing – OV17
Nitrogen flow rate	30ml/min
Threshold	10 µv
Volume injected	2 µl

Analytical Methodology

Sample (2 µl) oil was injected into the injection port by using the injection needle. In GC unit carrier gas used will ensure the migration of the components of the sample. The column used was 1/8 inch 55 packing OV-17. The ECD detector was used to observe free electrons entered by radioactive sources. The current produced by free electrons were detected by detector, when current is decreased. The response of the detector is plotted by the recorder which furnishes the chromatogram. Then the physical measurement like retention time, peak height and areas were measured.

Results and Discussion

Biomass and Bisurfactant Production by Bacteria

Water and hexane extracts enhanced the biomass and biosurfactant production and the results were significant when $(NH_4)_2HPO_4$ was amended in the medium. When water and hexane extract was used as energy source. In the absence of this nitrogenous source also using water and hexane extracts the bacteria's produced Biosurfactant significantly (Table 1).

Biomass and Biosurfactant production by Fungi

Both the fungi produced biosurfactant during the degradation process at normal laboratory temperature of 30c and the presence of nitrogenous sources expecting KNO_3 favoured Biosurfactant production. The best result were obtained in the presence of $(NH_4)_2HPO_4$ for both *Aspergillus oryzae* and *Penicillium chrysogenum.* But the comparison with the fungal species, bacteria showed better production. Hence, it has been taken for further analysis.

GC Analysis of Neem Oil

To find out the components present in the neem oil was analysed to compare the components of hexane extract of Neem oil. The results showed 14 peaks representing the presence of number of 14 components of which 4 peaks appeared to be significant (Fig. 3).

GC Analysis of Hexane Extact of Neem Oil

Since hexane extract of Neem oil supported the biomass and biosurfactant production to identify the components in the oil. About 11 peaks were identified and one significant peak with 21 percent

Table 1: Biomass and Biosurfactant production by Bacteria

Factors	Water Extract				Acetone Extract				Hexane Extract			
	P.putida		*P.aeruginosa*		*P.putida*		*P.aeruginosa*		*P.putida*		*P.aeruginosa*	
	BM	BS	BM	BS	BM	BS	BM	BS	BM	BS	BM	BS
C1	-	-	-	-	-	-	-	-	-	-	-	-
C2	-	-	-	-	-	-	-	-	-	-	-	-
C3	0.72	0.48	0.58	0.32	0.56	0.25	0.27	0.12	0.80	0.53	0.50	0.27
$(NH_4)_2HPO_4$	1.27	0.59	0.80	0.45	0.59	0.28	0.52	0.31	1.38	0.55	0.92	0.43
$(NH_4)_2SO_4$	0.94	0.30	0.27	0.13	0.30	0.16	0.28	0.09	1.02	0.48	0.63	0.38
$(NH_4)Cl$	1.24	0.52	0.68	0.34	0.25	0.10	0.43	0.20	0.47	0.25	0.28	0.12
KNO_3	-	-	-	-	-	-	-	-	-	-	-	-
pH_4	-	-	-	-	-	-	-	-	-	-	-	-
pH_9	0.26	0.13	0.32	0.16	0.15	0.07	0.33	0.17	0.24	0.12	0.32	0.18
25°C	0.31	0.10	0.23	0.11	-	-	-	-	0.32	0.15	-	-
30°C	0.76	0.46	0.58	0.32	0.56	0.25	0.29	0.13	0.80	0.53	0.51	0.24
35°C	-	-	-	-	-	-	-	-	-	-	-	-
40°C	-	-	-	-	-	-	-	-	-	-	-	-

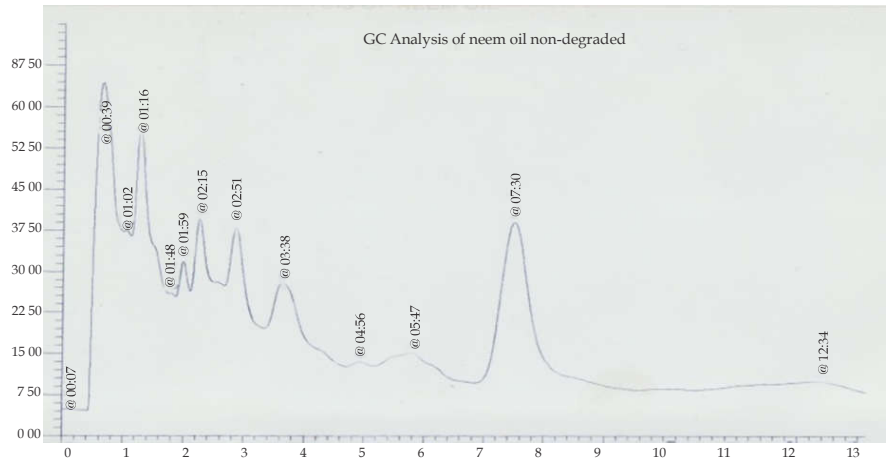

Fig. 3:

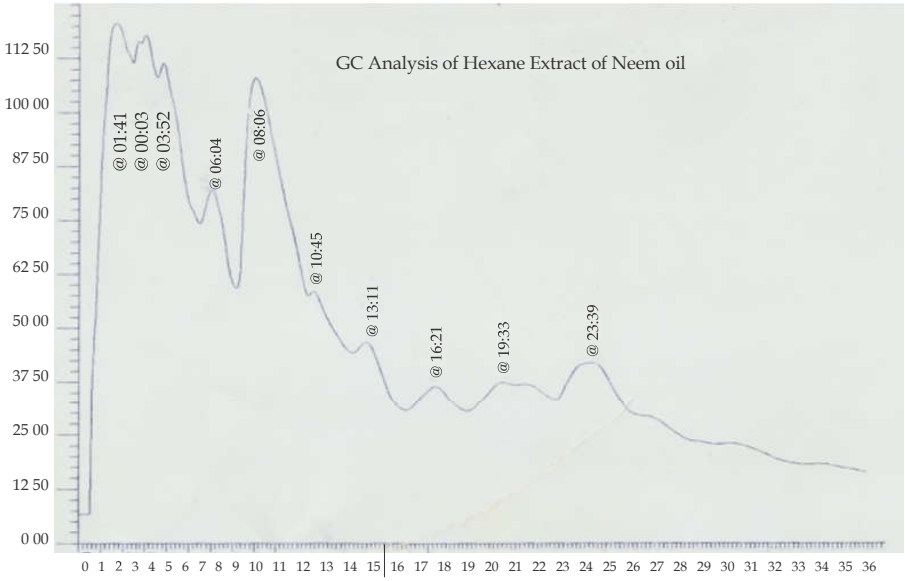

Fig. 4:

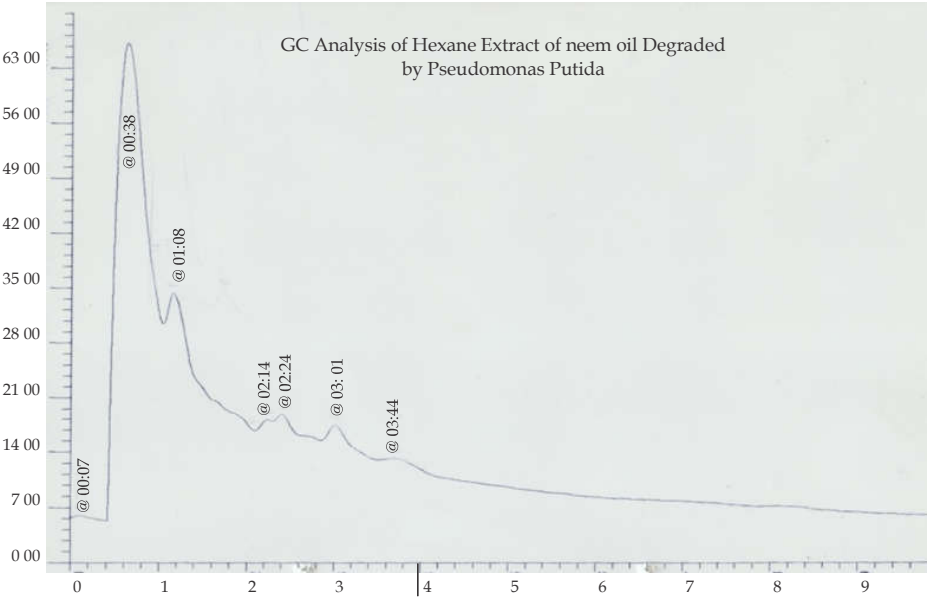

Fig. 5:

area of 21 appeared after 8 min (Fig. 4).

GC Analysis of Neem Oil Degraded by Pseudomonas Putida

This experiment was done to find out whether all the components in the results were significant when hexane soluble fractions of Neem oil was degraded and utilized by the microbes, as the results were significant when hexane soluble fractions were used as energy source. Only 7 peaks were recorded, which one peak appeared to be significant (Fig. 5).

Discussion

In the present investigation, efficiency of microbes is enhancing the biosurfactant production of neem oil has been tested. The experiment performed to find out the suitable medium suggested that in stone medium with water and hexane extracts of Neem oil as energy source. The oil degrading Bactria *Pseudomonas putida* and *Pseudomonas aeruginosa* could produce appreciable amount of biomass and biosurfactant than the fungal species. Presence of a nitrogenous source in the medium such as $(NH_4)_2HPO_4$ seems to play significant role under laboratory conditions and neutral PH. GC analysis of hexane extract of Neem oil degraded by *Pseudomonas putida* showed that almost all the components were progressively utilized by the microbes and therefore only one peak appeared in the GC analysis. Comparison of Neem oil and hexane extract of Neem oil showed no significant similarity. All the peaks in the Neem oil could be recorded by 15 minutes whereas the hexane extracts of Neem oil the peaks appeared for about 23 minutes.

Conculsion

As a result suggests *Pseudomonas putida* degraded most of the components, so *pseudomonas putida* is the best and safest oil degrading organism. Hence, Surfactants have several applications in agriculture and agrochemical industries. This study will help in replacing the harsh chemical surfactants with green ones. Several researchers indicate that variety of environmental niches such as soil, water, and leaf surface are explored for Biosurfactant producing bacteria. Plant associated microbes are known to produce biosurfactant indicating the potential role of biosurfactant in plant-microbe interaction and further application of biosurfactant in agriculture.

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Two Antibacterial Alkaloids from *Argemone mexicana*

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Abstract

The isolated alkaloids from *Argemone mexicana* seed showed considerable antibacterial activity against pathogenic bacteria, out of which one of them namely *Staphylococcus aureus* is Gram positive and remaining three Gram negative namely, *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Fractionation followed by TLC leds to the isolation of two known benzophenanthridine alkaloids namely and dihydroxysanguinarine and dimethylsanguinarine. The MIC was determined for each compound using a two fold serial dilution assay. The structures of these compounds are determined by $^{1}H_{1}$ and ^{13}C NMR analysis.

Keywords: *Argemone mexicana*; Alkaloids; Antibacterial activity.

Introduction

India has a rich flora that is widely distributed throughout the country. From ancient time plant and plant extracts are used for treatment and cure of various diseases (Dhar et al., 1968; Perumal Samy and Ignacimuthu, 1998, 2000; Dahanukar et al., 2000; Kumar et al., 2006).

Argemone mexicana L. (Papaveraceae), commonly known as prickly poppy, is used as a medicinal plant in several countries as antidote to snake venom, relieving tooth ache and its extract is also used to treat common colds, warts and itches, dropsy and even in curing jaundice (Chopra et al. 1986; Bhattacharjee et al. 2006).

In this study we report the antibacterial property of the main antibacterial compounds from Chloroform: Methanol (1:1) extract of *A. mexicana* seed on four pathogenic bacteria. We also identified the active compounds as dihydroxysanguinarine (1) and dimethylsanguinarine (2).

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Materials and Methods

Plant materials: Seeds of *Argemone mexicana*, collected from nearby site of Hatgobindapur College, Burdwan (23°16´N, 87°54´E), during spring (mid-March to mid-April 2017) and

herbarium sheet was prepared and Voucher specimen (voucher no. 110) submitted at Department of Botany, DBNDS Mahavidyalaya and taxonomically authenticated.

***Extraction and isolation of the antibacterial compounds*:** The air dried seeds (500 g) were extracted successively with 7 technical grade solvents (Merck) of increasing polarity, one after another on the same sample using Soxhlet apparatus following the method of Tang & Young (1982). The extract of chloroform: methanol (1:1, v/v) (21 gm) was further fractioned by column chromatography (Silica gel – G for TLC, Merck, India) for 72 h using n- hexane- ethyl acetate mixture of increasing polarity. The different fractions obtained were tested for antibacterial activity against all strains by direct bioautograhy on thin layer chromatography (TLC) plates (Lund and Lyan, 1975). The fraction was further purified by TLC on silica gel 60 F_{254} (Merck) with chloroform: methanol: water (65:35:5) as running solvent; resulting is the isolation and characterization of two active alkaloids, 1 (1.5 g) and 2 (1.2 g).

***Bacterial strains used*:** *Staphylococcus aureus, Escherischia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumonia,* having strain numbers MTCC 2940, MTCC 739, MTCC 2453 and MTCC 432 were obtained from Burdwan Medical College and maintined in Nutrient Broth M002 (Himedia, India).

***Antibacterial testing*:** MIC of the isolated compounds 1 and 2 was evaluated by the broth micro dilution method in Müller – Hinton broth according to the NCCLS standard (NCCLS, 1997). Gentamycin (5 µg/ml) was used as reference standard. Observations were performed in triplicate. The lowest concentration of 1 and 2 (6.2 – 28.1µg/ml) where no visible growth was recorded. Simultaneously, TLC bioassay using Bioautography technique (Hamburguer and Cordell 1987; Didry et al. 1990) was used to determine which compound in the $ChCl_3$: methanol (1:1, v/v) extract was active.

Results

The fractionation of the seed extract of the chloroform: methanol (1:1, v/v) of *Argemone mexicana* led to the isolation of two alkaloids, dihydroxysanguinarine (1) and dimethylsanguinarine (2). Compounds were identified by direct comparison of their $^{1}H_{1}$ and ^{13}C NMR spectral data with those found and described by (Chang et al. 2003).

Table 1 shows the Compound 1 and 2 displayed significant antibacterial activities with MIC's of 6.2 and 9.3 µg/ml respectively against *Klebsiella pneumoniae*, 12.5 and 15.6 µg/ml for *Staphylococcus aureus*, 6.2 and 12.5 µg/ml for *Escherichia coli* and 15.6 and 28.1 µg/ml for *Pseudomonas aeruginosa*.

Dihydroxysanguinarine (1) was the most active compound due to its inhibition of the growth of all the test bacterial strains. This is an indication of the compound as a broad spectrum antibiotics.

Table 1: Minimum Inhibitory Concentrations (MIC) (µg/ml) of active alkaloids from *A. mexicana*

Compounds	K. pneumoniae	S. aureus	E. coli	P. aeruginosa
Dihydroxy sanguinarine	6.2 ± 0.17	12.5 ± 0.06	6.2 ± 0.21	15.6 ± 0.04
Dimethyl sanguinarine	9.3 ± 0.12	15.6 ± 0.31	12.5 ± 0.18	28.1 ± 0.14
Gentamycin	2.5 ± 0.22	2.5 ± 0.08	5.0 ± 0.20	5.0 ± 0.32

Discussion

The chloroform: methanol (1:1, v/v) fraction of seed extract of *Argemone mexicana* showed antibacterial activity against four pathogenic bacteria. This activity has been identified as alkaloids dihydroxysanguinarine (1) and dimethylsanguinarine (2) using systematic fractionation guided by antibacterial assay. These compounds have also been isolated from plants of Papaveracea family (Sloviko et al., 1985: Daskalov et al., 1988: Chang et al., 2003: Novarro and Dalgado 1999). Alkaloids of this structural type have been shown to possess antibacterial activity against bacteria that produce oral infection (Gadawsky 1989; Harkrademet et al., 1990). From this study we noted that the active alkaloids from seeds of *A. mexicana* possess an antibacterial activity. This might explain the use of this plant for the treatment of various infectious diseases in folk medicine.

Conclusion

The only alternative to antibiotic is medicinal plants. The seeds of *Argemone mexicana* is considered as one of the nature's gifted property as it shows antibacterial activity against both gram positive and gram negative bacteria. But the seed extract when adulterated with mustard oil it causes dropsy. So one should be very careful about the way of extraction, method of application, duration of application and proper dose so that no resistance variety is created in bacteria.

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Potential of Lignocellulosics in Biothanol Production to Mitigate Energy Crisis

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Abstract

Ethanol is a simple alcoholic compound having high octane number. It is an oxygenated fuel. Bioethanol is ethanol that is derived exclusively from plant starch or cellulose as a byproduct of fermentation. The mixing of ethanol into petroleum base automobile fuels decreases the release of green-house gas emissions. Moreover ethanol is safer than common additive MTBE. Combustion of bioethanol results in cleaner emissions. The bioethanol from cellulosic feed stock is classified as second generation biofuel. Lignocellulosic biomass composed of different carbohydrate polymers (cellulose & hemicellulose), lignin, and small fraction of extractable acid, salt, minerals etc. It is the biodegradable portion of products, wastes, organic residues from agriculture and agroindustry, forestry and wood industry. Lignocellulosic biomass has been projected to be one of the main sources of economically alternative bioethanol production. Lignocellulosics can be obtained from corn stover, sugarcane bagasse, wheat straw, rice straw, oat hull, rice hull cotton stalk, parts of *Lantana camara*, water hyacinth etc. Bioethanol production based on lignocellulosic biomass requires multistep complex conversion technology. Milling, pretreatment, enzymatic hydrolysis, fermentation and distillation steps are involved. Genetically engineered strains of *Zymomonasmobilis*, *S. cerevisiae* are widely used. *P. stiptis* and *Candida shehataehaving* the ability of fermenting both hexose (glucose) and pentose (xylose) sugars to ethanol. Second generation bioethanol production fulfills the delusive gap of first generation bioethanol production from non-edible renewable feed stock. Production of bioethanol from lignocellulosics still requires considerable R &D before reaching the commercial production stage.

Keywords: Ethanol; Renewable energy; Bioethanol; Lignocellulosics; Fermentation.

Introduction

Commensurate with the Population growth and industrialization, energy consumption and requirements has increased steadily. To meet this energy demand crude oil is proven to be the major resources. World's dependency on fossil fuel has resulted in many unfavourable effects including diminishing crude oil reserve, deteriorating air quality, global warming, unpredictable weather changes etc. As a substitute of fossil fuel like gasoline ethanol, a renewable energy source

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is getting attraction these days. Ethanol, an oxygenated fuel having high octane (MON:90 and RON:109) value like that of petroleum based fuels. Ethanol is best known to run combustion engines at higher compression ratio thereby provides better performance (Wheals et al., 1999). Efficient mixing of ethanol into petroleum-based automobile fuels can concomitantly decrease petroleum usage and greenhouse gas emissions. Moreover, ethanol can be a safer alternative to the common additive, methyl tertiary butyl ether (MTBE), in gasoline. Toxic MTBE is awell-known contaminant in ground water (Wang et al., 1999). So, ethanol can be a safer substitute to alleviate the problems associated with the increasing energy demands across the world as well as a solution for an extent of 85% reduction in greenhouse gas emissions (Perlack et al., 2005).

Bioethanol, a form of quasi-renewable energy, can be produced from agricultural feedstocks. It can be made from very ordinary crops such as potato, sugarcane, cassava and corn etc. The usefulness of bioethanol in replacing gasoline is not beyond debate. Concerns about its production and use in relation to increased food prices as the large amount of cultivable land required for crops, especially from corn gain attention. Recent advancements with cellulosic ethanol production may soothe some of these concerns (Kinver and Mark, 2006). Cellulosic ethanol is a promising alternative as cellulose fibers, a ubiquitous component in plant cells walls, that can be used to produce ethanol (O.R. Inderwildi and D.A. King 2009). According to the International Energy Agency, cellulosic ethanol may play paramount role in the future (World energy outlook, 2015). Ethanol can be produced either from petroleum based products or from biomass. Most of the ethanol is producing from renewable resources this day. Although currently most of the ethanol produced from renewable resources mainly coming from sugarcane and starchy grains, meaningful efforts are being made to use lignocellulosic biomass as a source material for ethanol production (almost 50% of all biomass in the biosphere) such as agriculture residues (Bothast and Saha, 1997). Production of ethanol at very low cost from lignocellulosic biomass, are quite promising due to technological advancement in recent years.

Bioethanol production from starch enriched feed stocks such as potato, corn and sugarcane etc., are considered first generation process. It has already been developed. The long-term viability of this process is questionable as it will require significant amounts of arable land and consequently significant hike in food prices which will ultimately lead to food insecurity (Mitchell, 2008). First generation ethanol production process can not sufficiently meet the demand of global energy needs as estimates pointing out. As a result, second generation bioethanol production is gaining momentum. There are sufficient supplies of lignocellulosic materials. The production of bio-ethanol from lignocellulosic biomass [wheat straw,cornstover, sugarcane bagasse, rice hull, rice straw, oat hull and cotton stalk; crops such as Alfa Alfa and switch grass, various weeds such as *Saccharumspontaneum, Eichhorniacrassipes, Lantana camara* etc.] has become one of the best alternatives, because of their widespread abundance and relatively cheap cost of their procurement.

Lignocellulosic Biomass

Composition

High abundance, minimum cost and non-competitiveness with foodstuffs make Lignocellulosic biomass a promising resource for the production of bioethanol. Lignocellulosic biomass comprised of Cellulose, lignin and hemicellulose. Among them, Cellulose is the major component of lignocellulosic biomass, its concentration ranges from 40 to 50% of dry weight. Cellulose is a homopolysaccharide consisting of several hundred to more than ten thousand (β-1,4) linked D-glucose units. Cellulose binds tightly with lignin and hemicellulose. Efficient hydrolysis of cellulose needs lignin component separation to make cellulose more accessible to the enzymes (Selvi et al., 2009). The enzymatic hydrolysis of cellulose (cellulolysis and saccharification) is influenced by several factors, viz., degree of crystallinity, degree of polymerization, structural composition and availability of surface area etc. (Qi et al., 2009). For enhanced enzymatic saccharification of lignocellulosics efficient pretreatment is required. Monomeric sugars released after enzymatic saccharification can be converted into bioethanol. Hemicellulose concentration in lignocellulosic biomass is 25 to 35%. It is easily hydrolysable to produce fermentable sugars (Saha et al., 2007). Hemicellulose is a hetero polysaccharide comprising of pentoses (D-xylose and D-arabinose), hexoses (D-mannose, D-glucose and D-galactose) and sugar acids. Softwood hemicellulose mainly bears mannose as a major constituent whereas hardwoods mainly possessXylans (Balan et al., 2009). Lignin is the third major component of lignocellulosic biomass and its concentration ranges for 20 to 35%. It is a complex polymer

of phenyl propane (p-coumaryl, coniferyl and sinapyl alcohol). Lignin a cementing agent acts as an impenetrable barrier for enzymatic attack according to Howard et al. (2003). It provides plants with the structural support and impermeability. It also confers resistance against oxidative stress and microbial attack. These properties of lignin may be associated with amorphous nature, optical inactivity and water insolubility of it. The later properties also make it tough to degrade (Fengel and Wegener, 1984).

Sources

Sources of lignocellulosic biomass can be categorized as primary sources that includes crops and secondary sources includes residuals of production process such as straws, rice husks, baggase and tertiary sources as organic fraction of municipal solid wastes. (Fischer and Schrattenholzer, 2001). Agro-industrial biomass residues are byproducts of agriculture including coconut shell, rice straw, cotton stalk etc. (Demirbas et al. 2009). Forestry residues include wood chips, bark and saw dust. (Werther et al. 2000). Woody raw materials have long latency period and flexible harvesting times. Woody raw materials possess more lignin than agricultural biomass and have less ash content (Zhu and Pan, 2010). Agricultural residues are getting more reliance than woody stocks as they are more environment friendly than later one (Kim and Dale, 2005).

Lignocellulosics to Bioethanol conversion

Lignocellulosic biomass can be converted into ethanol via two major approaches –Thermochemical and biochemical approaches (Demirbas, 2007). Thermochemical approaches includes gasification of biomass first at high temperature (at 800^{o}C) which has given rise to production of syngases. Then syngases are converted to ethanol and water by using microorganisms like *Clostridimljungdahlii*. Subsequently ethanol are separated through distillation (Mu D et al., 2010). Biochemical conversion includes pretreatment step that can be mechanical, chemical or biological. This pretreatment step enhances surface area to optimize cellulose accessibility to cellulose. (Young and Wyman, 2008). Pretreatment stage is followed by acid or enzymatic hydrolysis often known as cellulolysis. It results in production of fermentable monomeric reducing sugar (Saccharification). Fermentation is the next step, involving conversion of reducing sugar to ethanol using yeast or bacterial fermentation. Produced ethanol are then purified via distillation (Mc Millan, 1994).

Use of genetic engineering in bioethanol production

Fermentative microorganisms have to be thermos tolerant. Biological treatment steps involving fungi which require high temperature and low pH such as basidiomycetes. As fungi act slowly, Enhancement in ethanol production requires production of potential lignocellulolytic fungi by mutagenesis, gene expression and co-culturing (Dashtban et al., 2009). Some genera of fungus (*Candida, Dekkera, Pichia* etc.) produce low ethanol and acetic acid which again acts as an inhibitor of fermentative yeast (Basilio et al., 2008). Some groups of bacteria can efficiently convert monomeric sugars to ethanol as *Zymomonasmobilis*. These bacteria are more vulnerable to inhibition than fermentative yeast (*S. cerevisiae)* (Chen, 2009). Simultaneous saccharification and fermentation (SSF) and simultaneous saccharification and combined fermentation (SSCombF) are cost effective as they reduce end product inhibition (Ho et al.,1998). The genetic modification of conventional *S. cerevisiae* strain is gaining attention as they are more optimally adapted to bioethanol production (Lilly et al., 2009). CBP combines hydrolysis and fermentation in a single reactor by utilizing genetically modified microoraganism that are capable of producing celluase enzyme (Lynd et al., 2005). *S. cerevisiae*, can also be genetically modified to express cellulolytic and hemicellulolytic heterologous enzymes. These type of modifications can be achieved through reassembling of all existing components of mimicellulosome on yeast's membrane surface from the thermophilic microoraganism C. *cellulolyticum* via a chimeric protein scaffold expression under PGK 1 regulation. The successful functionality of cellulosomein of *S. cerevisiae* and dockerin and cohesion of *C. cellulolyticim* proved that this genetic engineering based on minicellulosome model can be an attractive option for CBP process (Zyl et al., 2007).

Bio-ethanol production in world: an overview

Several countries throughout the world, have initiated new alternatives for gasoline from renewable feedstock (Goldemberg et al., 2007). In the North American hemisphere, bioethanol extraction has been done from starch based sources such as corn, while in the South American hemisphere, biofuel has been largely produced

from sugars including sugarcane and sugarbeets (Wheals et al. 1999). While European countries are taking extensive efforts to increase their 5% worldwide bioethanol production (Gnansounou et al., 2010), biodiesel produced in Europe primarily in Germany and France are substantial. They account for approximately 56% of the global biodisel production (EU, 2009). Although, most of the remaining countries in the world collectively account for only 5% of the global bioethanol production, China, Thailand as well as India are continuing to invest significantly in agricultural biotechnology sector and trying to be emerging as potential biofuel producers (Swart et al., 2008). In the U.S., biofuel-derived from corn has emerged as one of the primary raw materials for bioethanol production (DOE biomass 2009). According to the renewable fuels association statistics, the production of bioethanol was historically unparalleled in the U.S. by year 2009 capacity reaching 41.26 billion litres and representing 55% of the worldwide production. In the year 2010 corn-based ethanol operating productions generated a total of 12. 82 billion gallons (48.52 billion litres) with the largest nameplate capacity (28%) followed by Nebraska (13%) (Nebraska 2009). The world population is estimated to increases from 6.7 billion to 8 billion by 2030 (USCB, 2008). On the other side, global crude oil production is anticipated to decline from 25 billion barrels to 5 billion barrels by 2050 according to Campbell and Laherree (1998). Thus the energy demands of future are likely to play a key role in geo-political economics. Given this reality, nations throughout the world are now investing in alternative sources of energy, including bioethanol. The pioneer countries in bioethanol production are Brazil and the USA (as shown in Table 1). USA is the world's largest producer of bioethanol (Carere et al., 2008). Asian countries are altogether accounting for about 14% of world's bioethanol production.

Table 1: Leading bioethanol producers in the world

Country/group of countries	Ethanol produced in: Million liters	Ethanol produced in: MTOE
1. Brazil	19000	10.44
2. Canada	1000	0.55
3. China	1840	1.01
4. India	400	0.22
5. USA	26500	14.55
6. European Union	2253	1.24
7. Others	1017	0.56
8. World (Total)	52000	28.57

*Source: Data from OECD-FAOA glink-Casimo database (2007).
MTOE: Million tons of oil equivalents

Bio-ethanol as a renewable energy source

Renewable energy is energy that is obtained from renewable resources, are naturally replenished on a human timescale, such as sunlight, wind, rain, tides, waves, and geothermal heat (Ellaban et al., 2014). Renewable energy often provides energy in four important areas: electricity generation, air and water heating/cooling, transportation, and rural (off-grid) energy services (REN 21, 2010). Based on REN21's 2016 report, renewables contributed 19.2% to humans' global energy consumption and 23.7% to their generation of electricity in 2014 and 2015, respectively. This energy consumption is splitted as 8.9% coming from traditional biomass, 4.2% as heat energy, 2.2% is electricity from wind, solar, geothermal, and biomass 3.9% from hydro- electricity.

MTBE (Methyl tert-butyl ether) is an oxygenates (Fischer et al., 2005) and is a fuel additive that can raise the octane number. This water soluble chemical is a possible human carcinogenic (Belpoggi et al., 1995). To increase the octane number of the fuel, it should be substituted for other oxygenated substances. Presently, ethanol as an oxygenous biomass fuel is regarded as a most suitable substitute to MTBE for its biodegradable, low toxicity, persistence and regenerative feature(Cassada et al., 2000).

The United States gasoline supply is an ethanol blend and the importance of ethanol use is expected to increasety related health issues. Ethanol may be produced from many high energy crops such as sweet sorghum, corn, wheat, barely, sugar cane, sugar beet, cassava, sweet potato and etc. (Drapcho et al., 2008).

Conclusion

Focus on Lignocellulosic biomass as one of the main resources for economically attractive bioethanol production getting attention today. Agricultural wastes are renewable, less costly and abundantly available in nature. Agricultural wastes do not demand separate land, water, and energy requirements. They have no food value as well. For economically feasible bioethanol production, several hindrances are to be overcome.These refer to the four major aspects which are feedstock, conversion technology, hydrolysis process, and fermentation configuration. With regard to feedstock major obstacles are cost, supply, harvesting and handling. Conversion technology faces problems associated with biomass processing,

proper and cost effective pretreatment technology to liberate cellulose and hemicellulose from their complex with lignin. To achieve an efficient process for depolymerization of cellulose and hemicellulose to produce fermentable monomers with high concentration is the main challenge for hydrolysis process. In this case enzymatic hydrolysis may be the most potent alternative process for saccharification of complex polymer. To optimize the enzymatic hydrolysis process, several efforts have been made to reduce the cost of cellulase enzyme. Lastly in case of fermentation, the challenges involve xylose and glucose co-fermentation, and the use of recombinant microbial strains. In conclusion it may be said that to solve the technology bottle necks of the conversion process, novel science and efficient technology are to be applied, so that production of bioethanol from agricultural wastes may be effectively developed and optimized in the near future.

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Original Article

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Morphometric Studies of Some Diplodiniid (Diplodiniinae, Entodiniomorphida) Ciliates from the Rumen of Cattle (*Bos indicus*) in India

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Abstract

An investigation has been made to study the protozoan ciliates of subfamily Diplodiniinae (Order: Entodiniomorphida) from the rumen of cattle (*Bos indicus*). The present paper deals with the occurrence of some Diplodiniid ciliates from the rumen of cattle (*Bos indicus*) in India. The size and morphology of the species is compared to those previously reported in different geographical areas. In the present investigation *D. anacanthum* (Dogiel, 1927) and *D. triacanthum* (Dogiel, 1927) recorded first time in India from the rumen of cattle (*B. indicus*). *D. tetracanthum* (Dogiel, 1927) is reported from the rumen of cattle as a new host record in India.

Keywords: Rumen; Cattle; Ciliates; *Diplodinium.*

Introduction

The Rumen is anaerobic and the largest compartment of the stomach which occupies 80 per cent of the abdomen in the ruminant animals. The rumen does not secrete any enzyme but constantly receives the saliva. The pH of the rumen is in between 5-7.5 and temperature ranges from 38-41^{0}C. In this way the rumen favors for microbial fermentation. Rumen micro fauna includes viruses, bacteria, fungi and protozoa. Of them, protozoa have large bodies with characteristic shape and about 10^5, 10^6 per ml of rumen fluid. The impact of protozoa on the rumen digestion depends on their concentration and the generic composition of their population (J.P. Jouany and K. Ushida 1999).

Gruby and Delafond (1843) first reported the protozoa from ruminants since then a number of protozoan species have been reported from different parts of the world Dogiel (1927) Becker & Talbott (1927), Hsiung T.S. (1932) Clarke R.T.J. (1964), Ogimoto & Imai (1981) and Dehority (1993,2005) Gocman B. (1999a, 1999b, 2000) Gocman *et al.* (2005) Martenele I. *et al.* (2008), Gocman and Gurelli (2009), Dirk B. *et al.* (2010), Dirk B. and Dehority B.A. (2011) and Gurelli (2014), but very few studies have been made in India. Kofoid and Maclennan (1930, 1932, 1933), Dasgupta M. (1935), Banerjee A.K. (1955), Mathur C.S. (1963), Misra S.K.(1972), Mukherjee & Sinha (1989,1990) Sanghai P.K. and Kshirsagar H.S. (2015), Cedrola *et al.* (2016, 2017) studied rumen ciliates from different hosts. Kulkarni & Kshirsagar (2001) studied the genus *Entodinium* and reported 13 new species.

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Although many studies have been reported on the rumen ciliates of different hosts from all over the world no investigations have been made on Diplodiniid ciliates of cattle in India. The objective of this study was to identify and compare the information with earlier reports from different parts of the world.

Materials and Methods

During the present study rumen fluid samples were collected from 814 adult Indian cattle slaughtered at abattoirs of Kannad, Dist. Aurangabad of Maharashtra State (India). After the removal of the stomach the rumen was slit open and 10-15 ml of rumen fluid was collected in a glass vial then the immediately the glass vial was closed airtight and brought to the laboratory. It was centrifuged and preserved by adding 1:1 glycerine alcohol solution. To determine the intensity of the ciliates live specimen were examined under the microscope by taking drop of fluid on a clean glass slide.

The permanent slides of the sample were made in duplicate stained by wet Tungstophosphoric Haematoxylin stain. Identification of genera and species of rumen ciliates were based on description published by earlier workers (Dehority 1993). All the measures of the ciliates were based on a study of 50 specimens (n=50) with an ocular micrometer, line drawings were made with a camera lucida at magnification 10x X 40x.

Result and Discussion

Diplodinium anacanthum, *Dogiel 1927*

Description of the species: (Photomicrograph 1)

The body of this species is rounded and medium in size. The adoral ciliary zone is inclined ventrally encloses the mouth. The left ciliary zone is comparatively shorter than adoral zone. The operculum is distinct, broad and extended a short distance anterior to the oral area. The surfaces of the body are convex. The dorsal surface is slightly convex than the ventral surface. The posterior half of the body is tapering, not truncated as in *D. dentatum*. The most striking feature of this species is the absence of caudal spine or lobe posteriorly it is smooth rounded. A distinct cuticular ridge is arising from the anus extending anteriorly in mid-dorsal line.

The endoplasmic sack enclosed by the boundary line along with the body surfaces. It occupies almost all portion of the body. The boundary line is thin distinctly separates the ectoplasm.

The macronucleus is long, heavy, rod shaped body. It lies under the right dorsal surface of the body. Anterior third of the macronucleus slopes ventrally at an angle of 30-40^0. The anterior end is smooth rounded while; the posterior end is blunt and narrow. The micronucleus is an ellipsoidal body lies in a small depression produced in the dorsal surface of anterior third bent region of the macronucleus. The two contractile vacuoles found in the ectoplasm left the macronucleus. The anterior contractile vacuole found just at the level of micronucleus, while the posterior contractile vacuole in the posterior third region of the body.

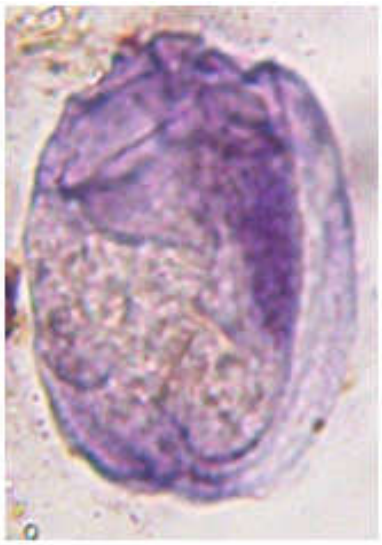
The body dimensions and other measurements of *Diplodinium anacanthum* are given in table 1.

Comments

Dogiel (1927) first described this species as *Anoplodinium denticulatum* f. *anacanthum.* Kofoid & Maclennan (1932) named this species as *Diplodinium anacanthum*. The comparison of the body dimensions recorded during the present study and those given by Dogiel (1927) are shown in table 1.

The table indicates that the length described here is smaller than the length given by Dogiel (1927). The width recorded during the present study is larger than the width reported by Dogiel (1927). The L/W ratio of the present species is 1.31, which is less against the L/W ratio 1.6 recorded by Dogiel (1927).

In the present studies *D. anacanthum* is recorded for the first time from cattle in India.



Photomicrograph 1: *Diplodinium anacanthum*

Table 1: The body dimensions and other measurements of *D. anacanthum*, *D. triacanthum* and *D. tetracanthum*. All measurements are in microns.

Sr. No.	Parameters	*D. anacanthum* (n=50)	*D. triacanthum* (n=50)	*D. tetracanthum* (n=50)
1	Body			
	Length	57.6-86.4 (71.02)	51.2-83.2 (62.52)	54.4-105.6 (75.71)
	Width	48-67.2 (54.30)	41.6-67.2 (52.80)	44.8-76.8 (62.97)
	L/W Ratio	1.15-1.56 (1.31)	1.04-1.38 (1.23)	1.04-1.38 (1.22)
2	Macronucleus			
	Length	22.4-51.2 (36.03)	12.8-41.6 (28.18)	19.2-54.4 (35.84)
	% Length to the Body	31.82-63.64 (50.63)	25-59.09 (45.04)	30.0-61.11 (47.19)
	Diam. Ant. End.	6.4-16.4 (10.97)	6.4-12.8 (10.68)	6.4-16.0 (12.48)
	Diam. Post. End	4.8-9.6 (6.66)	3.2-12.8 (6.75)	3.2-12.8 (9.47)
3	Micronucleus	1.6-6.4 (4.88)	1.6-6.4 (3.26)	1.4-6.4 (3.64)
4	Adoral ciliary zone (Mouth)	8-16 (11.30)	6.4-17.6 (11.04)	9.6-25.6 (16.12)
5	Left ciliary zone	6.4-9.6 (8.16)	3.2-12.8 (7.29)	6.4-12.8 (10.75)
6	Lobe/ Spine Ventral spine	--	3.2-12.8 (6.65)	3.2-12.8 (7.24)
	Middle spine (I lateral)		1.6-6.4 (3.36)	1.6-12.8 (4.57)
	II lateral		--	1.6-12.8 (4.41)
	Dorsal spine		3.2-9.6 (6.20)	3.2-6.4 (5.47)
7	Rectum	4.8-9.6 (7.40)	1.6-6.4 (4.03)	4.8-8.0 (6.36)

Table 2: Comparative body dimensions of *Diplodinium anacanthum*

Parameters	Authors	
	Dogiel (1927)	Present Study
Length	70-90 (80)	57.6-86.4 (71.02)
Width	40-60 (51)	48-67.2 (54.30)
L/W ratio	1.6	1.15-1.56 (1.31)

Diplodinium triacanthum, *Dogiel 1927*

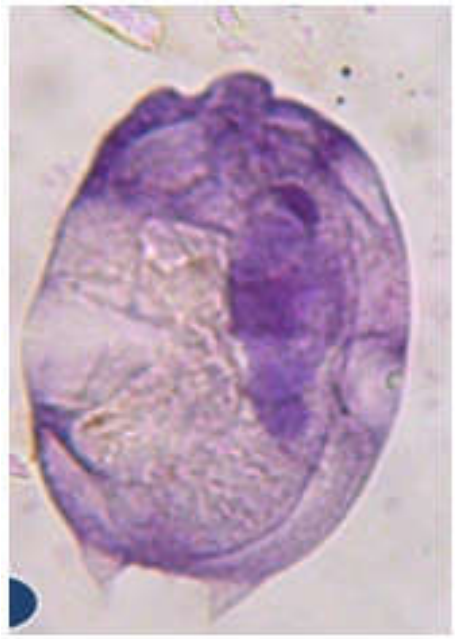
Description of the species: (Photomicrograph 2)

The body of this species is relatively short, heavy and rounded slightly tapering at posterior end. The adoral ciliary zone encloses mouth. The lips are weakly developed. The left ciliary zone is shorter than the adoral ciliary zone. Both the ciliary zones are separated by the distinct, broad operculum. The operculum projects only a short distance anterior top of the body. The surfaces of the body are convex gives maximum body width in the middle of the body. This species is identified by the presence of three caudal spines one ventral, one dorsal and one on the right side. The ventral spine is long and slightly incurved. The dorsal spine is nearly of equal size to the ventral spine while the third spine is very short. A distinct cuticular ridge arises from the anus extending anterior in mid dorsal line.

The endoplasmic sack starts anteriorly at the level of the anterior end of the macronucleus. It becomes tapering at posterior half of the body. The ectoplasm is thin. It is differentiated by a thin boundary layer.

The macronucleus varies from short, heavy to long rod shaped body. It lies under the right dorsal surface. The anterior third of macronucleus is bent ventrally at an angle of 30-45^0. The anterior end smooth rounded while posterior end is narrow with blunt end. An elliptical micronucleus lies in a notch of the dorsal surface of the anterior third bent region of the macronucleus. Two contractile vacuoles located at the left side of the macronucleus. The anterior contractile vacuole found situated at the level of micronucleus while the posterior vacuole placed at the level of the posterior end of the macronucleus.

The body dimensions and other measurements of *Diplodinium triacanthum* are given in table 1.



Photomicrograph 2: *Diplodinium triacanthum*

Comments

Dogiel (1927) firstly described it from the rumen of cattle from U.S.S.R. as *Anoplodinium denticulatum* f. *triacanthum*. Since then the species is described by many workers like Ogimoto K. & Imai S. (1981), Gureli G. (2016a) from water buffalo, and (2016b) from cattle in Turkey Cedrola *et al.* (2016) from sheep but the body dimensions are not given. The comparison of the dimensions of the species described here and those by earlier workers are shown in table 3.

The table reveals that the species described here is smaller than the species described by Dogiel (1927) and Clarke (1964) however; the maximum length of the species described here is close to the maximum length described by Dogiel (1927) and Clarke (1964). The width of the species recorded here is slightly smaller than the width described by Dogiel (1927) but it is larger than the width given by Clarke (1964). The L/W ratio described here is smaller than the L/W ratio given by Clarke (1964). The length of the macronucleus recorded here is shorter as compared to the length of the macronucleus given by Clarke (1964).

In the present studies, this species is described for the first time from the rumen of cattle in India.

Table 3: Comparative body dimensions of *Diplodinium triacanthum*.

Parameters	Authors		
	Dogiel (1927)	Clarke (1964)	Present Study
Length	70-85 (77)	65-81 (74)	51.2-83.2 (62.52)
Width	51-64 (55)	46-54 (49.5)	41.6-67.2 (52.80)
L/W ratio	--	1.33-1.65 (1.49)	1.04-1.38 (1.23)
Ma.nu.L.	--	35-40 (38.1)	12.8-41.6 (28.18)

Diplodinium tetracanthum, Dogiel 1927

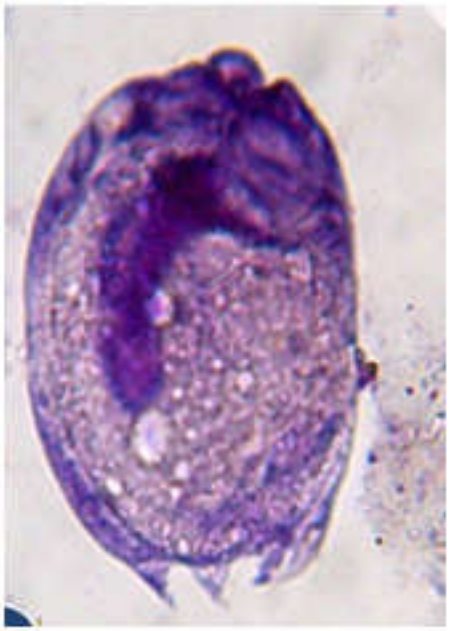
Description of the species: (Photomicrograph 3)

The body of this species is medium, spheroidal in shape and pointed at the posterior extremities of the body. The adoral ciliary zone comprises the mouth which is larger as compared to the left ciliary zone. The inner lips are continuous with the operculum which is well developed broad and runs forward short distance to the oral zone. The dorsal surface is slightly convex than the ventral surface. The maximum width of the body found in the middle of the body. The identifying character of this species is the presence of four caudal spines situated in posterior tapering part of the body. The ventral spine is the longest than all the spines, one dorsal and two lateral spines situated on the right side. A prominent cuticular ridge originating from the anus extending forward in mid dorsal line.

The endoplasmic sack originates near the anterior end of the macronucleus and occupies the posterior part of the body. The ectoplasm is slightly thick along the dorsal surface than the ventral surface of the body.

The macronucleus is heavy, long rod shaped body situated under the dorsal surface of the body. The anterior region slopes ventrally at an angle of 30^0-45^0. The anterior end of the macronucleus is slightly broader than the posterior narrow tapering end. The micronucleus is a small ellipsoidal body, which situated in the slight depression of macronucleus formed at anterior bent surface. The two ovoid contractile vacuoles located in ectoplasm at the dorsal side of macronucleus. Anterior contractile vacuole situated followed to the left ciliary zone and second one at the level of posterior end of macronucleus.

The body dimensions and other measurements of *Diplodinium tetracanthum* are given in table 1.



Photomicrograph 3: *Diplodinium tetracanthum*

Comments

Dogiel (1927) first reported *Diplodinium tetracanthum* from the rumen of cattle from U.S.S.R. as *Anoplodinium denticulatum f. tetracanthum*. Mukherjee & Sinha (1990) reported this species from the rumen of Goat in India but they have not

reported the body dimensions. A comparison of the body dimensions of the species described here and those given by earlier workers are shown in table 4.

The table reveals that the length of the species recorded during the present study is similar to the length given by Dogiel (1927) but it is slightly smaller than the length given by Clarke (1964). The width of the species described here is more than the width given by Dogiel (1927) and Clarke (1964) however the length of the macronucleus is less as compared to the length of the macronucleus given by Clarke (1964). The L/W ratio is also less than the L/W ratio given by Clarke (1964).

In the present studies, this species is described for the first time from the rumen of cattle as new host in India.

Table 4: Comparative body dimensions of *Diplodinium tetracanthum*

Parameters	Authors		
	Dogiel (1927)	**Clarke (1964)**	**Present Study**
Length	72-83 (76)	62-89 (78.8)	54.4-105.6 (75.71)
Width	52-61 (54)	40-59 (52.4)	44.8-76.8 (62.97)
L/W ratio	--	1.41-1.65 (1.51)	1.04-1.38 (1.22)
Ma.Nu. L.	--	27-51 (41.6)	19.2-54.4 (35.84)

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Original Article

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Inheritance of Tolerance to Rice Tungro Disease (RTD) in F2 Progeny of Cross Between Rice Varieties Radhunipagol and Pusa Basmati-1

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Abstract

Disease tolerance/resistance breeding depends on available genetic variability in the vast collection of germplasm as a source of disease tolerance/resistance. Rice varieties behave differently in tungro epidemic according to their susceptible and tolerant nature. From earlier investigations it was found that traditional rice varieties of West Bengal viz. Dumursail, Radhunipagol, Raghusail and Tulaipanja were tolerant varieties with zero yield loss. The genetics of tolerance in the traditional rice variety, Radhunipagol was investigated in this present study by crossing tolerant variety Radhunipagol with a susceptible variety Pusa Basmati-1. The F2 plants derived from this cross were evaluated in glasshouse and field experiments to determine the inheritance pattern of RTD resistance. A total of 14 F2 lines comprising of 683 F2 plants were evaluated for their reaction to rice tungro disease (RTD). A chi-square ($\chi 2$) analysis for assessing segregation from F2 led to the conclusion that the tolerance found in the F2 progeny of this cross is determined by a recessive gene. It indicates a typical monogenic recessive gene is governing resistance and susceptibility reaction against RTD in rice. The information obtained in this study could be valuable for rice tungro disease tolerance breeding using traditional rice varieties of West Bengal. Furthermore the data could be used in planning a systematic breeding programme to incorporate the RTD tolerance into the susceptible cultivars.

Keywords: Rice tungro disease (RTD); F2 segregating population; Chi-square ($\chi 2$) analysis; Goodness of fit; Inheritance.

Introduction

Breeding for resistance is the environmentally most sound and also most cost-effective approach to prevent losses caused by plant viral diseases. The green leafhopper (GLH) transmitted tungro virus results in one of the most economically important and wide spread viral disease of rice. Rice tungro disease (RTD) is one of the significant fears to sustainable annual rice productions in the world (Bunawan *et al.*, 2014). Management of RTD by the use of conventional tungro resistant rice cultivars has been the most important aspect of tungro research (Khush and Vinnani, 1985). It is a practical, cost-effective and environmentally sound way to stabilize rice yield and protect farmers' income.

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The first step in the study of genetics of viral resistance is to determine whether the resistant response is inherited, and if so, the number of genes involved and their mode of inheritance. (Shahjahan *et al.* 1990, Whitham and Wang, 2004.

Kang *et al.,* 2005). There have been several studies on the sources of host resistance to plant viruses and inheritance of resistance to plant viruses and viral disease (Diaz-Pendon *et al.,* 2004; Fraser, 1986, 1990). There have been several investigations on the underlying general trends or common mechanisms of virus resistance (Goldbach *et al.* 2003). Studies on the inheritance of tolerance/resistance have been carried out in some crop plants of economic importance viz. *Lycopersicon peruvianum* (Rosello *et al.* 1998), *Pisum sativum* (Provvidenti 1990; Provvidenti and Alconero 1988).

Host plant resistance

Host-plant resistance is the most effective and environment friendly approach to control the damage caused by insect pests and increase yield potential of cereal crops (Jena *et al.* 2006; De *et al,* 2012). Identification of genotypes resistant to tungro is part of the disease management programme (Latif *et al.* 2011). Localized outbreaks could then be managed by targeted deployment of relevant resistance genes to that particular environment (Azzam *et al.,* 2000).

Plant host resistance is achieved in two ways: one method involves dominant *Resistance* (*R*) genes and the other depends on recessive alleles of genes that are critical for plant viral infection (Masayosh *et al*, 2016).

Varietal tolerance

Varieties behave differently in tungro epidemic according to their susceptible and resistance nature (Dahal *et al.,* 1992). Extensive breeding programmes, conducted at International Rice Research Institute (IRRI), based on the screening of rice germplasm collections led to the identification of a number of rice cultivars resistant to RTD (Hibino *et al.,* 1990; Khush *et al.,* 2004). Among the land races of rice, Latisal, Dudshar, Ashanlaya and Nagra show mild symptoms. Indrasail, Rajmalati, Kalamkathi, Madhumalati and Dhushri remain symptompless (Mukhopadhyay, 1980). From earlier investigations it was found that traditional rice varieties of West Bengal viz. Latasail, Sonajhuli and Tulsibhog were found to be moderately tolerant with only 6%, 7% and 9% yield reduction respectively. Dumursail, Radhunipagol, Raghusail and Tulaipanja were tolerant varieties with zero yield loss (Dey and De, 2016). Tolernace was also observed in advanced rice breeding lines following mass screening and forced inoculation methods with *Nephotettix virescens* (Distant), the insect vector (Dey *at al.,* 2016). The tolerant varieties could be used as future tolerance donors in rice breeding programs.

More than 80% of reported viral resistance is monogenically controlled; the remainder shows oligogenic or polygenic control (Kang *et al.,* 2005). Many major resistance genes have been identified that condition race-specific resistance rice tungro (Azzam and Chancellor, 2002).

Rice Tungro Disease (RTD)

Tungro virus disease occurs if a susceptible variety, virus inoculum and the vector, green leafhopper that carries the virus are available in a rice field (Muralidharan *et al.*, 2003). The virus is transmitted mainly by leafhoppers *Nephotettix virescens* (Distant) and *Nephotettix nigropictus* (Stal) (Rivera and Ou, 1965, Azzam and Chancellor, 2002). The leafhopper transmitted tungro virus results in one of the most economically important and wide spread viral disease of rice. Rice tungro disease (RTD) caused by the co-infection of rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV) is a devastating viral disease of rice prevalent in Southeast Asia with outbreaks affecting thousands of hectares (Dai and Beachy, 2009).

The disease cause distinct stunting of plants, discolouration of leaves, reduction in tiller number and ultimately loss of yields. The discolouration starts from the tip and extend to the lower part of the leaf blade. Young leaves may have a molted appearance and old leaves show rusty specks of various size. The colour of leaves may be yellow or orange, slightly rolled outward and somewhat spirally twisted. The symptoms become more prominent in low nitrogen content of the soil (Mukhopadhyay, 1980). The disease adversely affects the yield components viz. height, tiller number, number of panicles/hill, number of grains, grain weight etc (Chowdhury and Mukhopadhyay, 1970). In case of severe infection by green leaf hopper (GLH) the plant look unhealthy, growth retarded, leaves turn yellow and the crop dried up. The plant may show reduction in height and tiller number when infested at tillering stage.

After high-yielding rice varieties were introduced in the early 1960s throughout South and Southeast Asian countries and double-rice cropping and staggered planting became more common in irrigated areas, tungro became an increasingly important disease (Hibino, 1996). At most of sites within each country, the genetic composition of the virus population was not significantly

different over the two or three cropping seasons. The result suggested that the geographically isolated populations are genetically stable over the sampling time (Azzam *et al.,* 2000).

The disease is caused by a complex of two viruses, RTBV (Rice Tungro Bacilliform Virus) and RTSV (Rice Tungro Spherical Virus). The RTBV is DNA virus, responsible for the development of the symptoms in leaves whereas RTSV is RNA virus, responsible for the transmission of the disease. Both the virus is non-enveloped. RTBV is a virus of 30-35nm in diameter and 160-220 nm long, whereas RTSV is of 30 nm in diameter. The virus infection results a drastic reduction in chlorophyll, amount of sugar increase whereas starch decrease in the grain. Enhanced understanding of transmission, inheritance pattern and biological control of these viruses makes tungro disease very significant in terms of plant virology, molecular biology and entomology, with the focus on achieving the ultimate goal of improved management strategies for control of RTD in order to reduce the economic damage to global rice production (Bunawan *et al*., 2014).

Materials and Methods

To determine the mode of inheritance of the rice tungro disease (RTD) tolerance, a tolerant landrace of West Bengal, Radhunipagol was taken as the male parent and crossed with the RTD susceptible Pusa Basmati-1 rice variety as the female parent. To identify gene(s) involved in RTD tolerance the association of genotypic and phenotypic variation for RTD resistance was examined in a F2 population derived from cross between a susceptible variety Pusa Basmati-1 and one tolerant variety Radhunipagol. The F2 plants derived from these crosses were evaluated to determine the inheritance pattern of RTD tolerance.

Rice population

Sowing of parent material and crossing program were performed during aman season. F1 seeds are collected and raised to build up segregating F2 population in rabi season. A total of 683 F2 plants planted in 14 lines were examined to analyze the pattern RTD tolerance inheritance. The screening was done according to IRRI Standard Evaluation System method (Gomez and Gomez, 1984) to assess their reaction against Rice Tungro Disease in Bose Institute Experimental Farm at Madhyamgram, West Bengal.

Evaluation of RTD reaction

The screening of the germplasm was done in three ways as follows:

A. Mass Screening in Seed Bed

1. The seedlings were sown directly on raised seedbeds in single lines flanked by one line of TN 1 (susceptible check). A spacing of 10 cm is left in between the lines. The seeds were sown in the 1st week of October so as to coincide with the peak level of natural occurrence of Nephotettix *virescens* (Distant) population. One line of TN 1 is sown lengthwise in both sides of the varieties. Three lines of tungro infected tillers of Jaya 1 (provided by Bidhan Chandra Krishi Visvavidalaya, West Bengal) were transplanted in the longitudinal channels between the beds. The disease score were taken from 30 – 60 days.

B. Mass screening in Field Condition

The same TN 1 encircled screening procedure was maintained for the transplanted plants in field condition.

C. Forced Inoculation method

The seedlings of different F2 lines were planted in 10 inch pots. The pots were placed inside mosquito nets to form insect-proof enclosures in the greenhouse of 6 x 7 ft area. Infected TN 1 plants were placed inside the mosquito net. 500 *Nephotettix virescens* (Distant) were released inside the mosquito net periodically. The pots were taken out after 7 days and observed for further development of the symptoms.

Statistical analysis

The F2 plants derived from this cross were evaluated in glasshouse and field experiments to determine the inheritance pattern of RTD resistance. The RTD occurrence was recorded among the plants of F2 population of the cross based on the visual scores. The numbers of resistant and susceptible plants among the F2 generation were counted per line. A total of 14 F2 lines comprising of 683 F2 plants were evaluated for their reaction to rice tungro disease (RTD). Observations recorded in segregating generations were subjected to the chi-square ($\chi 2$) analysis of goodness of fit, using standard formula at 5% level of significance. The Null hypothesis (H_0) was taken as 3:1 ratio (susceptible: resistance).

Results

To deduce the inheritance pattern of RTD tolerance rice (*Oryza sativa* L.) cross between tolerant traditional variety Radhunipagol × susceptible variety Pusa Basmati-1 were evaluated using Chi-square analysis ($\chi 2$) at 5% level of significance. The test was performed to analyze the expected deviation from the Mendelian segregation ratio in the segregating F2 generation. The results are presented in Table 1. All information pertaining to RTD tolerance confirmed that the F1s of the cross showed no symptoms to RTD and this marked clearly that the resistance was susceptible over dominance. With respect to observed: expected F2 segregation ratio for resistance: susceptible chi-square test showed non-significance chi-square value between the probability of 0.9 - 0.8. The Chi square ($\chi 2$) analysis confirmed that the expected ratio (Null hypothesis), is a good fit with 3:1 ratio (susceptible: resistance) in F2 progeny at 5% level of significance. It indicates a typical monogenic recessive gene is governing resistance and susceptibility reaction against RTD in rice for this cross combination.

The findings were as follows

1. The tungro tolerance seems to be governed by single recessive gene in the tolerant variety Radhunipagol.
2. The F2 segregated into susceptible: tolerant as 3:1 ratio and is supported by Chi square ($\chi 2$) test (Table 1).

Discussion

Recessive resistance, is also widely exploited in many crops (Truniger and Aranda, 2009; Wang and Krishnaswamy, 2012). In fact, about half of the alleles responsible for virus-resistance in crops are recessive (Kang *et al.,* 2005). Resistance with recessive inheritance, mostly acquired via the alteration of key host factors required for the viral infection cycle, is also recognized as an effective antiviral resistance mechanism (Robaglia and Caranta, 2006). Recessive resistance traits can be introduced into crop species by crossing, or random mutagenesis and selection (Piron *et al.,* 2010). Recessive resistance breeding has the practical advantages of not requiring the introduction of transgenes and not being restricted by the selection of naturally occurring traits only.

The result of genetic analysis for RTD tolerance in this study is consistent with other studies that resistance of tolerant Utri Merah rice variety to RTSV (strain A) is controlled by a single recessive gene, although additional genes in Utri Merah may also be involved in resistance against other strains of RTSV (Azzam *et al.* 2002).

For any monogenic trait, the segregation of plants in F2 generation should follow 3:1 ratio (resistant: susceptible). The goodness of fit was used to calculate Chi square ($\chi 2$) for 3:1 ratio at 5% level of signifinace. The ratio of susceptible and resistant plants in F2 generation of susceptible X resistant cross combination were tested for goodness of fit

Table 1: Chi – Square ($\chi 2$) Values of Segregation of Resistant/Susceptible F2 Plants to Rice Tungro Disease (RTD) from the Cross Pusa Basmati-1 ♀ X Radhunipagol ♂

Line No.	Total Plants	Sus.	Exp. Sus.	$\chi 2$	Res.	Exp. Res.	$\chi 2$	$\chi 2$ T	Probability
1	50	31	37.5	1.126	19	12.5	3.38	4.5	0.02- 0.05
2	50	36	37.5	0.06	14	12.5	0.18	0.24	0.05- 0.75
3	52	42	39	0.23	10	13	069	0.92	0.25-0.5
4	48	38	36	0.11	10	12	0.33	0.44	0.5
5	51	37	38.25	0.04	14	12.75	0.122	0.16	0.5-0.75
6	50	38	37.5	0.006	12	12.5	0.02	0.026	0.75-0.9
7	50	38	37.5	0.006	12	12.5	0.02	0.026	0.75-0.9
8	51	38	38.25	0.0016	13	12.75	0.004	0.005	0.9-0.95
9	54	44	40.5	0.302	10	13.5	0.907	1.209	025-0.5
10	54	43	40.5	0.154	11	13.5	O.46	0.61	0.25-0.5
11	51	37	38.25	0.04	14	12.75	0.122	0.16	0.75
12	46	31	34.5	0.35	15	11.5	1.06	1.41	0.1-0.25
13	49	35	36.75	0.083	14	12.25	0.25	0.33	0.5-0.75
14	29	24	21.75	0.23	5	7.25	0.69	0.92	0.75-0.9
Total	683	510	512.25	0.0099	173	170.75	0.0295	0.039	0.8-0.9

SUS=Susceptible, Exp Sus= Expected susceptible, $\chi 2$ = Calculated $\chi 2$ value,
RES= Resistant, EXP RES= Expected Resistant, $\chi 2$ T= Total $\chi 2$ value

to the expected segregation and all the crosses were in agreement with the expected 3:1 ratio with high degree of confidence (p = 0.8–0.9). This confirms the presence of one gene in the tolerant parent Radhunipagol.

Conclusion

The data generated here may be used as base line data in determination of the tolerant races against tungro disease. With proper serological and PCR based detection of the presence of RTV in the plants may help in screening these land races in search of resistance. The resistance against rice tungro disease may be transferred through breeding and a disease resistant plant may be developed which is evident from the crosses made. As the resistance is governed by a single gene, it may be relatively easy to raise a resistant plant against tungro with proper screening method.

Limitations of the study

The detection was based on the morphological symptoms, supported by forced inoculation technique only. The serological and/or PCR based detection was not possible due to lack of infrastructure.

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Clinico-Pathological analysis of Hb S/β+ Th Patients in Odisha: A Tertiary Care Hospital Based Study

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Abstract

India is ethnically a diverse country with marked regional variation. Due to migration, there is constant mixing of people from different regions. These migrations not only helped in creating variations leading to positive mutations but also caused many genetic abnormalities leading to inheritance of genetic disorders. The most common genetic disorder that occurs during the neonatal period is hemolytic anemia. Being multifactorial in nature, this disease is predominantly intrinsic to erythrocytic dysfunction. These dysfunctions are related to structural and functional abnormality of erythrocytes, thus leading to hemoglobinopathies. From clinical as well as epidemiological point of view, prevalency within hemoglobinopathic mutations are sickle cell anemia and thalassemia. These mutations affect population with origin in Africa, the Mediterranean region, Southeast Asia, the Middle East and the Far East. Around 1-2% of the global population is heterozygous for Hb S and 3% are heterozygous for β-thalassemia. Sickle cell disease (SCD) and thalassemia are the most common forms of hereditary hemolytic anemia. Apart from monogenic inheritance, co-inheritance is also seen leading to compoung heterozygosity within the population. Persons with Hb S/β- thalassemia major are almost never symptomatic at birth because of the presence of Hb F, but symptoms begin to develop by six months of age. They often die of cardiac complications of iron overload by 30 years of age. Beginning transfusion and chelation therapy are difficult challenges for parents to face early in their child's life. Therefore, this pilot study was attempted to observe the prevalence of compound heterozygosity within a particular population. If a bone marrow transplant is a possibility, the blood for transfusion should be negative for cytomegalovirus. Hematopoietic stem cell transplantation has cured >1,000 patients who have S-β-thalassemia major. We think that antenatal screening or screening of higher secondary school children to detect hemoglobinopathies, counselling of the individuals with hemoglobinopathies is expected to help in drastically reducing the incidence of the disease. We need to reduce the burden of genetic disease by implementing programs such as population screening, genetic counselling and prenatal diagnosis. The available data for Hb S/β- thal are mostly based on the patients attending hospitals for treatments and their immediate family members. This data will help in planning population screening programs and thus, consequently result in the reduction of genetic diseases.

Keywords: Hb S/β-Thal; Hemoglobinopathies; Transfusion.

Introduction

Human Hb is a globular tetramer formed by the combination of two "type α" (α or ζ) polypeptide (globin chains) with two "type β" (β, δ, Gγ, Aγ or ε) chains. Each chain has its own prosthetic heme group, which forms a reversible bond with the oxygen molecule (O_2), thereby fulfilling the primary function of Hb, which is to transport O_2 from the lungs to peripheral tissues (Old, 2007). Synthesis of each of the globins is controlled by

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distinct genes, which are arranged in two clusters; the genes that code for the α and ζ chains (α clusters) are located in the telomeric region of the short arm of chromosome 16 (16p 13.3), whereas the genes that code the β, δ, γ and ε chains (β *cluster*) are on the short arm of chromosome 11 (11p 15.5). During the embryonic period, the embryonic Hb variants Gower I (ζ2ε2), Gower II (α2ε2) and Portland I (ζ2γ2) are produced; during the fetal period these are substituted by fetal Hb or Hb F (α2γ2), which then cedes its place to Hb A (α2β2) and A_2 (α2δ2) in adulthood (Dhaliwal, 2004) (Fig. 1). Six months after birth, Hb A predominates absolutely, making up more than 95% of total cellular Hb, while Hb A_2 levels are around 2-3%, and Hb F levels are 0-2% (Catlin, 2003).

Hemoglobinopathies are the result of mutations that affect the globin genes and can be classified into two major groups namely

a) Structural alterations, which form anomalous Hb variants, and

b) synthesis alterations (thalassemia), where one or more types of globin chain are partially or completely suppressed. Less frequently, the two phenotypes can occur in combination. Less frequently the two phenotypes can occur in combination.

Structural hemoglobinopathies are generally caused by simple substitutions, small insertions or deletions of bases that affect coding regions of the genes and lead to amino acids in the protein chain being substituted (Khattab *et al*., 2006).

Notable among these is Hb S ($\alpha_2\beta S_2$), a variant that affects position 6 on the β chain, substituting Glutamic acid with Valine (β6 Glu→Val). It was described by Itano and Pauling (1949) as a form of Hb found in the red blood cells of patients with sickle-cell anemia (SCA), with electrophoretic migration that differentiated them from normal individuals. When Hb S is in its deoxygenated state (deoxy-HbS) and in elevated concentrations, it polymerizes, resulting in abnormally rigid and inflexible red blood cells (sickle red blood cells). These in turn lead to chronic hemolysis and vaso occlusion, , which are the pathophysiologic bases of the disease (Shah, 2004).

The thalassemia is the result of a reduction, or an absence of production, of one or more globin chain types, leading to a buildup of another type, the synthesis of which is unaffected. The excess chains are unstable and precipitate, leading to changes to the erythrocyte membrane and early destruction of the cell (Thein, 2004). Furthermore, the deficient hemoglobinization of erythrocytes results in hypochromia and microcytosis, which are characteristic abnormalities of this group of diseases. Thalassemia are classified as α, β, γ, δ, δβ or γδβ, depending on the type of chain whose production is affected. Thalassemia α and β are the most common, while the majority of the first are caused by deletions that remove α genes (Tolentino and Friedman, 2007). The β-thalassemias are generally the result of substitutions of bases on the exons, introns and promoter regions of β genes (Vekilov, 2007). It should be noted that if both the traits are inherited together, then this compound heterozygotic condition is denoted by HbS-β-thalassemia. However, co-inheritance of Sickle cell anemia and β- thalassemia (HbS-β thal) is mainly determined by β thal genes variants (Tefferi, 2004; Shah, 2004; Madigan and Malik, 2006).

During the process of mutation, LCR plays a vital role. In case of humans, LCR is physically defined by 5 HS region (Hypersensitivity region) which is responsible for transcription stimulation at high levels (Fig 1). Individual HS region have different roles in the remodeling of chromatin as well as globin gene switching and they are as follows:

i. HS_1 – It is located to the 5′ end of ε-globin gene and this region show no sign of hematological defect.

ii. HS_2 - It is a general enhancer element. It encodes for E-box sequences which forms the binding sites for the basic helix-loop-helix proteins such as USF and Tal1.

iii. HS_3 - It is a general weak enhancer element. It helps in chromatin remodeling.

iv. HS_4 - It helps in chromatin remodeling.

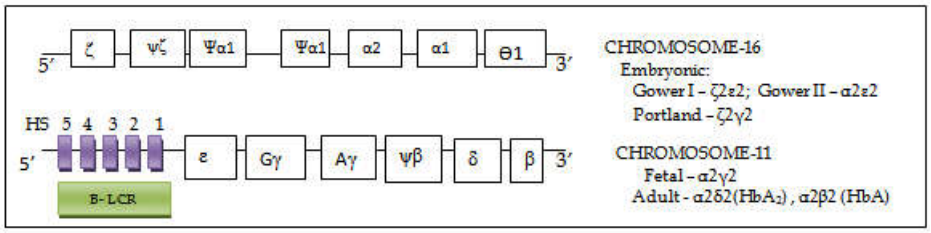

Fig. 1: Clusters of α and β-globin genes with β-Locus Control Region

v. HS_5 - It is a constitutive hypersensitive site which acts an insulator.

The abnormalities in the synthesis of Hb chain, both qualitatively and quantitatively can be assessed presently by using a number of conventional methods, such as HbF estimation, HbA2 estimation, CBC and so on (Urbinati et al., 2006).

Table 1: Variants in sickle β- thalassemia patients

Sl No	Variants of S-β-T	Efficacy
1	Mild Hb S/β Th	It is observed in about 15% of all cases in Southeast Asia. This group of patients maintains Hb levels between 9 and 12 g/dl and usually does not develop clinically significant problems. No treatment is required.
2	Moderately Hb S/β Th	The majority of HbE/beta-thalassemia cases fall into this category. The Hb levels remain at 6-7 g/dl and the clinical symptoms are similar to thalassemia intermedia. Transfusions are not required unless infections precipitate further anemia. Iron overload may occur.
3	Severe Hb S/β Th	The Hb level can be as low as 4-5 g/dl. Patients in this group manifest symptoms similar to thalassemia major and are treated as thalassemia major patients.

Methods and Methodology

A) Population study

While investigating the etiopathogenesis of 30 subjects with anemia, we came across with 10 subjects with Hb S/β Th. Herewith, we report the clinical findings of 10 patients with HbS received from tertiary care hospital. Present study relates to the results of clinical and hematological examination of 10 patients with HbS. Male to female ratio was 1:1. Six of 10 patients belonged to Balasore district; three other patients were from Banki, Nayagarh and Keonjhar of Odisha, India.

Age: Above 2 years and below 30 years.

Inclusion Criteria: Patients having high HbF, low/absence of HbA and not having more than 8 transfusions per year.

Exclusion Criteria: Transfusion cases of 21days, patients having any associated chronic illness.

B) Methodology

Venous blood samples were collected and smears were prepared. Blood amounting 2.5 ml was collected in a tube containing EDTA as an anticoagulant. It was mixed well and analyzed. Smears were stained by Leishman's stain and examined. Total hemoglobin was estimated by Sahli's method. Sickle cell test was also undertaken. It is a slide based test for sickling using sodium metasulphite. CBC and HPLC were followed using automatic cell counter

a) Cohort study (through clinical reports and questionnaire) was undertaken to analyze any symptomatic parameter (Vichinsky *et al.,* 2005; Wenning and Sonati, 2007; Wintrobe and Foerster, 2004).

b) PBS was carried out to study the morphology of blood cells. To prepare a PBS, 0.2 gm of powdered Leishman's dye was added to 100 ml of methanol (acetone free) and the mixture was warmed at 50^0C in the shaking waterbath for 15 min. The solution was then filtered and allowed to stand at room temperature for 24 h before use (Jison *et al.,* 2004; Vekilov, 2007).

c) Solubility test for HbS was performed following the standardised protocol. Sickling confirmatory test was performed by using 2% sodium metabisulfite solution (Itano and Pauling, 1949; Hustman et al., 1970; Vichinsky et al., 2005). Nestroft screening was followed to screen β-thal heterozygotes.

d) HPLC was undertaken by using Variant™ Hb testing system, Biorad.

C) Key parameters to be analysed in CBC

Structural hemoglobinopathies may have an impact on the red cell indices, and red cell indices are critical to the diagnosis of thalassemias. The key components of the CBC include Hb, red blood cell (RBC) number, mean corpuscular volume (MCV), and red cell distribution width (RDW) (Tefferi, 2004). The procedures were standardized by following the standardized protocol (Wintrobe and Foerster, 2004).

The **RDW** is a measure of the degree of variation in red cell size. Some causes of microcytic anemia, most notably iron deficiency, are characterized by an increase in RDW (Steiner *et al.,* 2007). Therefore, the RDW may provide information useful as an adjunct to diagnosis but is not useful as a lone indicator.The normal RDW level is 10.2 to 14.5%.

The **RBC count** is also useful as a diagnostic adjunct because the S-β-thalassemias produce a microcytic anemia with an associated increase in the RBC number. Other causes of microcytic anemia, including iron deficiency and anemia of

chronic disease, are more typically associated with a decrease in the RBC number that is proportional to the degree of decrease in Hb concentration.

Mean corpuscular volume (MCV) is the average volume of red cells in a specimen. MCV is elevated or decreased in accordance with average red cell size, i.e., low MCV indicates microcytic (small average RBC size), normal MCV indicates normocytic (normal average RBC size), and high MCV indicates macrocytic (large average RBC size). The reference range for MCV is 80-96 fL/red cell in adult.

The **Hb concentration** typically is decreased in S-β-thalassemia.

TThe **hematocrit** measures the volume of red blood cells compared to the total blood volume (red blood cells and plasma). The normal hematocrit for men is 40 to 54% whereas for women it is 36 to 48%. This value can be determined directly by microhematocrit centrifugation or calculated indirectly.

The **mean corpuscular hemoglobin** (**MCH**), or "mean cell hemoglobin" (**MCH**), is the average mass of hemoglobin per red blood cell in a sample of blood. It is reported as part of a standard complete blood count. **MCH** value is diminished in hypochromic anemias. MCH levels are between 27 and 31pg.

Mean cell hemoglobin concentration (MCHC) is the average concentration of hemoglobin in a given volume of blood. Normal MCH levels are between 27 and 36 g/dl for adults and between 32 and 34% for children. If the MCHC level is below 28%, this is considered to be too low. The MCHC level can be too low because of blood loss overtime, too little iron in the body, or hypochromic anemia.

Observation and Discussion

From the above study, following aspects have been observed:

a. The morphological picture of PBS in case of HbS-β thal patients is nearly similar to β-thal homozygotes showing moderate to severe aniso-poikilocytosis, hypochromia, microcytosis, polychromatic red cells and occasional target cells along with nucleated normoblasts. The presence of nucleated RBCs (NRBCs), polychromasia, sickle cells, target cells, Howell-Jolly bodies, and Pappenheimer bodies were also observed. Neutrophilia and thrombocytosis may also be observed (Fig. 2).

b. Sickling test shows the presence of sickling cells. Solubility test for the presence of HbS shows a positive turbidity resulting in visual impairment of the bold dark lines,thus, confirming the presence of HbS. NESTROFT test shows the presence of a partial visibility, thus, showing partial positivity (-/+) and confirming the presence of β-thalassemia heterozygotes (Table 3).

c. There is a significant decrease in MCV for all groups (26 fl - SS, 28.8 fl - S/Beta0 thalassemia and 20.8 fl - S/Beta+ thalassemia). Microcytosis resulting from the action of HU in S/Beta thalassemia patients measured by MCV, is lower than that is observed in homozygous S at six months of treatment but it reaches a comparative value after more than one year. Microcytosis and hypochromia due to the thalassemia mutation contribute to this effect (Table 2).

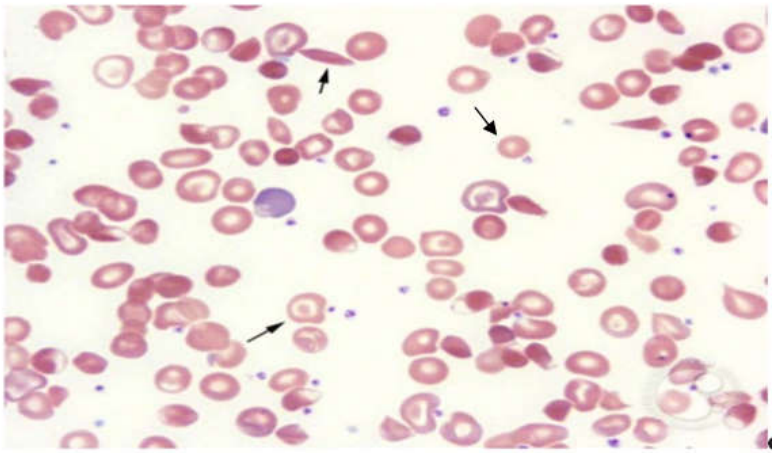

Fig. 2: Peripheral blood smear

d. The MCH concentration does not reduce significantly, but it is slightly higher in S/beta thalassemia. S/ß0 thalassemia results from the absence of ß-chain production that causes red blood cell (RBC) instability due to excess α chains, leading to abnormal erythropoiesis. S/ß0 thalassemia can be differentiated from sickle cell anemia based on RBC morphologic characteristics. S/ß0 thalassemia is characterized by microcytic, hypochromic RBCs, along with the presence of target cells and fewer sickle cells (Table 2).

e. The expected hemoglobin electrophoresis results in blood specimens from patients with sickle cell anemia show the following values: 80% sickle cell hemoglobin (HbSS), 1% to 20% hemoglobin F (HbF), 2% to 4.5% hemoglobin A2 (HbA2), and absence of hemoglobin A (HbA) if the patient has not recently received a transfusion. These cases had a large range of variation in hemoglobin level (5.2-13.0%), but the majority had moderate to severe anemia (Table 3).

Table 2: Various indices of clinical components

Case No	Subjects	Age (in yrs)	Sex	Hb (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW (%)	WBC ($x10^3$/µl)	RBC ($x10^6$/µl)
01	Father	37	M	13.7	45	68	28	28	7.8	11.6	5.9
	Mother	25	F	10.6	34	56	22	33	8.9	9.4	5.0
	Son	06	M	10.9	28.2	22	18.0	30	18.3	17.9	3.7
02	Father	29	M	12.7	40	70	28	27	8.4	8.2	4.5
	Mother	21	F	10.5	36	65	27	28	8.1	7.5	4.0
	Son	04	M	12.2	29.3	28.8	17.5	29	15.1	14.4	3.5
03	Father	37	M	13.9	45	65	31	35	9.5	9.5	4.6
	Mother	23	F	09.5	40	69	25	29	8.2	8.7	3.8
	Son	07	M	11.2	30.7	20.8	22.1	25	15.5	15.7	3.3
04	Father	41	M	13.9	50	80	30	36	9.7	11.4	5.0
	Mother	31	F	10.6	44	75	25	29	10.5	9.5	3.8
	Daughter	10	F	11.5	27.9	32.3	20.0	29	16.7	14.6	2.9
05	Father	33	M	14.1	51	65	29	36	11.4	10.5	4.8
	Mother	26	F	11.5	40	75	27	27	8.6	8.7	4.1
	Son	06	M	10.7	26.0	20.8	16.8	29	17.5	14.6	3.1
06	Father	29	M	13.8	55	71	30	32	9.5	9.5	4.5
	Mother	24	F	11.5	36	62	27	29	10.2	8.5	3.9
	Daughter	05	F	09.5	31.4	20.8	23.2	25	15.8	13.7	2.9
07	Father	34	M	12.6	51	80	31	36	8.5	10.7	5.0
	Mother	26	F	10.8	37	75	25	32	10.2	8.5	3.8
	Daughter	06	F	10.2	30.6	29.9	21.2	26	16.5	11.6	3.1
08	Father	38	M	14.2	50	65	31	29	10.4	11.7	4.9
	Mother	25	F	11.2	41	70	29	27	9.5	9.4	4.1
	Son	04	M	12.3	32.4	27.9	20.2	29	15.9	12.4	3.2
09	Father	42	M	13.7	49	80	29	35	9.5	11.5	5.0
	Mother	37	F	10.5	36	73	27	30	9.7	9.5	4.4
	Daughter	10	F	10.5	33.2	35.3	24.3	29	16.9	14.5	3.1
10	Father	30	M	13.5	55	91	28	30	10.4	10.5	4.9
	Mother	27	F	10.5	40	85	26	29	11.7	7.3	4.1
	Daughter	05	F	09.7	30.4	32.6	22.2	27	17.6	11.5	2.9

Table 3: HPLC data of 10 patients

Case No.	Subjects	Age (in yrs)	HbF	HbA	HbA_2	HbS	Sickling %
01	Son	06	23.9	2.8	2.9	61.3	Positive
02	Son	04	22.7	14.4	2.5	76.5	Positive
03	Son	07	17.8	7.3	2.4	69.5	Positive
04	Daughter	10	19.4	11.5	3.4	63.3	Positive
05	Son	06	17.6	8.8	2.5	69.0	Positive
06	Daughter	05	18.0	2.8	2.8	0	Negative
07	Daughter	06	28.9	2.4	2.7	65.3	Positive
08	Son	04	18.5	2.6	2.6	0	Negative
09	Daughter	10	23.7	2.2	2.4	63.2	Positive
10	Daughter	05	22.9	2.4	2.5	0	Negative

It is apparent that the majority of the sickle cell-β- thalassemia cases showed reduced values of red cell indices like HCT, MCV, MCH and MCHC manifesting hematological aberrations before blood transfusion (Table 2). The RBC values were either reduced or normal in the above 10 cases of sickle cell-β-thalassemia. HPLC also showed a predominant fluctuation in HbF, HbA_2, HbS and HbA values. Our study also projects the sickling percentage in the above subjects. Based on the above results, the effectiveness of screening can be compared with the clinical cases present previously for better knowledge and understanding. We also suggest that genetic counseling and prenatal diagnosis can be used for reducing the burden of increasing genetic disease.

Conclusion

Differentiation of sickle cell anemia, beta thalassemia and the sickle beta thalassemia syndromes need to be undertaken carefully due to close similarity of symptoms and laboratory findings, i.e., microcytosis, hypochromia, target cells and sickle cells in the peripheral smear. The hemoglobin electrophoresis pattern of the sickle-beta thalassemia consists of high HbS with an increase in HbF, HbA2 and low HbA value. The present study highlights the coinheritance of β-thalassemia and Hb S gene, which is wide spread in southern and western Odisha. Further it is assumed that a large number of such double heterozygote cases remain undiagnosed or misdiagnosed leading to premature death without proper treatment. Molecular diagnosis of Hb D, HbE or Hb S gene is required along with characterization of β-globin gene mutations in this region. The prenatal diagnostic facilities and services, genetic/ marriage counselling are the ultimate aims to be achieved. This is a preliminary study and we will carry out the beta globin gene mutation to establish the above facts in more detail.

Ethical Clearance

This study confirms the ethical principles of Medical research developed by World Medical Association, Declaration by Helsinki (1999). Ethical clearance was given by the Institutional Ethical Committee (IEC) of SCB Medical College and Hospital, Cuttack, Odisha to work in the Department of Clinical Hematology and Department of Pediatrics, SCB Medical College and Hospital, Cuttack. Vide letter number IEC/IRB No.-824/11.03.2019..

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Conflict of Interest: None

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