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Preface

Biotechnology is a research oriented field that encompasses a large number of intimately entwined disciplines. It is a fast growing area with huge potential and impacts in various fields like agriculture, healthcare, industries and environmental protection. To keep up with the rapid pace of the developments in the field, Department of Biotechnology, St. Joseph's College organized a National seminar on 'Recent Advances in Biotechnology'. The Seminar offered a unique platform for eminent researchers and experts to have intensive scientific deliberations and provided opportunity for young scholars to present their research innovations. Prominent resource persons from emerging fields of Tissue engineering, Regenerative medicine, Bioinformatics, Animal Cell culture and Aquaculture addressed topics of novel issues in respective fields. This volume consists of all the invited lectures and paper presentations. It is brought forth with the hope of promoting the novel ideas presented in the conference.

Department of Biotechnology, St. Joseph's College gratefully acknowledges the financial assistance from University Grants Commission. A special note of gratitude to the members of Advisory Board, resource persons and the delegates for their support, co-operation and active participation in the event.

Organizing committee

CONTENTS

KEYNOTE ADDRESS

Biotechnological applications in Aquaculture and Fisheries

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Introduction

Biotechnology is the application of science and technology to living organisms, as well as parts, products thereof, to alter living or non-living materials for the production of knowledge, goods and services. Biotechnology may include techniques such as bio-processing, bio-harvesting; bio-prospecting, bioremediation while applications may include: health, food, cosmetics, aquaculture & agriculture, fisheries, manufacturing, environmental remediation, bio-films and biomaterials etc. Aquatic biotechnology has continued to develop in recent years as a field of application of modern science and engineering of critical importance to the understanding, protection and exploitation of the resources for the progress of fundamental science and benefit of humanity. Priorities of research in aquaculture and aquatic biotechnology include improvement of fish and shellfish feed quality, genetic manipulation to improve nutrition, reproduction and growth in cultured species, development of useful transgenic varieties for better husbandry, development of prophylactic agents like vaccines, immunostimulants, probiotics, and assess biodiversity.

Indian fisheries and aquaculture is an important sector of food production, providing nutritional security to the food basket, contributing to the agricultural exports and engaging about fourteen million people in different activities. With diverse resources ranging from deep seas to lakes in the mountains and more than 10% of the global biodiversity in terms of fish and shellfish species, there have been considerable advances in the range of aquatic organisms, or molecules derived from aquatic organisms, that have been applied to a variety of problems in the medical and environmental fields. During the last ten years, through biotechnology and bio-informatics, information about the genetic structure of wild and captive populations of

several Indian fishes and traits of interest to aquaculture are now becoming available and our knowledge of these areas should increase greatly over the next decade, as should the applications of genetics to the aquaculture and fisheries.

Genetic modification and biotechnology also holds tremendous potential to improve the quality and quantity of fish reared in aquaculture. There is a growing demand for aquaculture; biotechnology can help to meet this demand. When appropriately integrated with other technologies for the production of food, agricultural products and services, biotechnology can be of significant assistance in meeting the needs of an expanding and increasingly urbanized population in the next millennium. Successful development and application of biotechnology are possible only when a broad research and knowledge base in the biology, variation, breeding, agronomy, physiology, pathology, biochemistry and genetics of the manipulated organism exists.

Applications in aquaculture

Increasing vigour of cultured species was always a challenge for improving productivity. Scientists have developed chromosome manipulations for higher growth of the fish. Chromosome set manipulation techniques to induce polyploidy (triploidy and tetraploidy) and uniparental chromosome inheritance (gynogenesis and androgenesis) have been applied extensively in externally fertilizing fishes especially in cultured freshwater fish, like salmonids, cyprinids, ictalurids and cichlids. Induced polyploidy refers to the production of fishes with extra sets of chromosomes. This can be done by treating fertilized eggs with either thermal shock, hydrostatic pressure or chemical treatments. If the treatments are applied shortly after fertilization, triploids can be produced due to retention of the second polar body of the egg. If the treatments are applied shortly before the first cleavage division, tetraploids can be produced. Current interest in induced polyploidy is mainly due to its potential application to fish farming. Triploid fish are sterile and this induced sterility could be useful in fish management and aquaculture as a method of preventing over-population and improving growth and survival of fish after the age of sexual maturity. By circumventing gonadal maturation, triploids of several species can grow faster than diploids (Thorgaard, 1987). Chromosome manipulation research

has been carried out in India since early eighties, leading to artificial induction of diploid gynogenesis and polyploidy in Catla catla, Labeo rohita, Cirrhinus mrigala, Oreochromus mossambicus, Beta splendens, Brachydanio rerio (Reddy et al., 1990; Kavumpurath and Pandian, 1990; Pandian, 1993, 2003). Considerable progress has been made in finding out optimum parameters with regard to the techniques of genetic inactivation of milt with UV irradiation, the exact thermal (cold and heat) and pressure shock regimes and the time and duration of their administration. This has resulted in the production of 100 percent gynogenetic and triploid individuals in O. mossambicus and B. rerio (Pandian, 1993).

Gynogenesis

Gynogenesis is a method to produce all female fish through induced chromosomal alteration. It involves producing diploid individual with both chromosome sets from the female. In other words, gynogenesis is the development of embryos from eggs without genetic contribution from penetrating sperm and is an example of total maternal inheritance. Induced gynogenesis involves inactivating the paternal (sperm) genome and then restoring diploidy either through suppression of the second meiotic division or suppression of the first mitosis. Most of the recent protocols for gynogenesis have used ultraviolet irradiation to inactivate the paternal genome, and temperature (cold or heat) shocks to restore diploidy. To date, gynogenesis has been artificially induced in over 20 fish species world wide. In India, gynogenesis has been induced in Labeo rohita, Cirrhinus mrigala and Catla catla at CIFA, Bhubaneswar using cold shocks (120C 10 minutes) and heat shock (390C, 1 minute) by John et al. (1984, 1988).

Androgenesis

Androgenesis is the method to produce all male fish species. In this technique, all of the genetic nuclear material is provided by the male parent and doubling of a haploid embryo can be achieved by interrupting the first cleavage in the developing egg. This process halts the actual division of the two cells while allowing the normal chromosome replication. This merging of identical chromosome sets can be achieved with heat or pressure shocks treatments applied a few minutes post fertilization. A number of androgenetic fish were produced using heat shock protocol. Though

survival rate is less in this process (0.5 to 10%), it would be possible to "recover" a population solely from cryopreserved sperm. This technique also useful, where male fish grow bigger and yield good productivity.

Transgenics in Aquaculture

Worldwide, the demand for fish continues to increase at a higher rate than wild fish population can support. A major problem facing in the aquaculture industry is outbreak of disease, as farmed fish are generally cultured at high densities and under stress, putting them at increased risk for bacterial infection. Current fishing practices are proving increasingly unsustainable because of the diminishing global fish stocks due to over exploitation and outbreaks of disease, thus threatening biodiversity. These concerns on depleting natural fish stocks, together with an ever increasing global market for food fish have led to a series of technological innovations such as transgenic to achieve increased production from aquaculture.

An organism that has a foreign or modified gene stably integrated in its genome using the in vitro genetic techniques of genetic engineering is called as transgenic or genetically modified organism (GMO). The introduction of this exogenous DNA into the genome of the recipient organism should be such that it is stably maintained in a heritable manner. This technology offers an excellent opportunity for modifying or improving the genetic traits of commercially important fishes for aquaculture. The driving force behind the application of transgenic technology to fish is the desire to produce genetically superior broodstock for food production. Asian scientists were the first to initiate research in transgenic fish and since then Asia is the hub of transgenic research (Pandian, 2003).

The first successful development of a genetically engineered or transgenic fish was reported in 1985, with human Growth Hormone gene microinjected into the fertilized eggs of goldfish (Carassius auratus). This was followed by successful development of transgenic loach (Misgurnus anguillicaudatus) with human Growth Hormone gene. Many genetically engineered fish species have been developed since 1985, along with various methods for inserting foreign gene to fish such as microinjection, electroporation, infection with pantropic defective retroviral vectors, particle gun bombardment and sperm and testis-mediated gene transfer methods. Till

now, fish including Indian major carps, goldfish, Atlantic, coho, and chinook salmon, rainbow and cutthroat trout, tilapia, striped bass, mud loach, channel catfish, common carp, Japanese medaka, northern pike, red and silver sea bream, walleye and zebrafish have been genetically modified to produce select traits such as increased growth, increased feed conversion efficiency, cold tolerance, and disease resistance. The Indian origin zebra fish Brachydanio rerio were genetically modified to different coloured fish using fluorescent genes from marine organisms and sold in market as 'glow fish'.

Biotechnology in fish breeding

Hormonal changes help the fish to breed and in many fishes the required hormone level do not produce in captive/ or non-native conditions. In such cases a hormone injection to female fish help to breed the fish and produce hatchlings. The production of synthetic GnRH hormone, Gonadotropin releasing hormone, by multinational company Glaxo International was great technology in the history of aquaculture. Synthetic GnRH is now the best available biotechnological tool for the induced breeding of fish and available in different commercial name from different companies. Depending on the structural variant and their biological activities, number of chemical analogues have seen prepared and one of them is salmon GnRH analogue profusely used now in fish breeding and marked commercially under the name of Ovaprim throughout the world.

Molecular markers are used for selective breeding in fishes. The gene construct of particular trait will be identified by sequencing from a population and that stock will be used to cross breed with other population, so that the targeted trait can be achieved in the offspring and thus productivity can be increased. In India, Central Institute of Freshwater Aquaculture, Bhubaneswar, Orissa successfully produced Jayanthi rohu, Labeo rohita, through selective breeding and it is having more than 14% growth over the ordinary rohu. Jayanthi rohu is being cultured in Andhra Pradesh in commercial scale.

Cryopreservation

Cryopreservation is a technique, which involve long-term preservation and storage of biological material at a very low temperature,

usually at -196 oC. It is based on the principle that very low temperatures tranquilizes or immobilize the physiological and biochemical activities of cell, thereby making it possible to keep them viable for very long period. The technology of cryopreservation of fish spermatozoa (milt) has been adopted for animal husbandary. The cryopreservation protocol of spermatozoa of Indian major carps, catfishes and many indegenous fishes have been developed by NBFGR. Cryopreservation overcomes problems of male maturing before female, allow selective breeding and stock improvement and enables the conservation. Gene banking through the cryopreservation of sperms, eggs and embryos is an important aspect of genetic conservation of fish stocks. Generally liquid nitrogen is used to store the sperm. These genetic resources may also become a valuable source of genes in support of the changing needs of the aquaculture industry. Cryopreservation has been demonstrated to be a successful means of storing fish sperms for extended period of time.

Cell culture

Cell culture has become one of the major tools used in the life sciences today. Tissue Culture is the general term for the removal of cells, tissues, or organs from an animal or plant and their subsequent placement into an artificial environment conducive to growth. This environment usually consists of a suitable glass or plastic culture vessel containing a liquid or semisolid medium that supplies the nutrients essential for survival and growth. The cells are removed from the organ fragments prior to, or during cultivation, thus disrupting their normal relationships with neighboring cells, and it is called Cell Culture. Cells are cultured in media with required nutrients. Generally foetal calf serum medium is used for cell culture. Successful primary cultures from a variety of tissues including fin, heart, kidney ovary tissue etc. of Indian major carp, stinging catfish, Heteropneustes fossilis, African catfish, Clarias gariepinus and rohu, Labeo rohita, denison barb, Puntius denisonii have been developed in India at National Bureau of Fish Genetic Resources (NBFGR), Lucknow. At present there is fish cell line repository in NBFGR with more than 45 fish cell lines developed by NBFGR and other institutions within India.

Cell culture is also used for reproduction in fish, particularly in spermatogenesis and oogenesis. The attempts to develop stem cell lines

from fish are successful. Sperm and egg producing cells from testis and ovary can be cultured and can be transplanted in the fish to produce viable sperm and eggs. This technique is also useful for producing a species through surrogation, i.e. production of sperm or egg of one species in closely related species. Yozhisaki et al., (2010) successfully demonstrated this technique for producing rainbow trout (Oncorhyncus mykiss) using masau salmon (Oncorhyncus masou). Embryonic stem (ES) cells are undifferentiated cell cultures derived from early developing embryos of animals, retaining their full developmental potential and their capability to differentiate. When introduced into host embryos, ES cells can participate in normal development and contribute to several host tissues including cells of the germline. Cell culture also has vast applications, such as toxicity test of drugs used in aquaculture and disease management of fishes.

Health Management

A major constraint in the development of aquaculture has been the loss due to diseases. A conservative estimate indicates that 10% of all cultured aquatic animals are lost as a result of infectious diseases. For example, shrimp production in several Asian countries declined sharply during the last decade due to viral diseases. Till recently, disease-management strategies were based mainly on chemotherapy. Biotechnological tools such as molecular diagnostic methods, use of vaccines and immunostimulants are gaining popularity for improving the disease resistance in fish and shellfish species world over for viral diseases, prevention of the pathogen in very important. In this context there is a need to rapid method for detection of the pathogen. Biotechnological tools such as gene probes and polymerase chain reaction (PCR) are showing great potential in this area. Gene probes and PCR based diagnostic methods have been developed for a number of pathogens affecting fish and shrimp. In case of finfish aquaculture, number of vaccine against bacteria and viruses has been developed. Some of these have been conventional vaccines consist of killed microorganism, but new generation of vaccine consisting of protein subunit vaccine and DNA vaccine are currently under development. Recently, scientists developed molecular based diagnosis for detection pathogenic virus using polymerase chain reaction (PCR) kits. These help farmers to identify the pathogenic virus in hatchery stage itself and can avoid infected stock from aquaculture.

Now PCR based kits are widely used for disease diagnosis in aquaculture by farmers. With the advent of bioinformatics, drugs formulation takes a quantum jump. As the sequence information is coming high throughput sequencers, bioinformaticians develop drugs using the information of host pathogen protein interaction.

Molecular markers and genetics

With the development of bioinformatics, researchers found molecular and genetic level changes in organisms and their manipulations for productivity. The particular construct of the genome is called markers, by which the organism can be identified or whole population can be segregated from another population. The different markers used in fisheries are protein markers and DNA markers. Protein markers are known as Allozymes and DNA markers again classified into mitochondrial DNA marker, nuclear DNA markers which is further classified as Randomly Amplified Polymorphic DNA (RAPDs) and Variable Number of Tandem Tepeats (VNTRs) loci such as minisatellites and microsatellites. In the past, allozyme and mtDNA (restriction fragment length polymorphism (RFLP)) markers have been popular genetics research. More recent marker types that are finding service in this field include, mtDNA sequence information, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite, single nucleotide polymorphism (SNP), and expressed sequence tag (EST) markers. mtDNA is mainly used for identifying a species and recently, sequence of Cytochrome oxidase-I (COI) gene is being used as barcode of the species. In the world there is huge project running in the name of "Barcode of Life Database" (BOLD) in which the scientists want to barcode whole species of the world. In India, National Bureau of Fish Genetic Resources (NBFGR), Lucknow generated barcode of more than 650 fish species so far. NBFGR is also carrying out project on barcoding of marine and freshwater fishes of India. NBFGR is also sequenced whole mitochondrial sequences of more than 10 fish species.

Other molecular markers such as RAPD and microsatellites are primarily used for differentiating the population at genetic level. Throughout the world more than 200 fish species have been characterized genetically using these markers, especially using microsatellites. In India, NBFGR characterized genetically more than 25 fish species by using RAPD and

microsatellite markers. Genetic characterization will help to identify the better population which can be taken up for husbandry practices and thus improving the productivity. Recently, scientist started developing whole genome sequencing of the fish species. In the world, whole genome sequences were produced for more than 42 species. Whole genome sequencing will help to understand genes which are useful for growth, disease resistance, abiotic resistance etc. This information will also be helpful for breeding and husbandry of the fish species.

Conclusion

Biotechnological research and development are growing at a very fast rate and there are many number of application of biotechnology in aquaculture. This was accelerated by the research on molecular markers and genomics. The biotechnology has assumed greatest importance in recent years in the development of fisheries, aquaculture and human health. The application of biotechnology in the fisheries sector is relatively a recent practice. Nevertheless, it is a promising area to enhance fish production through health management, selective breeding, chromosome manipulations and transgenesis. Biotechnological tools and products, such as synthetic hormones, cryopreservation, barcoding, microsatellite markers, cell culture etc. are very useful for characterization of the fish species, population and conservation. The increased application of biotechnological tools can certainly revolutionize our fish farming besides its role in biodiversity conservation.

INVITED LECTURES

In silico Analysis of Biological Sequences and Structures

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Bioinformatics is an emerging interdisciplinary branch of science that focuses on the analysis of various aspects of biology using the power of information technology. In other words, bioinformatics deals with the computational management of all kinds of biological information. At the core of bioinformatics are the biological databases. There are thousands of biological databases available online and most of them offer free access to their content. There are sequence databases (nucleic acid sequences, protein sequences), structure databases (protein structures, nucleic acid structures, other macromolecular structures and complexes) and specialized databases that can be effectively utilized to carry out bioinformatic studies. It is very essential for the budding bioinformatician to become familiar with all the basic tools available for bioinformatic studies. The sequences and structures can be searched for and retrieved from the databases using specific search and retrieval tools. The retrieved sequences, as well as experimentally generated sequences, can be analyzed using a variety of tools. The sequences can be compared with the ones in the databases to find similar sequences and to identify near relatives and far relatives of evolution. The sequences can also be subjected to the analysis of the motifs and patterns, as well as functional sites. The functional regions on DNA like coding regions, open reading frames, upstream and downstream regulatory elements, transcriptional elements, repeat sequences, restriction sites, melting temperature, CpG islands, etc. We can also identify the genes on the DNA sequences and the possible protein sequences coded by them. Using the protein sequences, we can predict the structure of the protein and thereby also the possible function of the molecule. Structural analysis of the protein targets also can give a lot of leads in the process of drug design and development.

Tissue Engineering – An Update

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Tissue engineering was defined almost two decades ago by Langer and Vacanti "as an interdisciplinary field of research that applies both the principles of engineering and the processes and phenomena of the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function". In fact it is trying to mimic nature to build a living system. For this the researcher has to understand the fundamental principles upon which living systems are created similar to functional subunit of a tissue, which contain different cell types and extra cellular matrix molecules. Recently many laboratories across the world have demonstrated the fundamental principles of successful tissue creation and some have shown the practical application to human therapy like bone, skin etc.

The discovery of stem cells other than the blood forming elements such as embryonic stem cells and other adult stem cells also points out potential application for human therapy.

Combination of the elements of Tissue Engineering and Stem Cell Science led to coin the term "Regenerative Medicine".

Major elements of Tissue Engineering consist of the triad Matrix, cells and regulators. Matrix can be a scaffold or biomaterial made of porous, absorbable synthetic or natural polymers. Decelluarised matrix is also of recent interest. In the case of bone tissue engineering, , the scaffolding material should have excellent mechanical strength, three dimensional interconnected macroporous microstructures, controllable biodegradation and bioresorption, suitable surface chemistry with good biocompatibility and biofunctionality

Cells can be stem cells, differentiated or other adult cells of autologous or allogenic origin. Cells can be used as such as isolated cells or cell substitutes, as encapsulated or as Cells on matrices (construct). Stem cells gained significance as they are the internal repair system, dividing

essentially without limit to replenish other cells and due to their ability to divide for indefinite periods in culture and to give rise to specialized cells. Popularity was enhanced as the Nobel Prize in Physiology or Medicine 2012 was awarded jointly to researchers Sir John B. Gurdon and Shinya Yamanaka "for the discovery that mature cells can be reprogrammed to become pluripotent state" with the incorporation of transcription factors Oct-4 & Sox2 for maintenance of pleuripotency and C-myC & Klf4 for ES phenotype & rapid proliferation.

Regulators include growth factors and cytokines, mechanical loading for bone remodeling, static or dynamic cultures using different types of bioreactors depending on the tissue type.

Electro spinning is applied more and more in tissue engineering scaffolds as it can mimic native ECM. Electro spinning provides a simpler and more cost-effective means to produce scaffolds with an inter-connected pore structure and fiber diameters in the sub-micron range. Electro spinning has been utilized to form Synthetic polymer scaffolds, Natural polymer scaffolds, Composite scaffolds and Functionalized scaffolds. Electrospun matrices are able to support the attachment and proliferation of a wide variety of cell types; as the cells are able to maintain their phenotypes on these nanofiber scaffolds

Cell sheet engineering is another important strategy for Tissue Engineering for Regenerative medicine. Poly (N-isopropylacrylamide) (PNIPAAm) is a well-known stimuli- responsive polymer, which responds to temperature by changing its hydrophilicity and swelling. Properties of PNIPAAm make it favorable to fabricate dynamic platforms to overcome the static features of previous technologies. Thermoresponsive substrates overcome static properties of previous cell-culture surfaces by providing the ability to form geometrically controlled and retrievable biomimetic tissue constructs in a temperature-dependent manner without the use of digestive enzymes. Two-dimensional thermoresponsive templates are useful to obtain intact monolayers of tissues with high cell-cell interactions. This can also be used for different regenerative therapies by creating thick tissues or prevascularized tissue constructs by stacking cell sheets. Three-dimensional thermoresponsive platforms give the opportunity to control tissue geometries in a 3-D manner by mimicking the native tissue architecture and

enable their further retrieval. However, creating shapes of native tissues and organs with preserved functionalities will be a challenge.

Our experiences in the field of cell sheet engineering with respect to corneal surface regeneration will be dealt as an example.

Localized Drug Delivery in Bone tissue Engineering

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Deep infection in bone, named "osteomyelitis", is one of the most difficult challenges encountered today, which can be characterized by progressive inflammatory degeneration of the bone. The total numbers of osteomyelitis cases are high – as approximately 112,000 orthopedic device-related infections occur per year at an approximate cost of $15,000 – $70, 000 per incident and 10 – 15% of infections are associated with open bone fractures. *Staphylococcus aureus* is the principle causative agent responsible for 70% of chronic osteomyelitis. Conventional treatment includes debridement of necrotic bone tissue coupled with the systemic administration of antibiotics for four to six weeks. However, systemic therapy remains a challenge because of the difficulty in achieving sufficient antibiotic concentration at the infection site, which can be attributed to the short half-life of the antibiotic; poor vascularisation at the infected area; or poor penetration into ischemic and necrotic tissue. Due to these complications, local controlled drug delivery has been instituted for the treatment of osteomyelitis. An example is polymethylmethacryalate (PMMA) loaded with antibiotics like gentamycin or tobramycin, but the main drawback of PMMA is its non biodegradability that necessitates revision surgery. This demands biodegradable carrier systems that can release antibiotics and simultaneously support bone regeneration in the infected area. The biomaterials like hydroxyapatite or its composites have gained much interest for these applications since it is osteoconductive and having similarity in chemical composition to native bone. Our groups' aims at the general requirements for localized drug delivery, with elaboration on how the selection of materials, configurations and processing affects drug delivery and regenerative efficacy both *in vitro* and *in vivo* for bone tissue engineering.

Animal Tissue Culture – An *In Vitro* Study

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Introduction

Considering the global scenario and global efforts to minimize animal sufferings, the present day biologists highlight on usage of alternatives to model organisms. The welfare of animals used in research is very important. There are good ethical, scientific, legal and economic reasons for making sure that animals are looked after properly and used in minimum numbers [1]. **Refinement**, namely any decrease in the incidence or severity of inhumane procedures applied to animals, has been extended to the entire lives of the experimental animals today. **Reduction** of the number of animals used to obtain statistically significant data may be achieved by improving experimental design and statistical analysis of data. **Replacement** refers to the development of validated alternative methods. Keeping in mind the above three terms, Refinement, Reduction and Replacement (the three R's), has emphasized on the use of alternative model organisms or systems [2,3]. Eventually, the desirability of preventing such suffering became recognised, and this led to legislation imposing a duty of care to prevent such suffering. In essence, a duty of care involves the anticipation of animal suffering occurring, and acting to avoid such suffering [4].

Model Organisms:

Several alternative model systems (precision cut goat liver slices, *Saccharomyces cerevisiae* cells, Drosophila melanogaster, Zebra fish etc) are being used in various studies as they mimic the in vivo systems. They are used to analyze the function of a given human disease gene [5]. Unicellular organisms such as yeast (*Saccharomyces cerevisiae*) can be used to analyze phenomena that involve important basic eukaryotic cell functions, such as metabolism, regulation of the cell cycle, membrane targeting and dynamics, protein folding, and DNA repair. Simple invertebrate systems such as *Drosophila melanogaster* or *Caenorhabditis*

elegans are excellent models for examining the coordinated actions of genes that function as components of a common molecular machine such as a signal-transduction pathway or a complex of physically interacting proteins. In contrast, mammalian systems such as the mouse, zebrafish, frog, and chicken are most likely to provide accurate models for the human disease state, which can be used to assess various strategies for intervening in the disease process [6].

Cancer cells lines have taken a giant leap in the research of model organisms. Nevertheless, they pose serious threats of contamination and various other laboratory maintenance issues which can be prevented by following any of the guidelines available. They would cover areas such as development, acquisition, authentication, cryopreservation, transfer of cell lines between laboratories, microbial contamination, characterization, instability and misidentification [7,8]. There are several laboratory terms relevant to tissue culture which one must be acquainted with. The table (Table 1) below shows light on some of the most frequent tissue culture terms.

Table 1: Common Cell Culture Terms and their meaning [7,8]

Common Terms	**Description**
Anchorage dependence	The requirement for attachment in order for cells to proliferate.
Anchorage independence	The ability of cells to proliferate in suspension, either stirred or suspended in agar or Methocel.
Authentication	Corroboration of the identity of a cell line with reference to its origin.
Cell concentration	Number of cells per ml of medium.
Cell density	number of cells per cm^2 of growth surface.
Cell line	the progeny of a primary culture when it is subcultured. A cell line may be finite (qv) or continuous (qv).
Cell strains	cell lines that have been purified by physical separation, selection or cloning, and which have specific defined characteristics, for example, BHK-21-PyY, anchorage-

	independent cells cloned from the BHK-21 cell line following transformation with polyoma virus.
Cloning	the generation of a colony from a single cell; subculture of such a colony would give rise to a cell strain. Because of potential confusion with molecular cloning, this term is probably better modified to 'Cell cloning'.
Confluence	a cell density at which all cells are in contact with no remaining growth surface.
Contact inhibition	Strictly, the loss of plasma membrane ruffling and cell motility on contact in confluent cultures, but often used to imply loss of cell proliferation after confluence, better referred to as 'Density limitation of cell proliferation'.
Continuous cell line	a cell line with an indefinite lifespan (immortal, over 100 population doublings; see also Immortalisation).
Density limitation of cell proliferation	the reduction or cessation of cell proliferation at high cell density.
Differentiation	Acquisition of properties characteristic of the fully functional cell in vivo.
DNA profiling	the assay of hypervariable regions of satellite DNA, usually by determining the frequency of short tandem repeats in microsatellite DNA using PCR of individual loci with defined primers.
Established cell line	the use of this term is discouraged because it is ambiguous; the preferred term is continuous cell line (qv).
Explantation	isolation of tissue for maintenance in vitro.
Finite cell line	a cell line that survives for a fixed number of population doublings, usually ~40–60, before senescing and ceasing proliferation.
Generation number	The number of population doublings of a cell line since isolation.
Growth curve	a plot of cell number on a log scale against time on a linear scale.

Immortalisation	The indefinite extension of lifespan in culture, usually achieved by genetic modification, but already acquired by some cancer cells.
Passage	the event of subculture (qv), used to define the number of subcultures that a cell line has gone through since isolation. If used ofcontinuous cell lines more usually the number of subcultures since last thawed from storage.
Primary culture	a culture from the time of isolation until its first subculture.
Primary explant	a small cellular fragment removed from tissue and placed in culture.
Provenance	details of the origin and life history of a cell line including various accidental and deliberate manipulations that may have a significant effect on its properties, latent or expressed.
Split ratio	The amount by which a culture is diluted before reseeding, usually a whole number.
Subculture	The transfer of cells from one culture vessel to another by dissociation from the substrate if a monolayer, or by dilution if grown in suspension.
Transformation	A heritable change involving an alteration in the genotype, usually subsequent to immortalisation. It is best reserved to describe an alteration in growth characteristics associated with malignancy (anchorage independence, loss of contact inhibition and density limitation of cell proliferation, and tumorigenesis in vivo).
Tumorigenesis	formation of a tumour in vivo, in the current context from implanted cells or tissue.

Further, for example, the LD50 test was used for many years to find out how toxic chemicals are. Scientists developed better tests, to do the same but using fewer animals and designed so that none intentionally received a fatal dose. The LD50 is now banned in the UK. And a recent review conducted by the pharmaceutical industry showed that much of the data from single dose acute toxicity tests in rodents can be collected from other tests, meaning that fewer rodents are required in the development of new

medicines [9]. The UK National Centre for the Replacement, Refinement and Reduction in Animals in Research (NC3Rs) is an independent scientific organisation tasked by Government to fund innovation and technological developments that replace or reduce the need for animals in research and testing, and lead to improvements in welfare where animals continue to be used [10].

Animal Tissue culture

In the pursuit of scientifically validating medicinal plants rich in antioxidants and chemopreventive agents, several plants are chosen to study the antioxidant status in vitro using new techniques. Many free radical scavenging assays and biomolecule protective effect (lipids, proteins and DNA) against oxidative damage has been studied in vitro [11, 12]. With the advent of Animal Tissue Culture (ATC) which is a boon to the field of biotechnology, immense work has been diverted to be carried out with ATC, provided sophisticated laboratories and experienced hands are available.

Cancer Biology Studies

Primary chick embryo fibroblasts and transformed cells (Hep2 laryngeal carcinoma) cells and other cell line studies have been carried out very efficiently using ATC techniques [13]. A differential effect was studied by a group [14], where it effectively protected the untransformed cells from oxidant-induced damage while rendering the cancer cells more susceptible to the cytotoxic action of the chemotherapeutic drug (etoposide) was observed. The parameters studied were MTT, SRB, LDH, Giemsa, ethidium bromide, propidium iodide and DAPI and DNA fragmentation [15]. It was used to scientifically validate the antioxidant and anticancer activity of herbal leaves and provide evidence for its potential use in the supportive therapy of cancer chemotherapy. Hence, animal tissue culture can be used in extensive research like cancer biology, drug designing and much more.

Conclusion

Knowledge about animals' physical and behavioral requirements is expanding rapidly and translating this into practical information is critical to minimise pain and suffering as well as ensuring the robustness and reproducibility of the experiments they are used for. The model organisms

are a wealth to the biological data and should be practiced more in the biological arena. It is the duty of each one of us working in this field to reiterate the thoughts on the 3R's and share the knowledge and practice the use of model organisms to reduce animal sufferings.

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Oral Presentations

An *In silico* evaluation on the druglikeness of phytic acid and its analogues

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Abstract

Inositol hexakis phosphate is known to be the phosphorous reserve in plants particularly in the seeds. Though it has been known for many of its negative impacts, current research works have identified it as a novel anticancer agent. The present study aims at investigating the drug likeness of phytic acid and its analogues through computational methods. Computer aided drug design has proven its significance in the past years due to its reduction in cost, time and side effects. Out of the 50 analogues of phytic acid selected for the study 42 gave very good docking results. Two potential cancer drug targets identified include mitogen activated kinase and 1, 4, 5-triphosphate receptor. The best docking scores obtained were -14.29 for 1-Diphosinositol pentakisphosphate and -14.02 for 2,3,4,5,6-pentaphosphonooxycyclohexyl dihydrogen phosphate for the above said targets. The negative binding energy obtained was -49.5kJ/mol for both; again affirms the protein ligand binding affinity. ADME results predicted the drug like properties of the compounds. Structure activity relationship model generated using GUSAR for the compounds with known experimental IC_{50} values included twelve compounds which gave significant statistical results.

Introduction

Phytic acid also known as InsP6 or inositolhexakisphosphate [1] was the foremost inositol phosphate discovered and also the most plentiful inositol phosphate in the world [2]. The major purpose of InsP6 in plants is that it acts as the phosphate storehouse in the seeds [3]. In nature phytic acid

($C_6H_{18}O_{24}P_6$) exists as free acid, phytate or phytin according to the physiological pH and metal salts [4]. The percentage of phytate in many of the plants have been quantified and the current investigations have demonstrated its activity in many cancer cell lines as well [5]. Works on the structure and activity relationship of phytic acid have never been carried out.

Methodology

Phytic acid and its analogous compounds were retrieved from the Pubchem repository of NCBI.

Ligand Preparation

The 2D structures of the preferred compounds were retrieved from Pubchem. Ligprep module was used for ligand preparation..

Identification of protein targets

In the present work one of the targets was selected with the help of PASS online server. The prediction results are analyzed based on the activity values. According to [7] the compound is very likely to reveal the activity in experimentation if the activity value is greater than 0.7. Based on the PASS results Inositol 1, 4, 5-triphosphate receptor protein (PDBid 1N4K) was selected as one of the protein target. Literature surveys explain that mitogen activated protein kinase can be used as a cancer drug target [8] [9].Based on literature survey PDBid 1PMQ was chosen as another target.

Protein Preparation

The crystal structures of two receptor proteins were prepared using Proteinprep wizard of Schrodinger. PDB-format file, includes a cocrystallized ligand, and does not include explicit hydrogens. The result is refined, hydrogenated structures of the ligand and ligand–receptor complex.

Grid Generation

Grid generation was done using the Glide module of Schrodinger. The ligand poses that Glide produce move across a chain of ranked screens that estimate the ligand's interaction with the receptor.

Docking

The process of docking a ligand to a binding site tries to mimic the natural course of interaction of the ligand and its receptor via a lower energy pathway. Here docking was done using Glide module.

Prime MM-GBSA

The ligand binding energies were calculated using Prime MM-GBSA. Molecular mechanics with generalised Born and surface area solvation (MM/GBSA) is a popular method to calculate the free energy of the binding of ligands to proteins.

Computational prediction of ADME properties

Absorption, distribution, metabolism, and excretion (ADME) properties of phytic acid and its derivatives were predicted by QuikProp which is a quick, accurate and easy-to-use prediction program of Schrodinger. The QuikProp predictions are for orally delivered drugs and for non-active transport. It also checks the Lipinski's rule of five.

QSAR studies

Quantitative structure activity relationship of phytic acid and its derivatives were predicted by using GUSAR software. GUSAR software helps to create QSAR models on the basis of the appropriate training sets represented as SDfile contained data about chemical structures and endpoint in quantitative terms [15]. GUSAR uses self-consistent regression for building of QSAR models [16].

Result and Discussion

In the biological activity spectra predicted the compound was very likely to be active against 46 targets. Perusing the results, it is evident that phytate is a Inositol 1,4,5-triphosphate receptor 1 antagonist. Therefore phytate can directly bind to a Inositol 1,4,5-triphosphate receptor 1. In a variety of cells the Ca^{2+}signalling process is mediated by the endoplasmic reticulum membrane associated Ca^{2+} release channel, inositol 1, 4, 5 triphosphate receptor. Mitogen activated kinase (1PMQ) was chosen as the second drug target based on literature survey.

Table **1**.Predicted biological activity spectrum of phytate.

Sl no:	Pa	Pi	Activity
1	0,986	0,000	Inositol 1,4,5-triphosphate receptor 1 antagonist
2	0,981	0,002	Tubulin antagonist
3	0,959	0,003	Angiogenesis inhibitor
4	0,954	0,000	Sphingosine 1-phosphate receptor 5 antagonist
5	0,909	0,004	Sugar-phosphatase inhibitor

Docking results

Docking was carried out using phytic acid and its analogues using both the targets 1N4K and 1PMQ. The results show that all the selected compounds are effectively binding with inositol 1, 4, 5 triphosphate receptor as well as mitogen activated protein kinase. 1-Diphosinositol pentakisphosphate (46173525) gave the best docking score of -14.29 for 1PMQ and 2,3,4,5,6-pentaphosphonooxycyclohexyl dihydrogen phosphate (178749) gave the best docking score of -14.02 for 1N4K. Table 2 and Table 3 show the top five docked poses. Out of fifty compounds fourty two gave docking scores less than -10 for 1PMQ and forty scored less than -10 for 1N4K which shows both the protein targets have good binding affinity towards the selected compounds.

Table **2**. Docked ligands in the order of XP score (1N4K).

Pubchemid	SP score	XP score	Emodel	glide energy	evdw	Ecoul
178749	-14.00	-14.02	-55.19	-101.78	-27.11	-103.71
4200706	-12.47	-12.50	-38.07	-83.45	-4.36	-83.01
16752671	-11.79	-12.46	10000.00	-47.57	-25.11	-75.10
46173525	-11.68	-12.36	10000.00	-43.96	-4.36	-63.20
46173281	-11.68	-12.36	10000.00	-43.96	-32.28	-63.20

Table **3**. Docked ligands in the order of XP score (1PMQ).

Pubchemid	SPscore	XP score	Emodel	Glide energy	glide evdw	glide ecoul
46173525	-5.27	-14.29	-79.45	-58.95	-19.44	-39.51
53380834	-6.12	-13.06	-0.08	-98.38	-28.90	-37.13
4487899	-6.41	-12.98	-95.79	-81.79	-20.02	-61.77
53379838	-7.11	-12.77	-73.91	-84.42	-27.11	-57.31
178749	-5.46	-12.48	-46.13	-51.94	-8.89	-43.05

MMGBSA prediction

The result distinguishes strong and weak binders. The following table shows some of the binding energies. The free energy of binding was least for (1r,2R,3S,4ss,5R,6S)-2,3,4,5,6-pentakis (phosphonooxy) cyclohexyltetrahydrogen triphosphate (53477671). The coulombic energy of the compound (-38.8kcalmol^{-1}), van-der-waal's energy (-40.1 kcalmol^{-1}), co-valent energy (-21.6 kcalmol^{-1}), coulombic binding energy (-11092.4 kcalmol^{-1}) and solvation binding energy (-2168.6 kcalmol^{-1}) were also significantly low. The compound 2, 3, 4, 5, 6-pentaphosphonooxycyclohexyl dihydrogen phosphate (CID178749) ranked in the top docked poses in both the protein targets have a satisfactory binding energy minima of -12.8 kcalmol^{-1}. Thirty one of the compounds gave minimum energy values. The negative binding free energies show that we have favourable ligand protein complexes.

Table **4**.MM-GBSA prediction.

Pubchemid	Binding free energy (kcalmol^{-1})	Coulombic energy of complex (kcalmol^{-1})	Van der Waals energy of the complex (kcalmol^{-1})	Covalent energy (kcalmol^{-1})	Coulombic binding energy (kcalmol^{-1})	Solvation binding energy (kcalmol^{-1})
53477671	-49.5	-38.8	-40.1	-21.6	-11092.4	-2168.6
53380834	-44.6	-51.4	-36.2	7.0	-10964.1	-2146.9

53380009	-40.3	-14.1	-49.0	-10.5	-10982.1	-2169.7
53380100	-37.0	-16.4	-49.9	-13.2	-10985	-2161.7
53380199	-34.5	-15.0	-35.7	-12.4	-10958.1	-2195.5

ADME screening

According to the analysis 44 physically significant descriptors and pharmaceutically relevant properties of the ligands like molwt, SASA, FOSA, FISA, volume etc were obtained. The following table shows some of the predicted properties.

Table **5**. ADME properties prediction.

Pubchem id	molwt	SASA	FOSA	FISA	QPlogBB	Volume	donorHB $	acceptHB $	QPlogPo/w
890	660.0	659.2	22.1	616.2	-6.3	1279.9	12	30	-1.6
46173525	740.0	709.2	14.7	675.3	-6.3	1401.3	10	32	-1.7
107758	500.1	598.6	38.8	545.5	-7.2	1072.8	10	23.4	-2.2
4487899	740.0	720.8	24.4	673.3	-5.8	1407.8	10	32	-1.7
45479488	820.0	750.4	21.9	701.9	-7.2	1506.0	8	34	-1.7

SASA- Total solvent accessible surface area (SASA) in square angstroms using a probe with a 1.4 Å radius. FOSA- Hydrophobic component of the SASA (saturated carbon and attached hydrogen). FISA- Hydrophilic component of the SASA (SASA on N, O, and H on heteroatoms).QPlogPo/w- Predicted octanol/water partition coefficient. QPlogBB - Predicted brain/blood partition coefficient.$- Estimated number of hydrogen bonds that would be accepted by the solute from water molecules in an aqueous solution. Values are averages taken over anumber of configurations, so they can be non-integer.

Quantitative structure Activity relationship study using GUSAR

In the consensus model the number of green atoms should be more which reflects the correlation between predicted activity and experimental activity.

In the present study majority of the selected compounds have a good number of green atoms and it explains the fact that the compounds contain atoms which contribute in increasing the activity of the structures.

Table **6**.Cytotoxicity prediction using QNA, MNA and Combinatorial model.

Pubchem id	IC_{50}	pIC_{50} (QNA)	pIC_{50} (MNA)	pIC_{50} (Combinatorial)
477	0.087	0.44	-3.35	-0.15
890	4.39	5.97	-0.71	3.81
107758	0.002	0.46	-3.38	-0.14
125004	20	17.68	18.64	15.38
439456	3.55	4.17	-0.23	2.85
443266	0.43	0.39	-3.03	0.04
10251645	2	0.95	-7.18	-0.01
14375662	0.268	0.95	-7.18	-0.01
16752673	19	17.89	18.12	16.67
44274820	0.009	-2.75	-27.9	-2.08
44332437	0.28	4.17	-0.23	2.85
46905360	0.172	-0.18	-4.32	-0.86

The statistical characteristics of the model are N= 12, R2= 0.976, F=27.059, SD= 1.600, Q2= 0.453, V= 3 where N is total number of molecules used, R is correlation coefficient, F is value of Fischer's parameter, SD is standard deviation, the cross-validated R2 and V is no. of variables used in the model building.

Conclusion

Lead based drug design is a constructive computational technique for identifying potential protein targets. Both the identified targets, mitogen activated kinase and inositol 1, 4, 5-triphosphate receptor gave extremely good docking results of -14.29 and -14.02. The minimum binding energy of -49.5kJ/mol for the compounds yet again confirms the binding affinity of

protein ligand complexes. ADME results predict the compounds satisfy the drug like properties like SASA, FOSA, FISA, QPlogBB etc. The structure activity relationship model generated using GUSAR reveals the various atoms of the compounds contributing in the increase of the activity as well as decrease of the activity.

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Microbial, Enzymatic and Biochemical Profiles of Ayurvedic Fermentation: Study on *Ashokarishta*

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Abstract

Arishta and Asava are herbal fermentation products of traditional ayurvedic systems. The method of preparation of Arishta and Asava is known as Sandhana Kalpanain Ayurveda, i.e, the method of aqueous alcoholic extraction bymicrobial fermentation where herbal juices or their decoctions undergo fermentation with the addition of sugars. The quality of final product depends on chemical structures released from plant parts, either directlyor aided by microbial degradation, undergone cosmetic and/or co-metabolic changes by microbes or the components may be of microbial origin like bacteriocins.The medicinal molecules, either formed during fermentation or pre-formed, are released in to the concoction via microbial fermentation. Ashokarishta is one of the most popular ayurvedic medicine used in bleeding disorders, inflammation, indigestion, etc. In the present work, Ashokarishta was prepared by traditional ayurvedic fermentation and the microbial, enzymatic and biochemical profile during fermentation was monitored. The parameters such as PH,Bacteral and Yeast profile, enzymatic activity and concentration of biomolecules such as carbohydrate, protein and lipid were analyzed in fermenting Ashokarishtain different time interval samples during the 30 day fermentation period.The results showed that Yeast growth and thus alcohol generation preceded Bacterial growth. By 30th day, the microbial count declined in the fermenting liquid column which might be due to the settling of biomass in the undisturbed fermenting liquid.The enzymatic profile (amylase, protease and lipase) of the fermenting samples correlated well with the concentration of various biomolecules (carbohydrate, protein and lipid) over the fermentation period.

Introduction

Ayurveda comprises of various types of medicines including the fermented forms namely *arishta* (fermented decoctions) and *asava* (fermented infusions). These are regarded as valuable therapeutics due to their efficacy and desirable features.The method of preparation of Arishta and Asava is known as Sandhana Kalpana in Ayurveda, i.e. the method of aqueous alcoholic extraction by fermentation. Thus Arishta and Asava are prepared by permitting the herbal juices or their decoctions to undergo fermentation with the addition of sugars.

Ashokarishta is extensively used for heavy menstrual bleeding and other gynaecological complaints. It contains 5 – 10 % of self-generated alcohol, which acts as medium for the herbal active principles. It is used in conditions like painful menstruation**,** heavy periods and fever, bleeding disorders such as nasal bleeding, bleeding haemorrhoids, inflammation, indigestion and lack of taste.

Materials and methods

Preparation of Arishta: The bark of ashoka is coarsely powdered and *kashaya* (water decoction) is prepared. The *kashaya* is strained and kept in the fermentation earthen ware pot. Other 14 drugs mentioned (Musta, Shunti, Ajaji, Daruharidra, Utpala, Haritaki, Vibhitaki, Amalaki – Amla, Amrasthi, Jeeraka, Vasa, Chandana, Jaggery, Dhataki)are powdered and jaggery is added and kept for 30 days.

The following parameters were analyzed in fermenting *Ashokarishta*by obtaining different time interval samples during the 30 day fermentation period.

a) Bacterial and yeast profile
b) Enzymatic activity
c) Quantitative estimation of biomolecules such as carbohydrate, protein and lipid

Results and Discussion

During fermentation, samples were obtained at 3 day intervals and the parameters such aspH, Bacterial and yeast profile, enzymatic activity

andconcentration of biomolecules such as carbohydrate, protein and lipid were monitored and are given below.

pH of *Ashokarishta* over 30 days of fermentation was found to decrease from 6 to 5 by 3rd day and then to 4.25 which remained the same till 24th day and then decreased to 4 till 30th day.

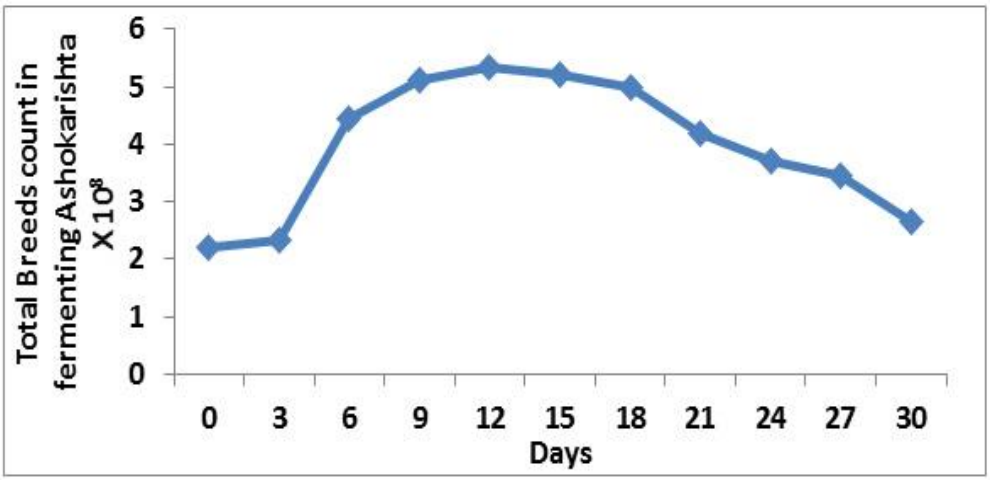

Figure1: Total Breeds count in fermenting *Ashokarishta*

Breeds count in *Ashokarishta* showed Gradual decrease from 12thto 30th day.

Yeast count showed peaks at 9th and 18thday of fermentation, Bacterial peak was on 15th day.

The results indicated Breeds count to be obviously higher than the corresponding CFU values. This is since the Breeds count tends to record dead cells as well as all that have not succeeded in growing as a colony in the scoring medium. Yeast growth and thus alcohol generation preceded Bacterial growth. By 30thday, their count declined in the fermenting liquid column. The decrease in microbial count might be due to the settling of biomass in the undisturbed fermenting liquid.

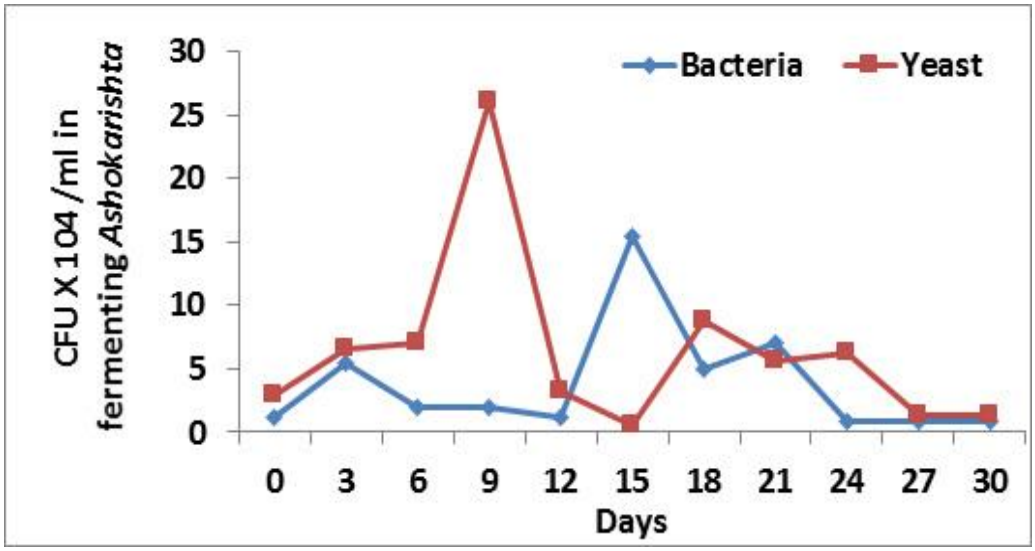

Figure2: Bacterial and Yeast count (CFU X 10^4/ml) in fermenting*Ashokarishta*

Table 4: Colony characteristics of various Bacteria and Yeast in fermenting *Ashokarishta*

Day	No	Size	Colony characteristics	Gram's nature
0	1	2-3	White, Opaque, Convex, Circular, Dried	Yeast
	2	3-2	White, Opaque, Flat, Irregular, Dried	Yeast
	3	2-4	Dirty white, Opaque, Convex, Circular, Dried	Gram + cocci
3	1	3-4	Off white, Opaque, Convex, Circular, Mucoid	Yeast
	2	2-3	White, Opaque, Convex, Circular, Slimy	Gram + bacilli
	3	3-4	Off white, Opaque, Flat, Irregular, Dried	Yeast
	4	2-4	Cream, Opaque, Flat, Circular, Dried	Yeast
	5	3-2	White, Opaque, Flat, Circular, Mucoid	Gram + cocci in cluster

6	1	2-4	White, Opaque, Convex, Circular, Dried	Yeast
	2	3-4	White, Opaque, Flat, Irregular, Dried	Yeast
	3	5-6	Dirty white, Opaque, Convex, Thinner edge, Irregular, Dried	Yeast
	4	4-3	Dirty white, Translucent, Convex, Circular, Mucoid	Yeast
9	1	3-4	Cream, Opaque, Convex, Circular, Mucoid	Yeast
	2	2-3	Off white, Opaque, Convex, Circular, Slimy	Yeast
	3	3-4	Light orange, Opaque, Convex, Circular, Slimy	Gram + coccobacilli
	4	2-4	White, Opaque, Flat, Circular, Flat	Gram + cocci
12	1	3-2	White, Opaque, Flat, Centre raised, Irregular, Flat	Yeast
	2	2-4	Off white, Translucent, Convex, Circular, Mucoid	Yeast
	3	3-4	White, Opaque, Convex, Circular, Dried	Gram - bacilli
15	1	3-2	Cream, Opaque, Flat, Circular, Dried	Yeast
	2	2-4	Off white, Opaque, Convex, Circular, Slimy	Yeast
	3	3-4	Dark yellow, Opaque, raised, Circular, Mucoid	Gram + diplo cocci
	4	2-3	Light rose, Translucent, Convex, Circular, Slimy	Gram + rods
18	1	3-4	Off white, Opaque, Flat, Irregular, Dried	Yeast

	2	2-4	Cream, Opaque, Flat, Circular, Dried	Yeast
	3	3-2	White, Opaque, Flat, Circular, Mucoid	Gram + cocci in cluster
	4	2-4	White, Opaque, Convex, Circular, Dried	Yeast
21	1	3-2	White, Opaque, Convex, Irregular, Dried	Gram + cocci
	2	2-4	Dirty white, Opaque, wrinkled, Circular, Dried	Yeast
	3	3-4	Off white, Opaque, Centrally raised edge, Circular, Slimy	Yeast
	4	2-4	Light red, Translucent, Flat, Circular, Mucoid	Gram + cocci
	5	3-4	Yellow, Translucent, Convex, Circular, Slimy	Gram - bacilli
	6	White cottony mycelium		
	7	2-4	Cream, Opaque, Convex, Circular, Dried	Yeast
24	1	3-4	White, Opaque, Flat, Irregular, Dried	Yeast
	2	2-3	White, Opaque, Convex, Thinner edge, Irregular, Dried	Yeast
	3	2-3	White, Opaque, Convex, Circular, Dried	Yeast
27	1	2-3	White, Convex, Flat, Circular, Dried	Yeast
	2	3-2	White, Opaque, Convex, Circular, Slimy	Yeast
	3	3-4	White, Opaque, Flat, Irregular, Mucoid	Yeast
	4	1-2	Dirty white, Opaque, Convex, Thinner edge, Irregular, Slimy	Yeast

	5	1-2	Dirty white, Opaque, Convex, Circular, Dried	Yeast
30	1	2-4	White, Opaque, Convex, Circular, Dried	Yeast
	2	3-4	White, Opaque, Flat, Irregular, Dried	Yeast
	3	5-6	Dirty white, Opaque, Convex, Thinner edge, Irregular, Dried	Yeast
	4	4-3	Dirty white, Translucent, Convex, Circular, Mucoid	Yeast
	5	3-4	Cream, Opaque, Convex, Circular, Mucoid	Yeast
	6	2-4	White, Opaque, Flat, Circular, Slimy	Gram + cocci

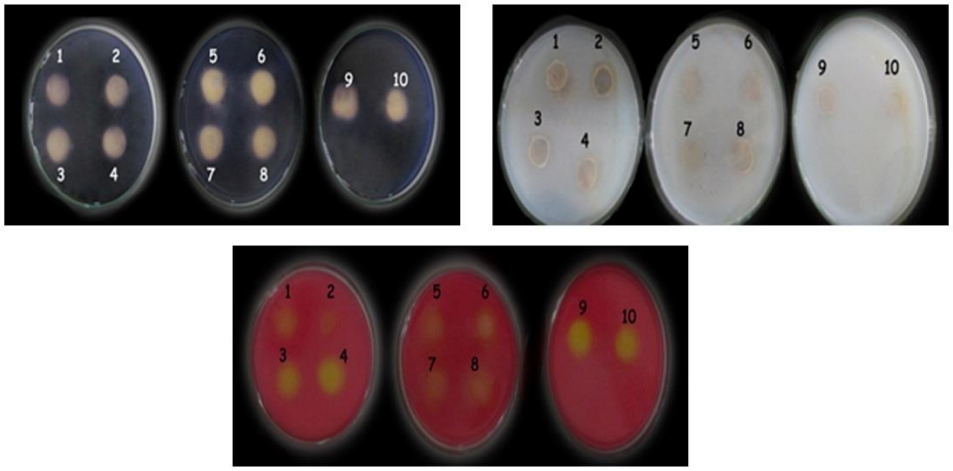

Figure 3: Enzymatic activities (amylase, protease, and lipase) in fermenting *Ashokarishta* over 30 days of fermentation. Amylase - The markings 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 in the plate corresponds to day 0, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 of fermentation. Protease - The markings 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 in the plate corresponds to day 0, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 of fermentation.Lipase - The markings 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 in the plate corresponds to day 0, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 of fermentation.

Amylase activityin starch agar mediumwas observed as halo around the discincorporated with *Ashokarishta*samples.Gelatin hydrolysis or protease activity in Gelatin agar medium was observed around disc incorporated with *Ashokarishta*. The Tween-20 agar plates were examined for the lipase activity and colour change (pink to golden yellow) was observed around the disc incorporated with *Ashokarishta*samples which indicates presence of lipase.

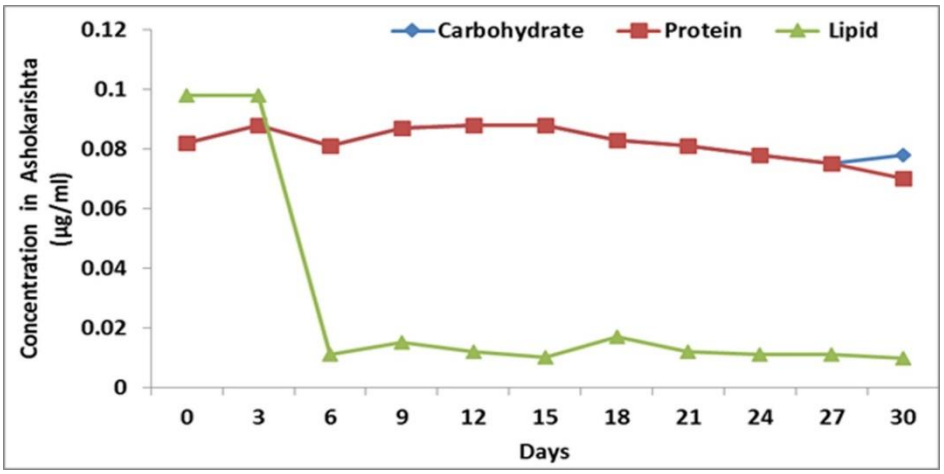

Figure6: Qualitative estimation of carbohydrate, protein and lipid in Ashokarishta

The results indicated that the carbohydrate and lipid concentration in *Ashokarishta* decreased as fermentation proceeds and this continued till 30th day. These results correlated well with the results of enzyme activity assay since the microbial/phyto enzymes may have contributed to the biodegradation of available carbohydrate and lipid in the fermenting liquid. The concentration of protein was found increase gradually throughout the fermentation period. This increase may have been contributed by the increasing biomassdue to yeast and bacterial growth.

Conclusions

*Ashokarishta*was prepared by ayurvedic fermentation.

The microbial, enzymatic and biochemical profile during fermentation was analysed.

In *arishta*, Yeast growth and thus alcohol generation preceded Bacterial growth. By 30th day, their count declined in the fermenting liquid column. The observed decrease in microbial count might be due to the settling of biomass in the undisturbed fermenting liquid.

The enzymatic profile (amylase, protease and lipase) of the fermenting samples correlated well with the concentration of various biomolecules (carbohydrate, protein and lipid) over the fermentation period.

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Biological Properties Of polysaccharides Isolated From The Wood Rotting Mushroom, Ganoderma lucidum

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ABSTRACT

Ganoderma lucidum belongs to division Basidiomycota. Ganocelium capsule is important ayurvedic medicine formed from mycelium of *G.lucidum*. The present study was focused on preparation of aqueous extract from Ganocelium capsule (mycelium), and partial purification of polysaccharides from the aqueous extract and also evaluation of antimicrobial and antioxidant activities of the polysaccharides isolated from the Ganocelium capsule. The Ganocelium capsule were collected and polysaccharides were obtained by defatting with petroleum ether and was isolated by hot water extraction at 90-100ºC,centrifugation at 20000 rpm for 20 minutes, & precipitated as dark brown powder. Phytochemical analysis was done with the help of Anthrone method and Ascending paper chromatography. The carbohydrate was quantitatively estimated in capsule by Anthrone method. The sample was treated by acid hydrolysis and was used for ascending paper chromatography. Antimicrobial activity was done with disc diffusion method and antioxidant activity was with Total antioxidant capacity and 2, 2-diphenyl 1- picryl hydrazyl (DPPH) radical scavenging assay. In Anthrone method, carbohydrate content was found to be 30.80 %.Monosaccharide present in the sample was estimated to be glucose with the help of Ascending paper chromatography. Ganocelium capsule showed antibacterial activity against four Gram negative organisms and not against the two Gram positive organisms which were tested. The maximum zone of inhibition was shown by *Pseudomonas* and *Klebsiella* at a diameter of 15 mm. Ganocelium capsule showed antifungal activity against the three tested fungal species. The maximum zone of inhibition was shown by *Alternaria* at a diameter of 32 mm. In Total antioxidant assay,

percent of inhibition was found to be 60.3 % at maximum concentration of 1000µg/ml, while in DPPH radical scavenging assay, it is found to be 59.36 %.

Introduction

Mushrooms are fleshy macro fungi belonging to the genus Basidiomycetes or Ascomycetes. Mushrooms are nutritionally functional food as well as a source of physiologically beneficial and nontoxic medicine (Janardhanan, 2001). Medicinal mushrooms have an established history of use in traditional oriental therapies. The longest tradition of using edible mushrooms for medicinal purposes is in China and Japan. *Ganoderma lucidum* (*G.lucidum*), commonly referred to as Lingzhi in China, is a fungus which has been widely used through the centuries for the general promotion of health and longetivity in Asian countries (Fig-1). Ganocelium capsules are derived from the mycelium of *G.lucidum*. Ganocelium is rich in oleic acid, cyclooctasulfur, LZ-8, polysaccharides, organic germanium, vitamins, etc. It can help improve body function and blood circulation. Ganocelium capsule is world widely produced by DXN group.Ganocelium capsule is an Ayurvedic preparation from the root extract viz., mycelium of medicinal mushroom *Ganoderma lucidum* that keeps the body healthy and enhances longevity. Strengthens immunity, improves blood circulation, relieves stress, promotes neurological health and normalizes/ corrects metabolic functions. It supplies nutrition and provides relief from various body disorders.

Objectives of the study

The present study was focused on the following aspects:

- ☞ Collection of Ganocelium capsules(mycelium of *G.lucidum*)

- ☞ Preparation of aqueous extract from the Ganocelium capsules and partial purification of polysaccharides from the aqueous extract.
- ☞ Preliminary phytochemical analysis of isolated polysaccharides.
- ☞ Evaluation of antimicrobial activity and antioxidant activity of the polysaccharides isolated from Ganocelium capsules.

Methodology

Isolation of polysaccharides -The Ganocelium capsules are obtained from DXN Malaysia and aqueous extract was prepared by hot water extraction at 90-100°C, by the method of Mizuno (2002). The polysaccharides were isolated from the aqueous extract by ethanol precipitation, centrifugation, followed by evaporation at 30-40°C.

Phytochemical analysis of isolated polysaccharides-The quantitative estimation of carbohydrate was carried out by Anthrone method using glucose as the standard. Polysaccharides were subjected to acid hydrolysis, followed by ascending paper chromatography. Standard monosaccharides like glucose, galactose, mannose, fructose and xylose were used as standards.

Antimicrobial activity- Disc diffusion method was used to study the antibacterial and antifungal activity of isolated polysaccharides from Ganocelium capsules.

Antioxidant activity-Antioxidant activity was studied using Total antioxidant capacity assay (TACA) and 2, 2-diphenyl 1-picryl hydrazyl (DPPH) radical scavenging assay. The total antioxidant capacity was measured according to spectrophotometric method (Negi et al). Various concentrations of polysaccharide (100-1000 µg/ml) dissolved in distilled water in apendoff tubes were combined with 1 ml of reagent solution containing 0.6 M sulphuric acid, 2.8 mM sodium phosphate and 4 mM ammonium molybdate. The tubes were capped and incubated at 95°C for 90 minutes. After cooling to room temperature, the absorbance was measured at 695 nm against blank. Determination of the scavenging effect of DPPH was carried out with different concentrations of the extracts prepared. In this

method, a commercially available and stable free radical DPPH (2, 2 – diphenyl 1- picryl hydrazyl) which is soluble in methanol was used (Aquino et al, 2001). In its radical form DPPH has an adsorption band at 515 nm, which disappear on reduction by an antioxidant compound. Different concentrations (100-1000 ug) of both the extracts were added to 1 ml of freshly prepared DPPH solution in methanol. Absorbance was measured at 515 nm, 20 minutes after the reduction has started.

Results and discussion

Isolation of polysaccharides and phytochemical analysis- The quantitative estimation of carbohydrate was done by Anthrone method using glucose as the standard. Polysaccharides from capsules produced characteristic colour reactions with Anthrone reagent, indicating the presence of carbohydrates. The carbohydrate content of fruiting body was 30.8 %. The Rf value of glucose was compared to that of the Rf values of the extract. The Rf value of the extract of capsule was closer to that of glucose. The Rf value of glucose was 0.73 and that of extract was 0.72.This indicated that extract contained glucose.

Antimicrobial activity of fruiting body-The Ganocelium capsules showed activity against four Gram negative bacteria, i.e., zone of inhibition was produced against *E.coli, Klebsiella, Proteus*, and *Pseudomonas* sp. It showed no activity against two Gram positive bacteria, i.e. *Bacillus* and *Staphylococcus* sp (Table-1). The maximal zone of inhibition was produced against *Klebsiella* and *Pseudomonas* sp. Drug at a dose of 1000 µg/ml produced an inhibition zone of 15 mm. Ganocelium capsule has showed antifungal activity against all the tested fungal species (*Aspergillus* sp., *Pencillium* sp., and *Alternaria* sp). In the case of *Aspergillus* sp., *Pencillium* sp., and *Alternaria* sp., the zone of inhibition was produced at a dose of 250, 500, 750, and 1000 ug/ml but not at 100 µg/ml. The maximal zone of inhibition was produced against *Alternaria* sp. Drug at dose of 1000ug/ml produced an inhibition of 32 mm.

Anti-oxidant activity of fruiting body-Anti-oxidant activities of capsules were tested using total antioxidant capacity assay. Ganocelium capsules showed total antioxidant activity. The percentage activity increased in a dose dependent manner (Table- 2). The capsules showed 60.3% activity at

1000 µg/ml. DPPH radical scavenging activity of fruiting body was studied. The polysaccharides showed DPPH radical scavenging activity in a dose dependent manner (Table- 3). The fruiting body of *G.lucidum* showed 59.36 % activity at 1000µg/ml.

Table-1. Antibacterial activity of Ganocelium capsules by Disc Diffusion Method

DRUG (µg/ml)	**ZONE DIAMETER (mm)**					
	E.coli	*Klebsiella*	*Proteus*	*Pseudomonas*	*Bacillus*	*Staphylo-coccus*
CAPSULE						
DMSO	NA*	NA*	NA*	NA*	NA*	NA*
100	NA*	NA*	NA*	NA*	NA*	NA*
250	NA*	6	7	7	NA*	NA*
500	6	7	10	11	NA*	NA*
750	10	11	11	12	NA*	NA*
1000	12	15	15	15	NA*	NA*

NA*=No activity, n=2

Klebsiella ***Pseudomonas***

Table-2. Antifungal activity of Ganocelium capsule by Disc Diffusion Method

DRUG (µg/ml)	**ZONE DIAMETER (mm)**		
	Aspergillus	*Pencillium*	*Alternaria*
CAPSULE			
DMSO	NA*	NA*	NA*
100	NA*	NA*	NA*
250	11	12	20
500	12	15	25
750	20	20	26
1000	23	25	32

NA*=No activity, n=2

Alternaria

Table-3. Total antioxidant capacity assay

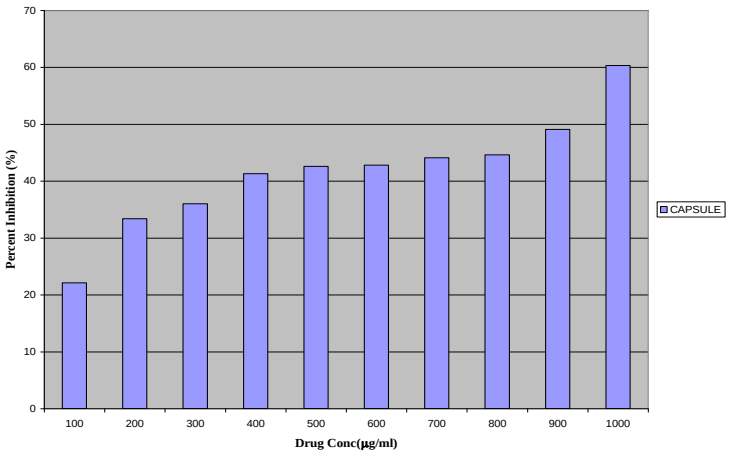
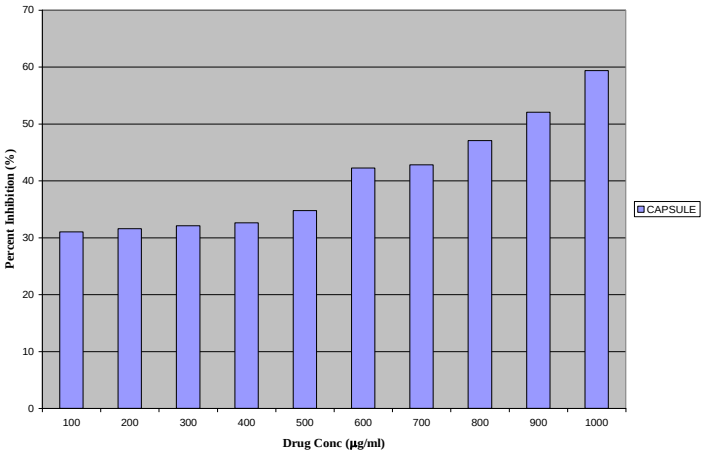


Table-4. DPPH radical scavenging assay



Discovery and evaluation of new polysaccharides from the various medicinal mushrooms as new safe compounds for treatment of various diseases has become a hot research spot. Polysaccharides are composed of repetitive structural features that are polymers of monosaccharide residues joined to each other by glycosidic linkages (Ooi & Liu, 2000).The broad spectrum of antimicrobial activity may be attributed to the presence of bioactive metabolites of various chemical types in mushroom compounds. The antioxidant mechanism may be due to the supply of anomeric hydrogen by capsules, which combines with radicals and it forms a stable radical to terminate the radical chain reaction.

Conclusion

Present study revealed the significant antimicrobial and antioxidant activities of polysaccharides isolated from the Ganocelium capsules. The isolation of polysaccharides from these capsules is simple, economical and can be carried out with minimal effort. In this context, the polysaccharides from capsules can be a suitable candidate as antimicrobial and antioxidant agent. However, further research is necessary to elucidate the structure and mechanism of action of these polysaccharides.

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Study on the prevalence of siderophore production in selected bacterial and fungal isolates

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Abstract

Siderophores, low molecular weight high affinity ferric iron chelators, are synthesized and secreted by many microorganisms in response to iron deprivation. These compounds solubilize and bind iron which is crucial for several metabolic processes and transport it back into the microbial cell, usually through specific membrane receptors. Potential linking of iron-binding molecules to microbial virulence and as antimicrobial agents for industrial, agricultural, clinical or chemotherapeutic applications have been of great academic and industrial interest.The current investigation aimed at qualitative identification of the prevalence of siderophores in some bacterial and fungal species using CAS agar plate assay. This was followed by the study of effect of pH and iron concentration on the siderophore production in CAS plate assay as well as in liquid medium. Siderophores were detected in *Aspergillus* and *Penicillium* species with *Aspergillus niger* being the most potent among them. The siderophores produced were detected to be of hydroxamate nature. Maximum siderophore production was observed after 9 days of incubation at pH 6 at 1 µM iron concentration and remained constant till 10 µM iron concentration, followed by a decrease at higher levels. This preliminary identification of potential strains further carried out by detailed characterization and purification can result in potent siderophore compounds with variety of specific applications in fields like medicine, biotechnology, agriculture, biomedical, clinical etc.

Introduction

Siderophores ("iron carriers") are low molecular weight, ferric ion specific chelating agents elaborated by bacteria and fungi growing under low iron stress (Neilands, 1995). They scavenge iron, an essential mineral

from the environment and make it available to the microbial cell. Iron is solubilized, bound and transported into the microbial cell, usually through specific membrane receptors. Siderophores with enormous diversity have been isolated from culture fluids of various bacteria and fungi (ferrichromes, ferrioxamines, fusarinines, aerobactin, enterobactin, pseudobactin etc.) (Jalal *et al.*, 1984). Microorganisms have been reported to produce different kinds of siderophores and their derivatives in varying proportions according to the substrate used. Potential linking of iron-binding molecules to microbial virulence and as antimicrobial agents for industrial, agricultural, clinical or chemotherapeutic applications have been of great academic and industrial interest (Syed *et al.*, 2013).

Materials & Methods

Screening for the presence of siderophores - CAS plate assay

The chrome azurol sulfonate (CAS) assay (Adriane *et al.*, 1999) was used employing CAS-blue agar as bottom agar plate which were overlayed with the appropriate nutrient medium i.e., Luria Bertani (bacteria) and Sodium succinate agar (Pseudomonas species) or Grimm Allen medium (fungi), to screen for the presence of siderophores. The bacterial and fungal cultures were spot inoculated and incubated at respective temperatures for the qualitative detection of siderophore production. The results were interpreted based on the colour change due to transfer of the ferric ion from its intense blue complex to the siderophore.

Quantitative estimation of siderophore production

Fungal cultures were grown in Grimm Allen medium at 28^0C for 12 days under shaking conditions (180-200 rpm) on rotator shaking incubator. Samples harvested at regular intervals were tested by CAS liquid assay and the percentage of siderophore units was estimated as the proportion of CAS colour shift using the formula $[(A_r-A_s)/A_r] \times 100$, where A_r is the absorbance of reference and A_s is the absorbance of sample, at 630 nm.

Detection of chemical nature of Siderophores - Tetrazolium Test

The chemical nature of siderophores were analysed based on their capacity of to reduce tetrazolium salt in presence of strong alkali. To a pinch

of tetrazolium salt, 1-2 drops of 2N NaOH and 1 ml of culture filtrate were added and the colour change noted (Snow, 1954).

Effect of pH And Iron concentration on siderophore production

The effect of pH on siderophore production was studied by using CAS plates with an overlay of Grimm Allen medium at different pH such as 5, 6, 7, 8, 9 and 10. For studying the effect of iron concentration, CAS agar plates were overlayed with Grimm Allen medium containing different concentrations of iron (0-10µM). The cultures were spot inoculated, incubated at 28^0C for 24- 48 h in dark and the results were interpreted based on the colour change. Further, Grimm Allen broth adjusted at different pH/iron concentrations were employed to corroborate the above results by CAS liquid assay.

Results and Discussion

Screening for the presence of siderophores

CAS agar petriplates inoculated with bacterial species in the current study showed no characteristic colour change after 24-48 h of growth (Table 3.1). The fungal isolates *Aspergillus* and *Penicillium* species showed positive responses with respect to rate of growth, reaction colour and rate of CAS reaction.

Microorganism	**CAS test**
Salmonella typhimurium MTCC 98	_
Salmonella typhimurium MTCC 1254	_
Bacillus thuringiensis MTCC 86	_
Aureobacterium flavescens MTCC3120	_
Escherichia coli MTCC68	_
Staphylococcus aureus MTCC3103	_
Pseudomonas aeruginosa MTCC2642	_
Klebsiella pneumoniae MTCC2653	_

Streptococcus thermophiles MTCC1938	_
Vibrio mereis MTCC4216	_
Pseudomonas spp.	_
Aspergillus niger	+
Aspergillus flavus	+
Penicillium spp.	+
Penicillium spp.	+

Table 3.1. Screening of microorganisms for siderophore production

A colour change from dark blue to purple was observed within 24 h in the bottom agar plates, surrounding *Aspergillus niger* (Figure 1). *Aspergillus flavus* showed a colour development from dark blue to orange yellow after 24 h and Penicillium species, after 48 h. The intensity of orange zone varied indicating different amounts of siderophore production being produced.

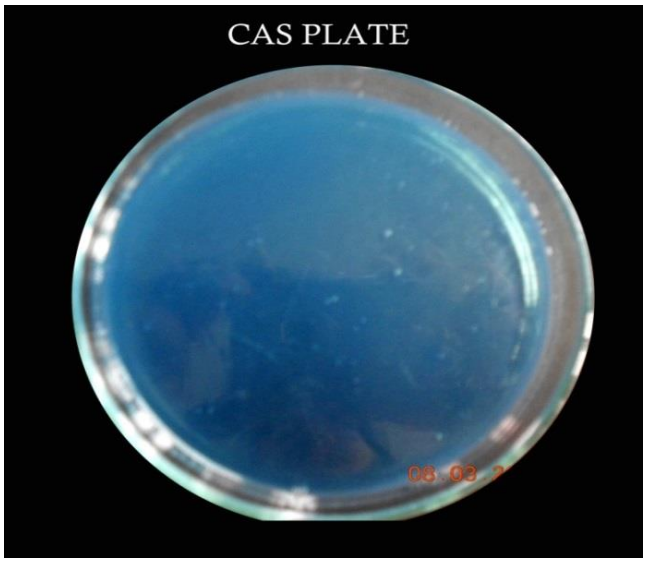

Figure 3.1. CAS Plate assay for siderophore production

Aspergillus niger showed stronger colour reaction compared to the other isolates (Figure 3.1). The siderophore secreted by microorganisms diffuses through CAS blue agar producing the colour change and the distinct responses in colour and intensity could be related to structural differences as well as the concentration of siderophore production. To measure the amount of siderophore production CAS-liquid spectrometric assay was carried out for all the cultures.

Quantitative estimation of siderophore production

The fungal isolates were grown in Grimm Allen medium for a period of 12 days with routine sampling done at 3 day interval. The siderophores produced were quantified using CAS liquid assay and the results were tabulated (Table 4.2).

Microorganism	**Day 3** **% SU**	**Day 6** **% SU**	**Day 9** **% SU**	**Day 12** **% SU**
Aspergillus niger	**27.81**	**31.5**	**84.37**	**51.56**
Aspergillus flavus	14.06	26.25	83.75	28.12
Penicillium	14.68	23.43	82.81	3.12
Penicillium 2	20.3	48.43	53.12	14.06

Table 3.2. Siderophores production by fungal isolates

The siderophore production over the fermentation period showed a characteristic pattern with an increase towards stationary phase of growth and a decline towards the final stages of culture growth in the case of Aspergillus and Penicillium species. Maximum total siderophore production was observed after 9 days of incubation, when the organism reached the stationary phase with the concentration of total siderophore in the medium decreasing considerably as the cultures grew older (Table 3.2). The results are in corroboration with earlier studies in various species of Aspergillus. All the four fungal species in the present study followed similar pattern of siderophore production. However, *Aspergillus niger* was found to be better among the four with respect to the yield and the colour development in CAS reaction and was chosen for further studies to determine the effect of various parameters like pH, aeration and iron concentrations on siderophore production.

Detection of chemical nature of Siderophores - Tetrazolium Test

Upon confirmation of the presence of siderophore by the CAS assay, a hydroxamate specific assay was used to determine its chemical nature. An immediate deep red colour formation on the addition of culture fluid from the CAS positive *Aspergillus* species to a pinch of tetrazolium salt and NaOH confirmed the hydroxamate nature of these compounds. The colour formation gradually decreased with incubation period which is in agreement with the siderophore production pattern.

Effect of pH on siderophore Production

One of the factors which play an important role in the solubility of iron is pH, thereby making its availability to the organism for its growth. The rate of growth and CAS reaction were affected by different pH (4-8) in the growth medium. After 24 h of incubation *Aspergillus* species showed good growth and CAS reaction on pH 4, 5, 6 and less growth and CAS reaction on pH 7, 8. *Penicillium* species also showed similar reaction of good growth and CAS reaction on pH 4, 5, 6 and less growth and CAS reaction on pH 7 and 8, but was comparatively slower.

The Grim-Allen medium adjusted at different pH was used to evaluate the effect of pH on siderophores production by *Aspergillus niger* over a period of 12 days using CAS liquid assay and the results were

tabulated (Table 3). There was a steady increase in growth and siderophore production at pH 6 till 9th day though further incubation resulted in a decrease in the siderophore yield. Alternatively, cells grown on pH 4, 5, 7, 8, 9 and 10 had significantly less growth and CAS reaction.

pH of the media	**Day 3 (% SU)**	**Day 6 (% SU)**	**Day 9 (% SU)**	**Day 12 (% SU)**
4	31.25	25	53.12	37.5
5	6.25	62.5	70.31	48.43
6	**31.5**	**57.81**	**84.37**	**51.56**
7	23.4	29.65	80.62	10.93
8	39.06	7.81	76.5	21.87
9	15.52	26.56	59.37	23.43
10	39.06	46.87	75	41.56

Table 3.3. Effect of pH on siderophore production by *Aspergillus niger*

Effect of Iron concentration on siderophore production

Biosynthesis and secretion of siderophores are mainly influenced by iron concentration. The effect of iron on growth and siderophore production by *Aspergillus niger* and *Penicillium* species was tested using CAS plates with Grimm Allen medium and different concentrations of iron from 0-10µM over a period of 120 h. The results showed that the growth increased as concentration of iron increased.

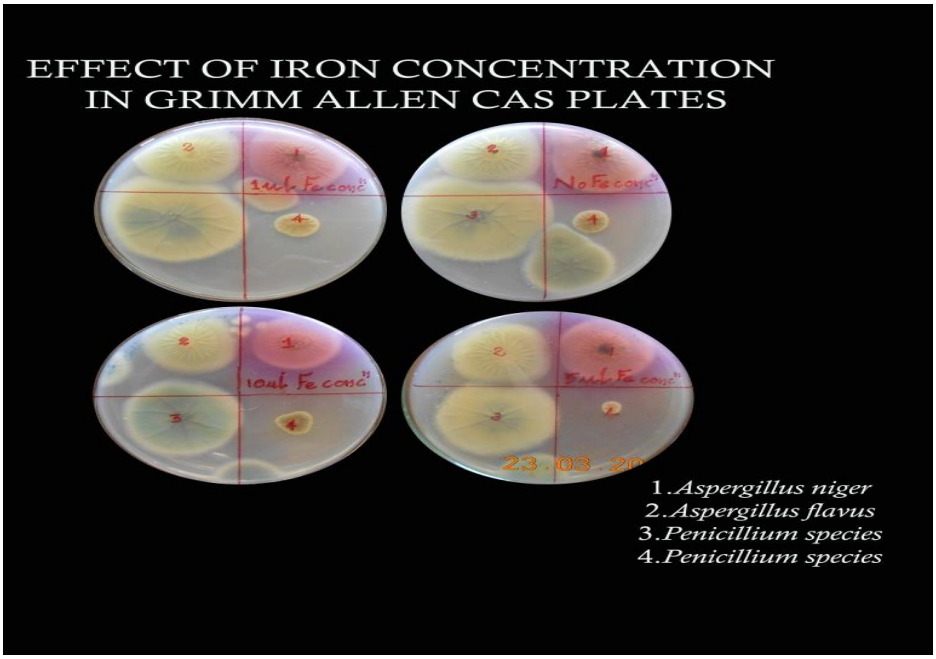

Figure 3.4. Effect of Iron Concentration on siderophore production

Siderophore productions by Aspergillus and Penicillium species were observed at all concentrations of iron and remained constant up to 10µM. *Aspergillus niger* showed maximum siderophore production in all iron concentrations and showed significant colour change from blue to purple, when compared with the other fungal cultures and hence was chosen further to study the influence of iron on siderophore production in liquid medium.

The effect of iron on siderophores production by *Aspergillus niger* over a period of 12 days was quantified with 0-50 µM iron concentrations using CAS liquid assay and the results were tabulated (Table 4.5). Maximum siderophore production was observed at 1 µM iron concentration and it remained the same till 10 µM iron concentration.

Concentration of Iron (µM)	**Yield (% SU)**
0	95.16
1	96.31

5	91.78
10	91.15
20	82.41
30	56.29
40	32.20
50	25.71

Table 3.5 Effect of Iron on siderophores production by *Aspergillus niger*

A notable decrease in the rate of siderophore production was seen from 10 µM to 50 µM concentration of iron but the growth was maximum at high iron concentration. It has been reported that iron concentration of 50 µM is high and generally results in excellent growth with only modest yields of siderophores (Neilands, 1984). Thus although cell growth reached a maximal value above 10 µM added Fe (III), siderophores biosynthesis was lowered in this concentration, since cell growth and the siderophores production are inversely proportional responses.

Conclusions

Siderophores are specific ferric iron binding compounds produced by microorganisms which promote the rate of Fe^{3+} transport. Since iron is crucial for several metabolic processes, microorganisms have developed different mechanisms to overcome this limitation, an important being production of ferric ion specific chelators termed siderophores. The current investigation was aimed at studying the prevalence of siderophores in some bacterial and fungal species. The CAS agar plate assay as a qualitative methodology for evaluating siderophore production as a function of time was used to screen siderophore producers and further to evaluate the effect of pH and iron concentration on the siderophore production. It was also attempted to correlate the siderophore production in liquid medium with the CAS plate assay. Siderophores were detected in *Aspergillus* and *Penicillium* species with *Aspergillus niger* being the most potent among them. The

siderophores produced were of hydroxamate nature. Maximum siderophore production was observed after 9 days of incubation at pH 6 at 1 µM iron concentration and it remained the same till 10 µM iron concentration, followed by a decrease at higher levels. This preliminary identification of potential strains further carried out by detailed characterization and purification can result in potent siderophore compounds with variety of applications of academic and industrial interest.

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Study on Plant Growth Promoting Activity of Endophytic Plant Growth Promoting Rhizo - Bacteria from Root Nodules of *Mimisa Pudica*

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Biotechnology has opened up new possibilities concerning the application of beneficial bacteria to the soil for the promotion of plant growth and the biological control of soil – borne pathogens. As the large scale release of genetically engineered bacteria to the environment faces a number of regulatory hurdles, the need to isolate and select superior, naturally occurring rhizosphere bacteria continues to be of great interest. Plant-associated bacteria that live inside plant tissues without causing any harm to plants are defined as endophytic bacteria. Endophytic bacteria reside within the plant tissues and have often been reported to promote plant growth. Rhizobia are particularly known for their symbiotic relationship with legumes.

Endophytic rhizobacteria which promote plant growth are involved with host plants in mutual interaction. They promote plant growth directly or indirectly, via production of phytohormones, biocontrol of host plant diseases or improvement of plant nutritional status. Rhizobia are perhaps the best known beneficial plant associated bacteria because of their importance in nitrogen fixation.Forthcoming **Bergey's manual of systematic Bacteriology** identifies 5 genera of rhizobia, namely Rhizobium, Sinorhizobium, Azorhizobium, Bradyrhizobium and Mesorhizobium. They all belongs to the α – class of proteobacteria, where they are distributed in to four distinct phylogenetic branches.

The present study describes the isolation of a bacterial strain from surface – sterilized root nodules of Mimosa pudica; which the bacteria was found to be Gram – negative, capsulated, non- endospore forming rod with free nitrogen (N) fixation ability.

Production of bacterial cellulose from paper hydrolysate and coconut water as dual cheap Carbon source

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Abstract

Bacterial cellulose was produced through fermentations of coconut water containing media using the bacterium *Gluconacetobacter SCET 7* isolated from grape juice. The yield of cellulose was compared with standard production media. The BC yield in standard medium reached 4.1 g/l after 7 days and in coconut water medium reached 5.3 g/l after 4 days of cultivation. The optimum fermentation conditions for the production of cellulose by a newly isolated *Glucanobacter* sp. SCET 7 were determined by static cultures. The strain was able to produce cellulose at 20 –35 °C with a maximum at 30 °C. Cellulose production occurred at pH 4.0–7.0 with a maximum at pH 5.0. Under these culture conditions, 8.8 g/l cellulose was produced after 4 days of cultivation, although this strain produced only 5.2 g/l in the standard medium. The addition of 1.5 % (v/v) ethanol to the medium enhanced cellulose production up to 15.8 g/l in coconut water medium ,which was about two times higher than that without ethanol .Thus industrially important polymer production can be accelerated by using coconut water. Thus the utilisation of coconut water for biocellulose production makes the wide variety of applications in medical, enzyme, cosmetic and electronic industries possible at the same time increasing the value of coconut cultivation in industrial level.

Introduction

Microrganisms produce unique compound and polymers during their life cycle which can be harvested to decrease the exploitation of conventional natural resources and as a better alternative in many fields. Bacterial cellulose is one such highly useful polymer is secreted by many species of

bacteria mainly *Gluconacetobacter, Agrobacterium, Aerobacter, Azotobacter, Rhizobium, Sarcina, Salmonella, Enterobacter, Escherichia* and several species of Cyanobacteria (Barbara *et al*., 2008).

The well ordered structure of bacterial cellulose is accounted due to its formation from microfibril fibres of nanometer size and this peculiar structure makes it adaptable for wide variety of applications like biopaper production, matrix for electronic equipments and transducers in acoustic applications, health food, cosmetics, and as biobandages. It is its high water retaining capacity, biocompatibility, low thermal expansion and high wet tensile strength that makes it most suitable for such a massive application sequences (Keshk and Sameshima, 2006).

Unlike cellulose polymer obtained from plant sources, which is composed of lignin and hemicelluloses, the bacterial cellulose is more advantageous from its purity aspect increasing its production demand (Klemn et al., 2001).Such high purity graded cellulose is also a promising hope for efficient bioethanol production and thus biofuel production from non food crop resources becomes possible thus diminishing one of the major disadvantages of biofuels.

At present BC is also utilized as a paint additive, membrane application, and in biofuel cells (Iguchi and Yamanaka, 1997). At the same time utilization of this versatile polymer for all these applications become possible only when its profitable bulk production come in to play. Traditionally, BC is produced from expensive culture media, containing glucose as carbon source and other nutrient sources resulting in very high production costs, which limits the use of this material to very high value added application (Sherif, 2008).The use of cheap carbon and nutrient sources is an interesting strategy to overcome this limitation and therefore to increase the competitiveness of this unique material.

The Exploration of other suitable cheap media alternatives can bring the hidden utilities of BC in to market. This paper investigate the utilisation of mineral rich industrially under utilisedcoconut water as cheap Carbon source for BC production . The coconut water is good source of vitamins and minerals to enhance the bacterial polymer production. The use of

microorganisms and for the complete utilization of these industrial wastes will produce biopolymers.

Materials and Methods

Isolation and Identification of Microorganism

2 ml of grape juice samples were incubated statically at pH 6.0 and 30ºC for 7 days in 60 ml of Hestrin-Schramm (HS) medium composed of 4.0% D-glucose, 1.0% peptone, 1.0% yeast extract, 0.54% Na2HPO4 and 0.23 citric acid [Dreywood R (1946)]. After incubation, the cultures were streaked onto HS-agar plates. The growth of the colonies was observed during incubation at 30ºC for 3 days. White to cream colonies with mucous structure was purified by repeated streaking onto agar plates. Morphology of the cells was examined under light microscope. Gram staining was performed to select gram negative strains. The motility of cells was observed by hanging drop method.Purified cultures were streaked onto CaCO3-agar plates to confirm acid production and investigate over oxidation of acetic acid by formation and disappearance of clear zones around colonies [Napoli C,1975]. CaCO3-agar medium was composed of 0.05% D-glucose, 0.3% peptone, 0.5% yeast extract, 1.5% CaCO3, 1.2% agar and 1.5%(v/v) ethanol. Microbial growth was examined during incubation at 30ºC for 2-7 days. Acid forming colonies were subjected to further biochemical tests. Catalase, oxidase and indole tests were performed using BD reagent droppers. Acid production from fructose, galactose, glucose, lactose, maltose, sucrose and xylose was investigated by using 1% of tested sugar solution as the only source of carbon. Urea broth was used to determine urea utilization. Citrate utilization was tested using citrate broth.

The isolate producing large quantity of cellulose was characterized byPCR using 16S rRNA as template. The 16S

rRNA gene was amplified by PCR with two primers:20F (59-GATTTTGATCCTGGCTCAG-39, positions 9–27)and 1500R (59-GTTACCTTGTTACGACTT-39, positions 1509–1429). The purified PCR products were sequenced directly by using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit and an ABI PRISM 310 genetic analyser.

Cellulose Production

Seed culture was prepared by inoculating1% of culture in to 10 ml of HS broth. The tubes were incubated statically at 30°C for one week. Cellulose production was carried out by the addition of 1% (v/v) seed broth to the coconut water media as well as standard fermentation medium. 100 mL of coconut water containing 10% of paper hydrolysate was used as cheap medium for cellulose production and 100mL of fermentation medium consist of 100mL of distilled water, 4g of yeast, 45 g of peptone, 1.035g of citric acid, 2.43g of disodium phosphate and 0.45g of magnesium sulphate was prepared and sterilized by autoclaving. The inoculated flasks were incubated at 30 ^{0}C for 9 days. Cell growth was evaluated by measuring the absorbance at 660 nm using a spectrophotometer. Cellulose formation was monitored by the appearance of a white pellicle on the surface of culture broth.

Extraction and Identification of Cellulose

The cellulose pellicles produced by acetic acid bacteria was confirmed by treating the pellicles with 0.5N NaOH and by cellulase hydrolysis and estimation of reducing sugar by DNS assay. The broth was centrifuged for 10 minutes at 4,000*g*. After washing three times with distilled water, pellicles were subjected to boiling for 15 minutes with 0.5 N NaOH. Cellulose is resistant to this treatment and thus remaining material was accepted as cellulose free from microbial cells and medium components. Cellulose was washed three times with distilled water and dried at 105ºC then weighed. The residual glucose concentration was determined quantitatively by DNS assay.

Optimization Of Cellulose Production In Low Medium

The optimization of cellulose production in coconut water media was carried out for different incubation temperature of 20°C, 25°C, 30°C, 35°C, 40°C, pH medium of 3.0 ,4.0, 5.0, 6.0, 7.0, 8.0 and concentration of coconut water of 40%, 50%, 60%, 70%, 80%, 90% and 100% .The influence of cellulose production by ethanol was also evaluated.

Result and Discussion

The isolation of *Gluconobacter* from grape juice was done on selective media containing ethanol .Only *Gluconobacter* will grow in this media.White to cream colonies with mucous structure were observed after incubation and they were purified by repeated streaking on to agar plates.

Three Gram negative rod shaped bacteria were observed when viewed under light microscopy. They were named as SCET 4, SCET 7 and SCET 8.These Gram negative cultures were transferred onto CaCO3-agar plates for visualization of acid production. The isolate formed CaCO3 clear zones. The isolates SCET 7 had given maximum clear zone .These colonies were selected as acetic acid bacteria and were subjected to further analysis. The biochemical properties of the isolates capable of cellulose production and reference strains are summarized in Table 1.Molecular characterization of isolate SCET 7 was performed by 16SrRNA analysis.Similarity search was performed using NCBI BLAST.The given sequence had given 98% similarity with Gluconacetobacter xylinus

Table.1 Biochemical characteristic of bacterial isolate SCET 7 isolated from grape juice

Characteristics	MTCC Culture	Isolate
Motility	+	+
Catalase	+	+
Indole	–	–
Cellulose production	+	+
Oxidation of acetate	+	+
Citrate	+	+
Acid production from		
D-glucose	+	+
Sucrose	+	+
Fructose	–	–
Lactose	–	–
Galactose	+	+
Maltose	–	–
Mannose	+	+
Xylose	+	+

The bacterial cellulose production by isolates SCET 7 in coconut water containing paper hydrolysate media was compared with standard production media. The turbidity of the culture broth increased within hours of inoculation and a cellulosic film was obtained on the air-liquid interface after the 4th day of incubation in low cost media inoculated with SCET 7 and after 7th day of incubation in standard medium.(Figure 1). Incubation was lasted for a week and cellulose production was monitored. After 8 days of incubation the cellulose yield was calculated. The BC yield in standard production medium reached 4.1 g/l after 7 days and in coconut water medium reached 5.3 g/l after 4 days of cultivation.The pH value of the media decreased during the cultivations, from around pH 5 to around pH 3.

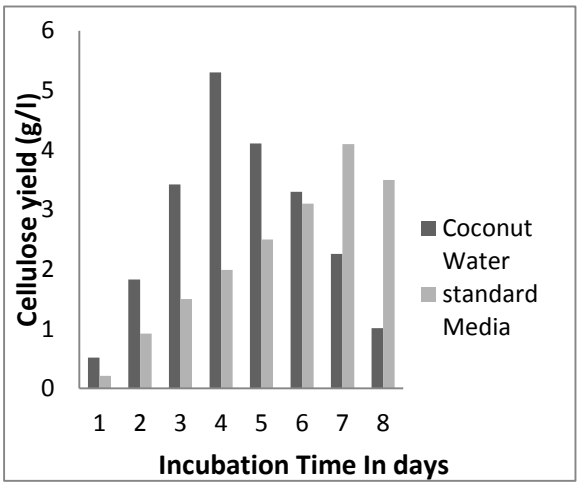

Figure 1: Comparative study of Bacterial cellulose production by SCET 7 isolates in low cost coconut water paper hydrolysate and cellulose production medium.

The isolated bacterial pellet was confirmed by treating with cellulase enzyme as well as by NaOH treatment. The bacterial cellulose was used as substrate for cellulase enzyme. Bacterial cellulose and enzyme was mixed with acetate buffer and incubated at 50^{0}C for 1 hr. After incubation the reaction was stopped by heating for 5 min and DNS assay was carried out. The DNS assay has given positive result for reducing sugar. In this way confirmed the presence of bacterial cellulose in production media.The amount of sugar present in the natural carbon sources was estimated by DNS method. Carbohydrate analysis of coconut water showed presence of 18 3.0 % of total sugar respectively.

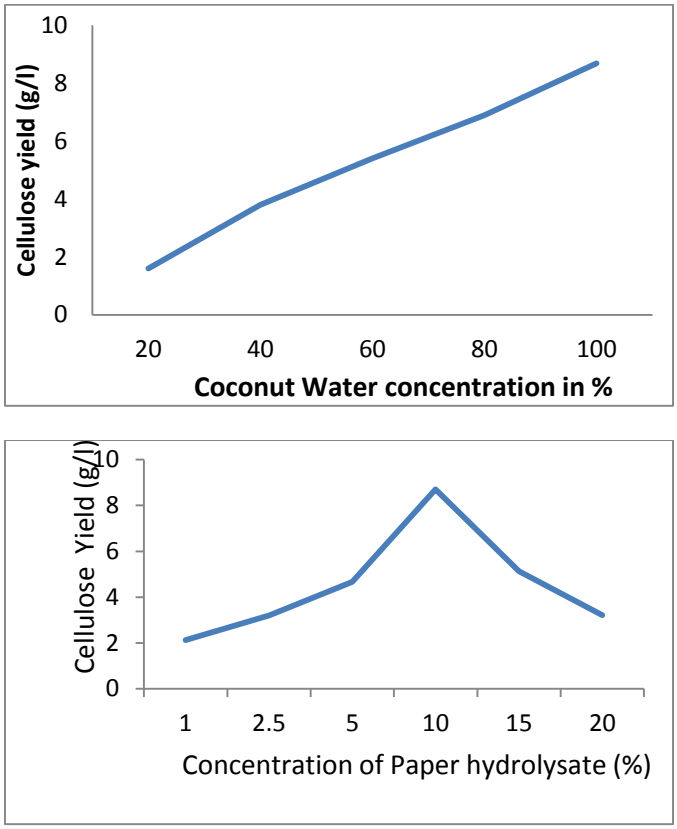

Figure 2: Effect of coconut water concentration (2a) and paper hydrolysate concentration (2b) for cellulose production by SCET 7 isolates at temperature 30^0C and pH 5.0

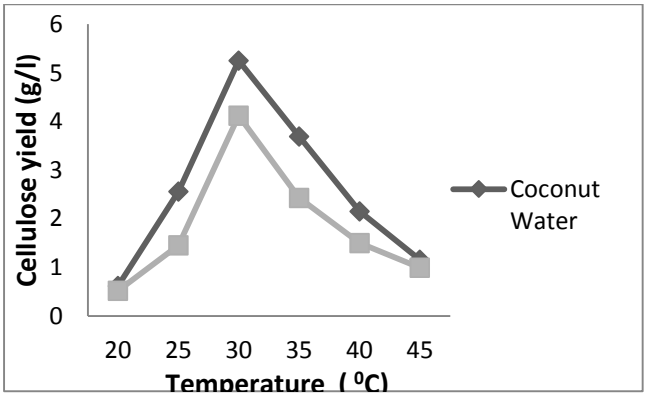

Figure 3: Effect of temperature on cellulose production in low cost media containing 100% of coconut water waste water and standard production media by SCET 7 strain at pH 5.

The effect of initial pH on cellulose production was tested in the range of pH 2.0–7.0. A high level of cellulose production was observed over a broad pH range between 4.0 and 6.0, and was maximum at pH 5.0 (Figure

4). It is generally accepted that the optimal pH range for cellulose production by *A. xylinum* is 4.0–7.0 .According to Chawla *et al*., 2008, cellulose production by *Acetobacter xylinum* is more in acidic condition because it is a type of acetic acid microbe that need acetic condition for growth.

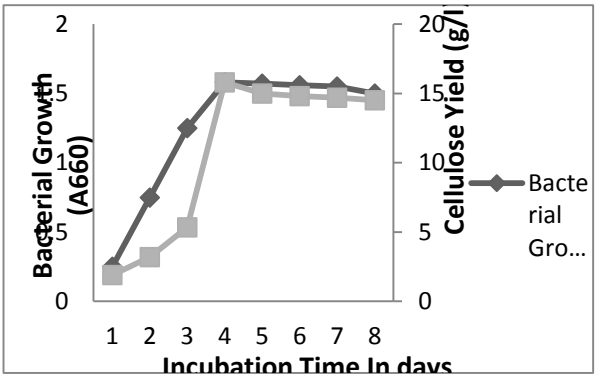

Figure 4: Time course of bacterial growth and cellulose production inlow cost media containing 100% of coconut water 10% paper hydrolysate and 1.5% of ethanol by SCET 7 isolates at temperature 30^0C,pH 5.0.

Figure 4 shows the time course of growth of *Glucanobacter* sp. SCET 7 under the optimal cultural conditions. Cellulose production increased with cell growth. Thus we confirmed that cellulose production by *Glucanobacter* sp. SCET 7 was associated with cell growth. It was expected that enhanced growth would lead to enhanced cellulose production. However, Kamide et al. [25] reported from the synchronous culture of *A. xylinum* IFO13693 grown on glucose medium in static culture that cellulose was produced when the cell number in the medium was constant.

Conclusion

Coconut water is rich in carbohydrates, proteins, and trace elements; they can be used as good nutrients for the production of food grade bacterial cellulose. The coconut water was found to be the best cheap media it does not involve toxic and hazardous materials in producing BC, which is excellent and suitable for safe environments such as medical and cosmetic applications. Use of this cheap substance should provide economical sources of nutrients for the production of bacterial cellulose. It aids the polymer production at the same time give better yield with in less time than

other media. Thus the utilisation of coconut water for biocellulose production makes the wide variety of applications in medical, enzyme, cosmetic and electronic industries possible at the same time increasing the value of coconut cultivation in industrial level.

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Evaluation of antimicrobial and ANTIOXIDANT POTENTIAL of ethnolic and chloroformic extracts of *Biophytum sensitivum*

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Abstract

Plant materials remain an important resource to combat serious diseases in the world. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain arts of the country. Various crude extract of this plant have shown multifarious activities which include antioxidants, anti-inflammatory, and antitumor activity. Medicinal plants become the main source for cancer drug development. The crude plant material ethanolic and chloroformic extract was prepared by using in a Soxhlet apparatus. The hot water extract of the powdered material was also prepared, in a water bath at 80-90°C for 8-10 hours. Percentage yield of ethanolic, chloroformic and aqueous extract of the plant extract is 8.35%, 4.25% and 3.9% respectively. Phytochemical screening revealed the presence of tannin, saponin, phenol, flavonoid, alkaloid and steroid in the ethanolic extract. Chloroformic extract contain tannin, phenol, alkaoid and steroid. Aqueous extract showed the presence of tannin, anthraquinone, phenol, flavonoid and alkaloid. Optical density method and well diffusion method are used for the analysis of anti bacterial activity. Ethanolic extract of *Biophytum sensitivum* was found to be effective against *Bacillus thuringiensis*, *Staphylococcus aureus*, *Pseudomoas aeruginosa*, *Salmonella typhi* and *Escherichia coli*. The antifungal activity of the extract was checked by well diffusion method and growth inhibitory assay. The results showed inhibition of the growth of *Aspergillus niger.* The three extracts of *B.sensitivum* showed strong hydroxy radical scavenging, reducing power, total antioxidant capacity and DPPH activities. Thus they can be used in the treatment of infectious diseases caused by resistant microbes.

Introduction

Plants are the important part of the biodiversity system which is vital to the survival of life on earth. Plants and plant-based medicaments are the basis of many of the modern pharmaceuticals. Although medicinal plants give slow recovery, the therapeutic use of medicinal plant is becoming popular because of their lesser side effects and low resistance in microorganisms (Renu Solanki, 2010). Medicinal activity of plants can be attributed to the secondary metabolites such as alkaloids, flavonoids, tannins and phenolic compounds. These phytochemicals present in the plants play a vital role in contributing them the antimicrobial action. Phytochemicals function as antioxidants that react with the free oxygen molecules or free radicals in our bodies. Free radicals can damage the cells of our bodies and must be removed. Biophytum sensitivum, is a very small flowering plant belongs to the Family Oxalidaceae. It is a perennial herb of Indian origin. It used for chest complaints, convulsions, cramps and inflammatory tumours. Decoction of leaves and roots is given for diabetes, asthma and phthisis. Fibromyalgia, leg cramps, leg pains, osteoarthritis, rheumatoid arthritis, tennis elbow are also treated using this plant in Ayurveda.

Materials and Methods

Bacterial cultures: *Bacillus thuringiensis* MTCC869, *Staphylococcus aureus* MTCC3103, *Streptococcus thermophiles* MTCC1398, *Escherichia coli* MTCC68 *and Salmonella typhi* MTCC1254 cultures were provided by MTCC, Chandigarh. *Pseudomonas aeruginosa* culture was kindly provided by Poly Clinic Pvt Ltd , Thrissur , Kerala , India

Fungal cultures**:** *Aspergillus niger* , *Aspergillus flavus* and *Penicillium chrysogenum* cultures were isolated form soil**.**

Media**:** Muller Hinton Agar , Sabouraud's Dextrose Agar and Nutrient Broth. All chemicals used were of analytical grade and provided by Hi media Laboratories Pvt Ltd, Mumbai.

Collection of plant material: The fresh and healthy leaves, stem, root and flower from the plant were collected from open fields of Mulankunnathu Kavu, Thrissur district, Kerala. The plants were identified at the Department of Botany, St .Mary's College, Thrissur District, Kerala, India.

The fresh plant parts were dried and crushed into powder using mortar and pestle.

***Preparation of Biophytum plant extracts*:**

The crude powdered plant material of 20 g was extracted with 70% ethanol and chloroform in a Soxhlet apparatus for 8-10 hours. The hot water extract of the powdered material was also prepared, in a water bath at 80-90°C for 8-10 hours. The extracts were then concentrated at 40-45°C and air dried. The dried powder samples were then stored in air tight bottles at 4°C. Percentage yield of ethanolic, chloroformic and aqueous extract of the plant extract is 8.35%, 4.25% and 3.9% respectively.

***Phytochemical Screening*:**

The crude ethanolic, chloroformic and aqueous extracts were analyzed for the presence of various phytochemicals by the standard procedure of Sofowara (1993) and Harborne (1973).

***Determination of antibacterial activity*:**

Antibacterial tests were carried out by **well diffusion method** and optical density method with some modifications. In well diffusion method, culture was uniformly distributed on to Muller Hinton agar plates. Different concentration of drugs such as 100, 500, 1000, 1500µg/ml and a control (2% DMSO) were poured on each well. Then the plates were incubated at 37° C to 16-18 hours. After incubation zone diameter was measured.

In **optical density method** cultures were inoculated into nutrient broth, and each extracts were added to the same. DMSO was used as the solvent to dissolve the extracts. For bacteria, the media were incubated at 37°C for 24 hrs. After incubation samples were measured spectrophotometrically at 600 nm. The percentage of inhibition was calculated using the following formula

- % inhibition = C-T/Cx100

Where, C is the absorbance of control and T is the absorbance of test sample.

Determination of antifungal activity:

Antifungal tests were carried out by well diffusion method and growth inhibitory assay. Drugs of different concentrations and a control (2% DMSO) were added to each well. Then the plates were incubated at room temperature for 3-4 days and the incubation zone diameter was measured. In

growth inhibitory assay, agar disc of test species was cut from one week old cultures on SDA plates and placed mycelial surface down on the center of SDA plates containing drugs of various concentrations. The plates were then incubated in dark at 26° C the extension diameter of hyphae from the center to the sides of the dishes was measured every 24 hour for 5 days. Growth inhibition of treatment against control calculated by:

% inhibition = C-T/Cx100, Where, C-average of hyphal extension of control, T-average of replicates of hyphal extension of test

Evaluation of antioxidant activity:

DPPH radical scavenging assay: DPPH (1,1 diphnyl-2-picryl-hydrazyl) is widely used to test the ability of the compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity. Freshly prepared methanolic solution of DPPH was incubated at ambient temperature with various concentrations of the extracts and absorbance was measured at 517 nm. The percentage inhibition of DPPH reduction was calculated by the formula.

% DPPH radical scavenging activity = [Absorbance of control –Absorbance of test sample)/ (Absorbance of control)] x 100

***Total antioxidant capacity assay*:** Total antioxidant capacity was measured according to spectrophotometric method. Extracts (50mg/ml) dissolved in distilled water and added 1 ml of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated at 90°C for 90 minutes. After cooling to room temperature, the absorbance of the each extract was measured at 695nm. The activity of the drug was compared with that of the control.

***Hydroxy radical scavenging activity assay*:** Hydroxy radicals generated from Fe3+ ascorbate –EDTA –H2O2 system (Fenton's reaction) was estimated by its degradation of deoxy ribose that resulted in thio barbituric acid reactive substance (TBARS). The reaction mixture contained deoxyribose (28 mM); FeCl3 (1mM); KH2PO4-KOH buffer (20 Mm; pH7.4); EDTA(1mM); ascorbic acid (0.1mM) and various concentrations of extracts in a final volume of 1.0 ml. The reaction mixture was incubated for 1 hour at 37° C. The TBARS formed was estimated by TBA method. The hydroxyl radical scavenging activity was determined by comparing absorbance of control with that of treated one.

% hydroxyl radical scavenging activity = [(Absorbance of control – Absorbance of test sample)/ (Absorbance of control)] x 100

Total reducing power activity assay: Different concentrations of extracts were mixed with 2.5ml of phosphate buffer (200 mM, p^H 6.6) and 2.5ml of 1% potassium ferric cyanide. The mixtures were incubated for 20 min at 50^0C. After incubation, 2.5ml of 10% trichloro acetic acid were added to the mixtures, followed by centrifugation at 650rpm for 10min. The upper layer (5ml) was mixed with 5ml of distilled water and 1ml of 0.1% ferric chloride and the absorbance of the resultant solution were measured at 700nm. The activity of the drug was compared with that of control

Results and Discussion:

***Phytochemical Screening*:-** Phytochemical screening revealed the presence of tannin, saponin, phenol, flavonoid, alkaloid and steroid in the ethanolic extract. Chloroformic extract contain tannin, phenol, alkaoid and steroid. Aqueous extract showed the presence of tannin, anthraquinone, phenol, flavonoid and alkaloid. Thus compared to other extracts, more number of metabolites were detected in ethanolic extract. This may be due to high solubility of active compounds of *Biophytum sensitivum* with ethanol during the extraction process compared to aqueous solvent. This justifies the traditional use of ethanol in extracting the plant components, to control the pathogenic organisms (Pandit & Langfield., 2004).

***Antibacterial activity assay*:** Antibacterial testing of the extracts from *Biophytum sensitivum* was done by well diffusion method. After incubation the zone diameter was measured. (Table). Ethanolic extract is found to be effective against five bacterial species and has a maximum activity against *Bacillus thuringiensis*. Highest activity against *Staphylococcus aureus* was shown in the highest concentration (1500µg/ml) of the chloroformic extract. The maximum inhibition zone produced by *Staphylococcus aureus* was 14 mm. The higher concentration of extract (1500µg/ml) produced highest activity against *Streptococcus thermophiles.* The maximum inhibition zone produced is 14mm.

The results of optical density showed effective inhibition of bacterial growth. Ethanolic extract showed highest activity against Gram positive *Bacillus thuringiensis*. The percentage inhibition of *Bacillus thuringiensis*

increases with drug concentration. It showed 65% activity against 1500µg/ml. The chloroformic extract of *Biophytum sensitivum* showed activity only against Gram positive *Staphylococcus aureus* and showed highest activity**.** Percentage of inhibition was 55% in 1500µg/ml. Aqueous extract of *Biophytum sensitivum also* showed activity only against Gram positive *Streptococcus thermophilus* and showed highest activity**.** Percentage inhibition was 49% activity in 1500µg/ml. Tannins bind to proline rich proteins and interfere with the protein synthesis. Flavanoids are hydroxylated phenolic substance known to be synthesised by plants in response to microbial infection and it should not be surprising that they have been found in vitro to be effective antimicrobial substances against a wide array of microorganisms. Steroids have been reported to have antibacterial properties, the correlation between membrane lipids and sensitivity for steroidal compound indicates the mechanism in which steroids specifically associate with membrane lipid and exerts its action by causing leakages from liposomes (Amita *et al*.,2011).

***Antifungal activity assay*:** Antifungal activity was evaluated by well diffusion method and it was observed that the ethanolic extract inhibited the growth of *Aspergillus niger*. By the Growth inhibitory assay, the ethanolic, chloroformic and aqueous extracts highly inhibited the growth of *Aspergillus niger*. The maximum percentage inhibition of *Aspergillus niger* was 82.35% in the ethanolic extract, 49.01% in the chloroformic extract and 47.05% in the aqueous extract.

Antioxidant Activity assay

DPPH radical scavenging activity: DPPH radical scavenging activity of ethanolic extracts of *Biophytum sensitivum* was studied. It showed Free radical scavenging activity in a dose dependent manner (Fig 2). Ethanolic extract showed an inhibition of 16 % at lowest tested concentration (0.3125mg/ml) and 91 % at highest tested concentration (10mg/ml). IC_{50} value of ethanolic extract by DPPH radical scavenging activity was obtained as 1.25mg/ml that of the cholroformic extract was 1.9 mg/ml. IC_{50} value of aqueous extract by DPPH radical scavenging activity was obtained as 3.5mg/ml**.**

Hydroxyl radical scavenging activity: The maximum inhibition at a concentration of 10mg/ml which showed of 82% was shown by 10mg/ml of chloroform extract (Fig 3). IC_{50} value of ethanolic extract by hydroxyl radical scavenging activity was obtained as 8mg/ml and that of the cholroformic extract is 1.5mg/ml.

Total antioxidant activity: The ethanolic extract of Biophytum sensitivum showed maximum activity in a concentration of 10mg/ml that is 91% total antioxidant activity (Fig 4). IC50 value of the ethanolic extract was reported as 1mg/ml and that of the chloroformic extract was 0.94mg/ml .The IC50 of the aqueous extract was found to be 1mg/ml.

Total reducing power: The ethanolic extract of *Biophytum sensitivum* showed potent antioxidant activity (Fig 5). Concentration of 10mg/ml of the extract showed maximum antioxidant activity of 94%. The percentage activity increased in a dose dependent manner. IC_{50} value of the ethanolic extract was reported as 0.95mg/ml and that of the chloroformic extract was 1.5mg/ml .The IC_{50} of the aqueous extract was found to be 4mg/ml.

The presence of flavonoids and tannins in all the plants is likely to be responsible for the free radical scavenging effects observed. Flavonoids and tannins are phenolic compounds and plant phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers (G A Ayoola, 2008). Antioxidant action of phenolic compounds is due to their high tendency to chelate metals. Phenolics possess hydroxyl and carboxyl groups, able to bind particularly iron and copper (A Michalak, 2006)

Conclusion:

B.sensitivum ethanolic, chlororformic and aqueous extract exhibits antibacterial, anftifungal and antioxidant activity. Thus they can be used in the treatment of infectious diseases caused by resistant microbes. The three extracts of *B.sensitivum* showed strong hydroxy radical scavenging, reducing power, total antioxidant capacity and DPPH activities. The bioactivity of the various extracts of *B. sensitivum* corresponding to its traditional application can validate the traditional folk medicinal usage of the plant. Furher phytochemical studies are required to isolate the types of compounds responsible for the antimicrobial effects.

Table. Antibacterial activity of B. *sensitivum* by optical density method

Drug (µg/ml)	Percentage Inhibition (%)					
	Bacillus	***Staphylococcus***	***Streptococcus***	***Pseudomonas***	***Salmonella***	***Escherichia***
ETHANOLIC EXTRACT						
100	43	22	4	38	27	32
500	58	39	6	42	39	38
1000	60	42	9	54	43	50
1500	65	54	11	58	54	55
CHLOROFORMIC EXTRACT						
100	5	34	4	11	6	7
500	11	48	7	13	12	9
1000	21	51	12	24	19	24
1500	23	55	19	30	25	28
AQUEOUS EXTRACT						
100	3	7	20	6	2	6
500	9	8	32	9	5	9
1000	15	12	37	14	12	13
1500	19	15	49	17	17	18

Fig.1. Graph showing the effect of ethanolic extract of B.*sensitivum* on bacteria

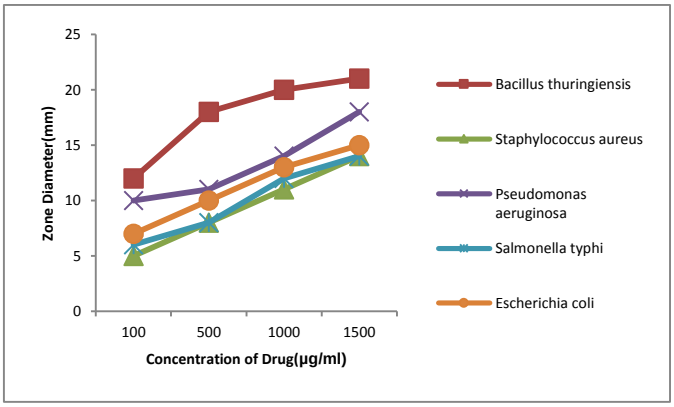


Fig 2 .DPPH radical scavenging activity of sequential extracts of B.*sensitivum*

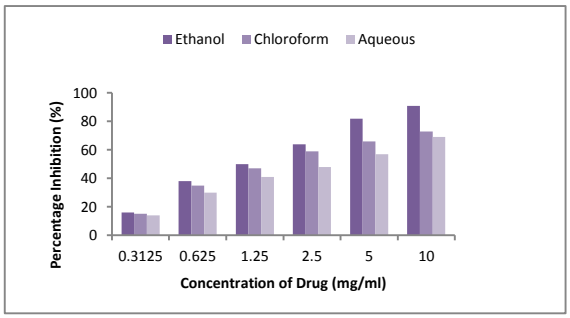


Fig. 3. Hydroxyl radical scavenging activity of sequential extracts of B.*sensitivum*

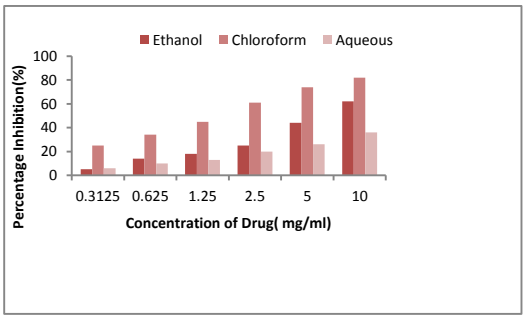


Fig 4.Antioxidant activity of sequential extracts of B.*sensitivum* By Total Antioxidant assay

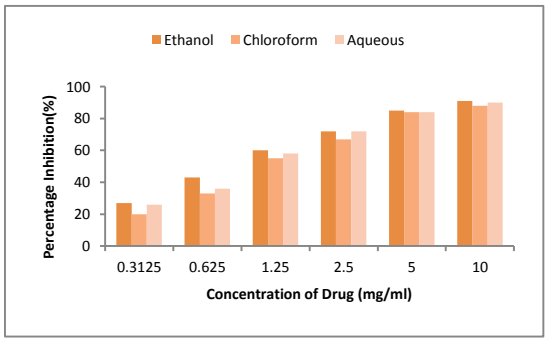
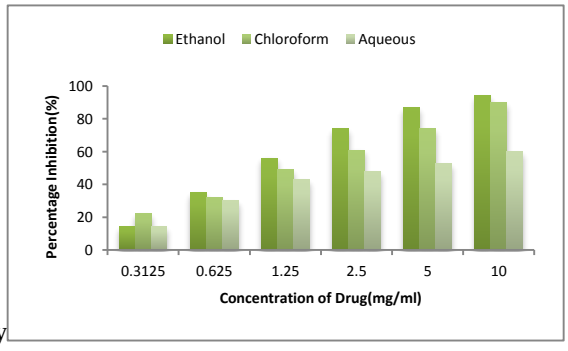


Fig 5. Antioxidant activity of sequential extracts of B.*sensitivum* By Total Reducing Power



Assay

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In vitro mutagenic studies on Stevia rebaudiana

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Diabetes currently affects 246 million people worldwide and India has the largest number of people with diabetes i.e. 40.9 million. Some people switch to artificial sweeteners, but these man-made chemicals cause more health problems than they cure. These chemicals attack vital organs that could lead to serious complications after prolonged use. Considering the rapid increase in diabetic patients in India due to change in food habits and lifestyles, *Stevia* will be sole alternative for sucrose as a natural sweetening agent in future. *Stevia* helps to treat many ailments like high blood pressure, hyperlipidemia, obesity, skin diseases and digestive. It has no side effects and is safe for consumption. As an exclusive source of natural sweetening agent and has wide range of medicinal values, there will be huge demand in the market for planting material. The major hindrance in breeding programme is low seed production and poor viability. In vitro mutagenesis and molecular breeding approaches are good alternative for crop improvement.

Spontaneous or induced variability can be used in functional genomics as well as for crop improvement. Various attempts were made in past to improve the crop by induced variability using physical and chemical mutagens. The vast information and sequence data bases accumulated during last a few years facilitated the gene directed modifications and gene discovery using reverse genetics tools. TILLING (Targeting Induced Local Lesions IN Genome) is a reverse genetics approach which make high through put screening of the single nucleotide variations and allows the direct identification of beneficial nucleotide and amino acid changes in genes with known functions and their use as the genetic markers for selection. Induced mutagenesis or accessions containing natural polymorphisms (Eco-TILLING) combined with TILLING provides a powerful tool for non-transgenic method for crop improvement as it works within the genome of the plant itself and thereby, is free of the regulatory hurdles imposed on genetically modified organisms. The range of alleles that can be developed via TILLING in a short time is unparalleled and

unlikely to be found elsewhere in the pool of germplasm accessible to plant breeders. The current approach includes *in vitro* mutagenesis combined with genomic screening in relation to phenotypic variations in *Stevia*.

HET-CAM: A Novel Approach to replace Animal Model

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The Hen's Egg Test – ChorioAllantoic Membrane (HET-CAM) is proposed to provide information on the effects that may occur in the conjunctiva following exposure to a test substance. Chicken-embryo models have long been used as models for embryotoxicity by virologists. The CAM is a vascularized respiratory membrane that surrounds the developing bird embryo. The CAM is composed of an ectodermal layer that consists of epithelium that is two to three cells thick; a mesodermal layer that consists of connective tissue, ground substance, and blood vessels, and an endodermal layer. The blood vessels that are present in the mesodermal layer of the CAM are branches from the embryo-allantoic arteries and veins. These vessels contain erythrocytes and leukocytes that are believed to be involved in the inflammatory response following exposure to external stimuli. The denaturation of proteins (observed as coagulation) is proposed to be an indicator of effects on epithelial cells in the CAM. Based on these factors of HET-CAM test, in our present research, we tested the inflammatory responses of different commercially available biomedical products that have been in daily use in the hospitals for the patients. Main aim of our research is to identify the human friendly biomaterials. The chick CAM model allows for rapid, simple and low cost screening of tissue reactions to the different biomaterials. The CAM model is a true *in vivo0*system that can be used as an intermediate step between a cell culture and a more complex mammalian model. The CAM can be used for the evaluation of both acute and chronic inflammatory responses to biomaterials. In addition, the CAM model used in the study presented the ability to continuously visualize the implant site without having to sacrifice the test animal.

Poster presentations

The Study Detecting the Presence of Phytochemicals, Antibacterial, Larvicidal and Insecticidal Activity of *Eupatorium Odoratum* Flower Extracts

Jofeena joy(1): and Dr Deepa G Muricken (2);

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Abstract

The Eupatorium odoratum is included in sun flower family Asteraceae it have many properties as anti-bacterial, anti-mycobacterial properties. It also contains many phytochemicals. It used as tea in many places. It used as a medicinal as well as ornamental plant. Successive extracts from dried flowers of *Eupatorium odoratum* were investigated for the presence of phytochemicals. Methanol extract was used for the Phytochemical and antibacterial studies. Aqueous extracts of Eupatorium *odoratum* was used for the insecticidal and larvicidal activity. As a result it was found that the plant flowers have insecticidal and larvicidal activity and a slight range of antibacterial activity.

Introduction

Eupatorium odoratum Linn or *Chromolaena odorata* is a fast growing perennial and invasive grass or sub shrub also known as Siam weed, Christmas bush and common Floss flower native to south and Central America. It has been introduced into the tropical regions of Asia, Africa and other parts of the world. It is a multi-stemmed shrub to 2.5 m tall in open areas. It has soft stems but the base of the shrub is woody. In shady areas it becomes etiolated and behaves as a creeper, growing on other vegetation. *Eupatorium odoratum* is a species of flowering shrub in the sun flower family, Asteraceae. It suppresses young plantations, agriculturalcrops and smothers vegetation as it possesses allelopathic potentialities and growth inhibitors (Ambika and Jaya Chandra; 1980). The weed is poisonous to

livestock as it has exceptionally high level of nitrate (5 to 6 times above the toxic level) in the leaves and young shoots; the cattle feeding on these die.

In recent times, there is an increase in global utilization of herbal medicine in treatment of various diseases, affecting humans (Vande Broek et al, 2004; Cragg & Newman 2001). *Eupatorium odoratum* were used as a traditional medicinal plant for centuries. Young leaves are crushed and the resulting liquid can be used to treat skin wounds. The phytoprostane compound chromomoric acid c-l has been identified from *Eupatorium odoratum* as a strong inducer of the activity of transcription factor NFE2L2 (Nrf2), a master regulator of a range of gene with defensive, anti inflammatory & detoxifying functions (Heiss et al 2014).

The antioxidant activities of ethanolic extracts of leaf, stem, root and defatted flower parts were evaluated by β-carotene bleaching and 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assays. Leaf and flower revealed the good antioxidant activities in both methods. In β-carotene bleaching assay, ethanol extracts of leaf and flower exhibited the relative antioxidant activity values as 0.95± 0.029 and 0.86±0.05, respectively. The DPPH activity of ethanol extracts of leaf and flower was increased in a dose dependent manner, which was found in the range of 5.65 - 87.93 and 13.98 - 91.4% inhibition, respectively, as compared to butylated hydroxyanisole (23.29 - 91.05). The IC50 of leaf and flower extracts in DPPH radical was 44.5 and 50.9 µg mL-1, respectively. This study suggests that leaf and flower parts of E. odoratum could be pharmaceutically exploited. (Amatya et al 2011).

Methods

Antibacterial Activity Analysis Of Eupatorium Odoratum Flower Extract

Agar diffusion method:

The agar diffusion method is usually used to check the sensitivity of antibiotics against microorganisms. The activity of *Eupatorium odoratum* flower extract was tested against six microorganisms (*E.coli*, Staphylococcus, Streptococcus, Bacillus, Proteus, Pseudomonas, and Klebsiella). 100ml of nutrient broth was inoculated with the test organisms and incubated at 37°C over night. 0.1ml of culture were swabbed over six plates containing solidified sterile nutrient agar medium and allowed to dry.

Required number of wells was cut upon each plate with equal distributions using a sterile gel puncture and the agar plugs were removed carefully. In each wells methanol extracted samples (0.083g/ml of DMSO) of 10µl, 15µl, and 20µl were loaded. It was incubated over night at 37°c. The diameter of zone of inhibition was measured using scale.

Phytochemical Analysis Of Eupatorium Odoratum Flower Extract

Phytochemical screening is one of the necessary steps to find out the chemical constituents which lead the isolation of compound. Since 19th century different bioactive phytoconstituents have been isolated and purified. Many of these are used as the ingredients of modern medicine (Vijayalakshmi & Ravindhran 2002) and for many other industrial purposes.

Insecticidal And Larvicidal Activity

White mealy bug and mosquito larvae were collected and kept in 10 ml of aqueous extract of flower and 10 ml of water was placed as control. The extract was taken in two small beakers (10 ml each) and 5 numbers of each organism were added separately, and kept several hours to obtain mortality rate.

Result

Antibacterial activity of flower extract of eupatorium odoratum:

The antibacterial activity of methanol extract of *Eupatorium odoratum* flower was studied using agar diffusion method. Out of 7 *(E.coli*, Staphylococcus, Streptococcus, Bacillus, Proteus, Pseudomonas, and Klebsiella) microorganisms were tested 2 of them (Staphylococcus and Bacillus) shows zone of inhibition. The obtained zone of inhibition was measured using scale. zone of inhibition was produced against staphylococcus and Bacillus which shows the anti bacterial activity of extract. The maximum zone was obtained for Staphylococcus and a minimum for Bacillus. Other organisms are not showing any antibacterial activity.

Phytochemical analysis of eupatorium odoratum flower extract:

The methanol extract of *Eupatorium odoratum* gave positive results for Tannin, Phytosterols or Triterpinoids, Flavanoids, Coumarins, Saponins, Quinones, Cardiac Glycosides, Terpenoids, Sreroid, Acids, And Phenols.

Anthraquinones were gave negative result in phytochemical analysis of methanol extract.

Insecticidal And Larvicidal Activity

The aqueous extract of *Eupatorium odoratum* flower showed insecticidal activity against white mealybug and larvicidal activity against mosquito larvae. One of the white mealy bug were died in 3hours, two of them were in 4½ hours and another two of them were died within 5 hours of time. In the case of mosquito larvae two were died in 30 minutes one was in 40 minutes and the last two were in 45 minutes of time. The bugs and larvae in distilled water were not showing any mortality rate compared to those in the extract. This data sheds light to use this source as a means of larvicidal and insecticidal activity.

The Effect Of Fipronil Pesticide On Soil And *Pisum sativum*

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Abstract

A Pesticide is any mixture of substances intended for preventing, destroying, repelling or mitigating any pest. Fipronil [5-amino-1-(2,6-dichloro-α, α, α -trifluoro-p-tolyl)- 4-trifluoro methyl sulfinylpyrazole-3-carbonitrile] is a new generation phenyl - pyrazole broad spectrum insecticide. It is used to control insect attack in a wide range of crops. Fipronil is known to have moderate acute toxicity to people and mammals, but its effect on soil and leguminous plant *pisum sativum* was unknown. The aim of our study is to evaluate various effects of the insecticide "fipronil" on Pisum sativum seed germination and the soil in which the pea plant grows. Our study points out that treatment with fipronil have positive effect on the *Pisum sativum* seed germination. Properties of fipronil treated soil were significantly altered, some of these changes are beneficial for plant growth but others are not. The increase in the pH of soil due to fipronil treatment will be due to adsorption, abiotic and biotic degradation processes. The increased moisture content after fipronil treatment indicates the increased soil water holding capacity. The significant reduction in the total number of microorganism in the soil after fipronil treatment and antibacterial property of fipronil is detrimental for the soil health. For the better soil health, fipronil can be applied at medium to low dose, discontinuously in combination with other pesticides to achieve effective pest control.

Introduction

Fipronil is unique in that it is a slow-acting poison which allows the poisoned insect time to return to the colony and spread the poison to others, resulting in more effective colony elimination. It acts by blocking GABA and glutamate gated chloride channels in the central nervous system of insects It is effective against a wide variety of insects. Fipronil is used to

control insect pests in more than100 crops including rice, wheat and legumes.In higher concentrations fipronil is toxic to fishes, bees and birds. It is classified as a possible human carcinogen

The aim of the study was to assess the effects of different concentrations of Fipronil on soil, *Pisum sativum* seed germination and growth.

Materials And Methods

The pea plant - *Pisum sativum* was used for all experiments. The *Pisum sativum* planted trays are divided into the following groups.

- a) **Group 1**: Control (C) - given tap water
- b) **Group 2**: Low concentration of Fipronil treated (L) - 10 mL of a 1% solution/day
- c) **Group 3**: Medium concentration of Fipronil treated (M) - 10 mL of a 10% solution/day
- d) **Group 4**: High concentration of Fipronil treated (H) - 10 mL of a 100% solution/day

The plants were regularly watered on a daily basis, depending on the soil moisture

Determination of pH - pH of each group was recorded using pH meter.

Standard plate count - Pour plate method is used to enumerate the total number of microorganism in the soil

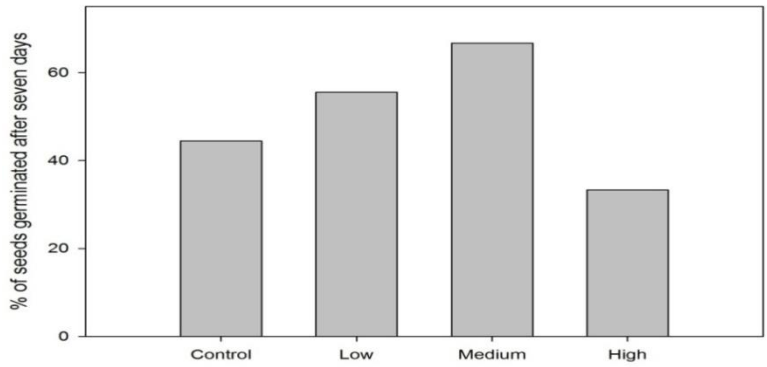
Moisture Content - complete evaporation of moisture from a pre weighed amount of soil is used to measure the moisture content

Determination of the presence of pesticide degrading bacteria - Enrichment medium was prepared with Fipronil as the sole carbon source.

Determination of antibiotic activity – Disc diffusion method is used to measure the antibacterial activity of fipronil

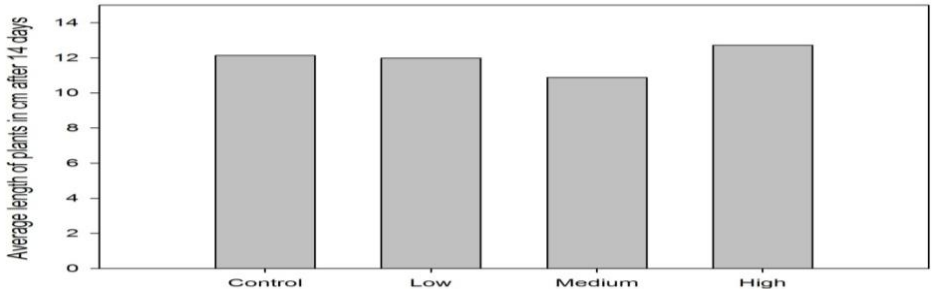
Result and Discussion

Effect of Fipronil on the growth of Pisum sativum



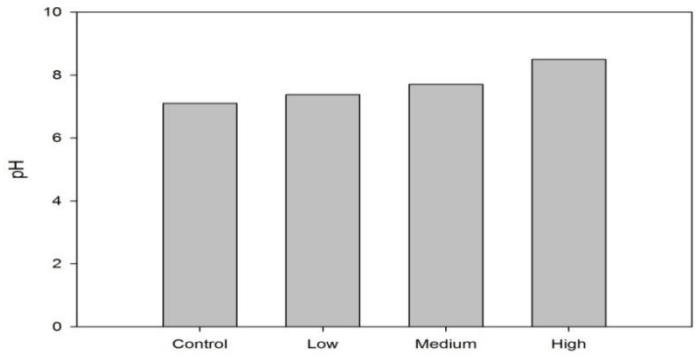
The percentage of seeds germinated after seven days of planting. The low and medium dose of treatment with Fipronil gave better germination when compared with both control and high dose treated group

Average length of plants after 14 days



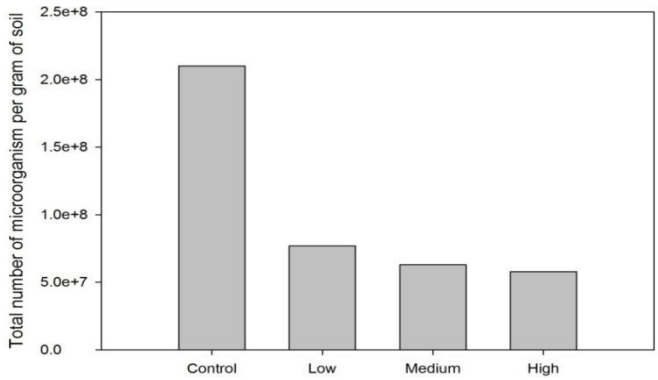
Average length of plants after 14 days didn't show any significant change

pH of soil samples from control and experimental groups



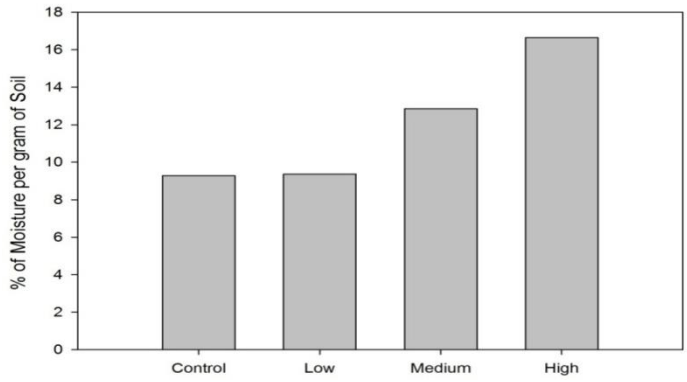
pH of the soil tent to increases as with the concentration of the Fipronil treatment when compared with control..

Determination of total number of microorganism in the soil



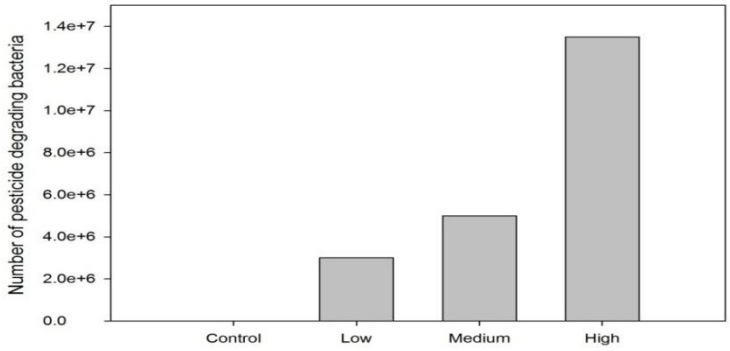
The pour plate method is used to determine the total number of viable microorganisms in the soil. The total number of aerobic and anaerobic microorganisms is found to be decrease as the concentration of the Fipronil increases.

Moisture Content of a Soil samples



The percentage of moisture content in the soil increases with the increase in the pesticide concentration.

Number of pesticide degrading bacteria in the soil samples



The number of pesticide – Fipronil degrading bacteria increases with the increase in the dose of treatment

Antibiotic activity of Fipronil

Streptomycin, Klebsiella and Proteus was found to be sensitive to Fipronil and a clear zone was observed. *E. coli* was found to be resistant and hence no clear zone was obtained around the disc.

For the better soil health, fipronil can be applied at medium to low dose, discontinuously in combination with other pesticides to achieve effective pest control.

This research project contributes to the understating the effect of fipronil on *Pisum sativum* seed germination, in addition to laying the foundation for the effective use of fipronil as a pesticide without affecting soil health.

Partial Purification of Lipase from *Eupatorium Odoratum* Leaf Extract

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Abstract

Eupatorium odoratum is a fast-growing perennial and invasive weed native to South and Central America. It is a species of flowering shrub in the sunflower family, Asteraceae. It is also known as *Chromolaena odorata*. It is sometimes grown as a medicinal and ornamental plant. This study involved the detection of lipase from the leaf extract of *Eupatorium odoratum.* There are no reports of lipase enzyme from *Eupatorium odoratum.* The current investigation shows the *Eupatorium odoratum* leaf exhibited lipase activity. Here we use a cost effective and a simple method for the detection of this protein. The principle behind this method is monitoring the change in color of phenol red, a pH indicator when lipase will release free fatty acids. This colorimetric method helps us to detect the lipase qualitatively and we determined its molecular weight using SDS-PAGE. The molecular weight of lipase was found near to 20kDa. Lipase protein also has wide spread applications in the field medicine and industry

Introduction

Eupatorium odoratum is a species of flowering shrub in the sunflower family, Asteraceae. It is used as a traditional medicine in Indonesia. The young leaves are crushed and the resulting liquid can be used to treat skin wounds. The economic value of the plant is low. In recent decades, it has become a serious pest in the humid tropics of South East Asia, Africa and Pacific Islands. Common names of Eupatorium odoratum includes Siam weed, Christmas Bush and common Floss flower.

Lipases, collectively known as "lipolytic enzymes," are characterized by their ability to hydrolyze hydrophobic long- and short-chain carboxylic acid esters, respectively. Lipases catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase, whereas esterases catalyze the hydrolysis of ester bonds of water-soluble

substrates. Lipases are versatile group of enzymes that not only hydrolyze the esters of long chain aliphatic acids from glycerol at oil/water interface but also involved in the transesterification reaction. Lipases have preference to hydrolyze triacylglycerol, diacylglycerol and monoacylglycerol to glycerol and free fatty acids. Lipases are widely distributed in plants, animals, insects and microorganisms. In plants, lipases are mostly present in food reserve tissues of growing seedlings especially in those which contain large amounts of triacylglycerols. In animals, the lipases are found in pancreas, and on the surface of mucous cells of the gastric mucosa. In insects, these enzymes are found mostly in plasma, salivary glands, muscles and fat bodies. In case of plants, lipases are mostly present in oil seeds, cereals and lactifers. Plant lipases have to principal functions: to provide energy by hydrolyzing the oils stored in seeds and protection (Shah, 2005).

Materials And Methods

Plant material

The plant leaves were collected from various locations in Thrissur. The leaves were washed thoroughly with tap water and shade dried at room temperature. The dried plant leaves were finely powdered using an electric grinder.

Lipase Activity Of Eupatorium Odoatum Leaf Extract

Lipase activity in *Eupatorium odoratum* leaf extract was analyzed using a simple method (Rajini Singh, 2011). Constituents used for lipase activity test are given in the table.

Table 2. Composition of Lipase assay medium

Components	Quantity
Phenol red	0.01%
Substrate	1%
Calcium chloride	10mM
pH	7.3-7.4
Agar	2%

Substrate: Coconut oil

Two main methods were used for the detection of lipase activity

Plate method

0.01% phenol red and 10 mM calcium chloride were mixed in 100ml distilled water. The pH was adjusted to 7.3-7.4 with sodium hydroxide. 2% agar was added to the media and dissolved by heating. 250µl substrate (coconut oil) was added to the media and mixed thoroughly. The media was poured into petriplates and allowed to solidify. Wells were punched on the agar and the leaf extract was poured to the wells. Incubated the plates at 37^0C overnight.

Tube method

The above mentioned method was modified in our laboratory and developed new method as tube method. Media composition was same as in the plate method without agar. After the substrate was added to the tube, mixed thoroughly/motorized. The pH was adjusted to 7.3-7.4 with sodium hydroxide. One tube considered as blank in which no substrate was added. In case of control tubes, only media was added. All tubes were incubated at 50^0C for 10 minutes. After incubation the reaction was stopped by adding 95% ethanol. The colour difference was monitored with respect to control and test tubes. The visible spectrum of the blank, control tubes and the reaction was taken separately and the difference in the absorption maxima of phenol red was compared.

Table 3. Contents of the test tubes.

Sample	Medium	Substrate	Leaf extract
Blank	5ml	-	-
Test	5ml	75µl	75µl
Control	5ml	-	75µl

Purification Of Lipase From Eupatorium Odoratum Leaf Extract

Buffer extract of *Eupatorium odoratum* was used for the purification of lipase. 10gms of leaf powder was mixed in 100ml tris buffer pH 7.0 and stirred for 8-9 hours using a magnetic stirrer. It is then filtered through a muslin cloth and centrifuged at 10000rpm for 10 minutes. The supernatant was collected and performed chromatographic purification.

Most of the time, a single chromatographic step is not sufficient to get the required level of purity. Hence, a combination of chromatographic steps is required. Ion exchange chromatography is the most common chromatographic method used for the purification of lipase.

Ion exchange chromatography

Ion exchange chromatography involves two primary steps, first the binding of a protein to a charged resin and second the elution or displacement of the protein from the charges of the resin. Critical to the former are the pH of the buffer used to equilibrate and load the proteins onto the column. Factors that control the elution are pH or ionic strength. Common ion exchangers include the positively charged anion exchanger - DEAE (diethylaminoethyl) and the negatively charged cation exchanger - CM (carboxymethyl).

Purification of lipase using ion exchange chromatography

1. The empty column was washed and fixed to the stand.
2. The top cap was removed and packed the column with 15 ml DEAE cellulose.
3. It is equilibrated with equilibrium buffer (Tris HCL pH 8) 2ml of leaf extract was loaded to the equilibrated DEAE cellulose column. The sample was allowed to enter the column before starting the washes.
4. The column was washed with 50-100ml of equilibration buffer.
5. The enzyme was eluted out from the solution using 50ml of elution buffer (different moles of Nacl)
6. Collect the elute in test tubes as 2ml fractions. Optical density at A_{280} was monitored.
7. Follow up with a high salt buffer wash to remove any tightly bound proteins and regenerate the resin for the next use.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE separates proteins according to their molecular weight, based on their differential rates of migration through a sieving matrix (a gel) under the influence of an applied electrical field.

SDS-PAGE is used to determine the molecular weight of lipase from *Eupatorium odoratum* leaf extract. SDS-PAGE at alkaline pH (8.3) was carried out according to the method of Laemmli (1970) in a discontinuous buffer system.

Reagents:

- **A.** Acrylamide (29.2 g) and bis-acrylamide (0.8 g) were dissolved separately in a minimum amount of water and mixed and made up to 100 ml, filtered and stored in a dark brown bottle at 4^0C.
- **B.** *Separating gel buffer:* Tris (18.15 g) was dissolved in water and pH was adjusted to 8.8 with HCL (6N), solution made up to 100 ml and stored at 4^0C.
- **C.** *Stacking gel buffer:* Tris (3 g) was dissolved in water and pH adjusted to 6.8 with HCL (6N), solution made up to 100 ml and stored at 4^0C.
- **D.** *10% Sodium dodecyl sulfate:* SDS (10 g) was dissolved in water (100 ml).
- **E.** *Ammonium persulfate:* It was freshly prepared by dissolving 50 mg in 0.5 ml of distilled water.
- **F.** *Tank Buffer:* Tris (0.3 g), glycine (1.44 g) and SDS (0.1 g) were dissolved in 100 ml of distilled water.
- **G.** *Staining solution:* Coomassie brilliant blue (CBB) R-250 (0.1 g) was dissolved in a mixture of methanol: acetic acid: water (25:10:65, v/v). The reagent was filtered and stored at 25 ± 2^0C.
- **H.** *Destaining solution:* Methanol: acetic acid: water (25: 10: 65, v/v).
- **I.** *Sample buffer:* Prepared in solution C diluted 1:4, containing SDS (4% w/v), β-mercaptoethanol (10% v/v), glycerol (20% v/v) and bromophenol blue (0.1% w/v).

Initially contents of the separating gel were mixed (Table 4), degassed and poured between the assembled glass plates, the bottom edge sealed with agar (1% w/v). The gels were layered with 0.5 ml of distilled water and allowed to polymerize at 25 ± 2^0C for 30 minutes.

After polymerization of the separating gel, contents of stacking gel were mixed and layered above the polymerized separating gel. The gels thus prepared were of the size 10.5 × 9 cm and thickness 0.8 mm.

Samples were prepared by dissolving protein (60 µl) in solution "I" (20 µl). The samples were heated in dry blocks at 100^0 C for 5 minutes. Cooled samples were then layered in the wells immersed in solution "F" (Tank buffer) and were run at constant voltage (50 v) until the tracking dye, bromophenol blue entered the lower tank buffer.

Medium range protein M_r markers (Phosphorylase b, 97.7 kDa; BSA, 66.3 kDa; ovalbumin, 43.3 kDa; carbonic anhydrase, 29 kDa; soyabean Kunitz inhibitor, 20 kDa and lysozyme, 14.3 kDa) was used. The markers were supplied as a solution having a total protein concentration of 3 mg/ml. The markers were diluted 1:1 with the solution 'I'and boiled for 5 minutes.

Staining: The gels were stained for protein with reagent 'G' for 3-4h at 25 ± 2^0C and destained in the reagent 'H'.

Table 4. Preparation of separating gel and stacking gel

Solution	**Separating gel (ml)** **(12.5% T, 2.7%C)**	**(10% T, 2.7%C)**	**Stacking gel (ml)** **(5% T, 2.7% C)**
Solution A	3.33	2.66	0.83
Solution B	2.00	2.00	-
Solution C	-	-	1.25
Distilled Water	2.55	3.22	3.03
Solution D	0.08	0.06	0.05
TEMED	0.01	0.01	0.01
Solution E	0.03	0.03	0.03

Total	8.00	8.00	5.00

Silver staining

Silver staining is used to detect proteins after electrophoretic separation of polyacrylamide gels.

1. Fixing solution

60ml acetic acid, 25ml methanol and 250µl formaldehyde. Make the volume up to 100 ml. Keep the gel in this solution for about 45-60 minutes(if the gel is destained after coomassie staining, this step can be avoided).

2. 50% ethanol wash for 30 minutes (twice).

3. Pretreatment

20mg/100ml $Na_2S_2O_3$. Keep the gel in this for 1 minutes with continuous shaking.

4. Wash with distilled water 3 times.

5. Impregnation

Silver nitrate 200mg/100ml and 25µl formaldehyde. Keep the gel in this solution for 20 minutes with shaking.

6. Development

Sodium carbonate 6g/100ml, 75µl formaldehyde and 1mg $Na_2S_2O_3$. Keep the gel in this solution till the band appear (reduce silver ions to metallic stain).

7. Wash with distilled water twice.

8. Stop solution

6ml acetic acid and 2.5ml methanol. Make up to 100ml. Wash the gel in this solution.

Results and Discussion

Lipase Activity Of Eupatorium odoratum

Plate method

After the lipase assay, colour change was produced around the well. Colour changes from pink to yellow. This denoted that the leaf extract have oil degrading activity or lipase activity. This is the first report for the presence of lipase in *Eupatorium odoratum* leaf.

Tube method

The tube shows colour change after incubation. Read the optical density and the results obtained colorimetrically showed the degradation of oil by lipase enzyme present in the leaf extract of *Eupatorium odoratum*. Coconut oil was used as the substrate to check the lipase activity. This method is a simple and cost effective method to check the presence of lipase. The presence of protein can be analysed in a short period of time.

Partial Purification Of Lipase

Ion exchange chromatography

After ion exchange chromatography, absorbance was read at 280 nm of all the collected fractions. The graph given below shows the absorbance of each fractions.

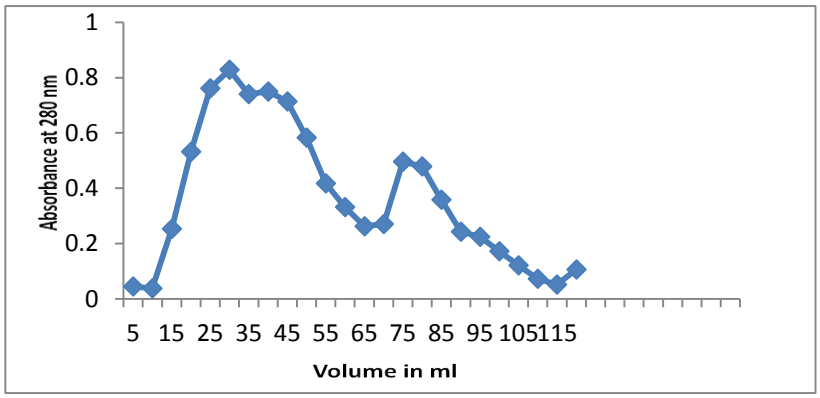

Fig: 4. Purification profile of lipase on DEAE Cellulose ion exchange chromatography

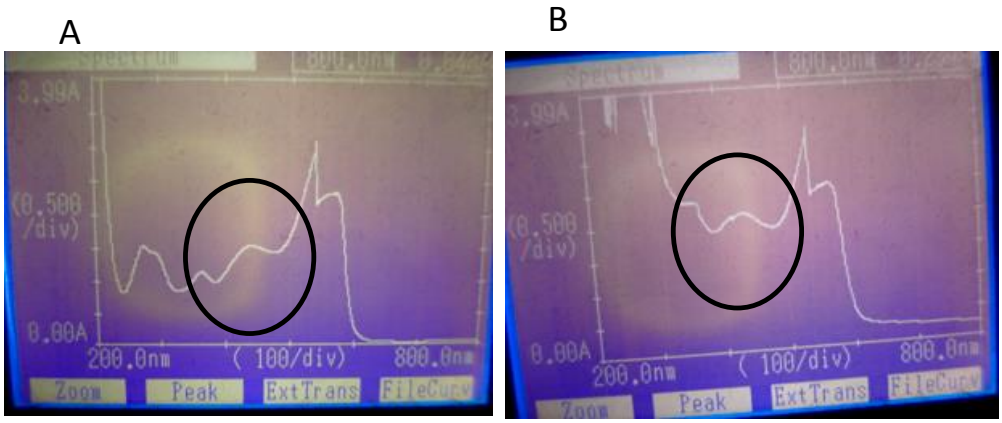

Fig: 5. Spectrum of *Eupatorium odoratum* leaf extract showing lipase. A) Control tube without leaf extract. B) Leaf extract showing lipase activity.

Phenol red shows absorption maximum at 550 nm at alkaline pH. In the figure B, the shift in peak near yellow-orange range is visible due to lipase activity.

The fractions showing maximum lipase activity were pooled and loaded on to SDS-PAGE to check for purity and also the molecular weight of the protein. Since the purified protein was low in concentration, silver staining was done.

Sds Polyacrylamide Gel Electrophoresis

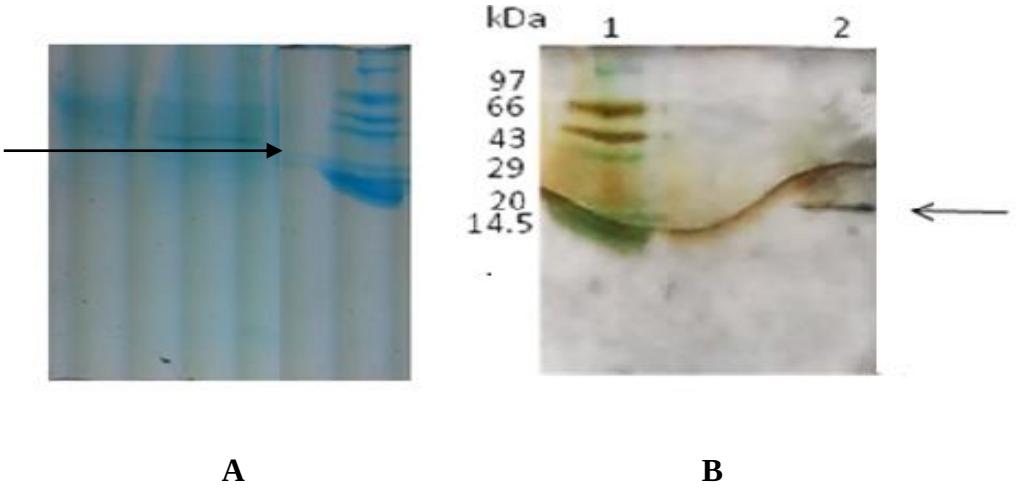

Fig: 6. SDS PAGE profile of lipase purification. Fig (A) is crude extract of lipase after coomassie staining. Fig (B) silver staining of purified protein.

The gel shows molecular weight of lipase enzyme present in *Eupatorium odoratum*. The molecular weight of lipase was found near to 20 kDa. The molecular weight have to be confirmed further using other techniques like MALDI.

Lipase enzyme gains attention due to their wide variety of industrial applications. This enzyme is wide spread in occurrence, i.e., it is seen in unicellular life forms like microbes to the highly evolved plant species, both dicots and monocots and also seen in animals including primates like humans. Lipases can be used in cosmetics, detergents, biodiesel preparation and variety of pharmaceutical applications. Application of lipases in detergents marked a new era in detergent industry. Traditional fossil fuels like petroleum products becoming a limited source made us to think in different way to create new fuels. This led to the development of bio-fuels which employs the potential of lipase in the field. Employing this enzyme, bio-fuels can be developed even from things we considered as waste. Thus this enzyme paves an efficient way in waste management which is the current major issue of the era.

Conclusion

There are no previous reports of lipase enzyme from *Eupatorium odoratum.* Our preliminary studies have shown that *Eupatorium odoratum* exhibited lipase activity and we partially purified this enzyme from the leaf extract. We also determining the molecular weight of the enzyme and it was found near to 20 kDa. Due to its lipase activity, it can provide newer leads and clues for modern drug design and for industrial application.

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Aerobiological Investigation of Aspergillus Species from Indoor and Outdoor of Poultry Farm

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Aerobiological survey was carried out from indoor and outdoor poultry farm field at Hessaraghatta village, Bangalore. Samples were collected fortnightly for a period of one year (January 2011 to December 2011) by using Andersen two stage sampler and petri plates with Malt extract agar media, sampling time was limited to 5 minutes. A number of fungal species had been recorded, among these Aspergillus species is found to be present all round the year. A total percentage of Aspergillus species from indoor environment of poultry farm were Aspergillus niger 2.01 %, Aspergillus terreus 1.22 %, Aspergillus fumigatus 1.09 % and Aspergillus flavus 0.74 % followed by outdoor Aspergillus terreus 5.62 %, Aspergillus niger 2.47 % and Aspergillus flavus 2.07 % were recorded. Identification is done on the basis of colony colour, microscopic morphology, measurement of conidia and conidiophores by comparing with manual of Aspergillus and other standard literature. Majority of the Aspergillus species are present throughout the calendar in monthwise appearance.

Polymerization of Bio-friendly Bone Implant Model against Biofilm forming Bacteria

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The polymer chitin fibre (CHF) was found in the nature as marine wastes from crustaceans. The research literatures explain the antimicrobial potentiality of these polymers and one of its derivative called chitosan (CS) which were used to make bone and dental implants. Medical implants the most unavoidable medical device of nowadays has many potent advantages. One of its important limitations in medical usage is colonization of biofilm forming microbes on the surface of the implants and produce high risk to the patients. This study involves the evaluation of these polymers against one such high rate biofilm forming bacteria, *Staphylococcus epidermidis* which can prevail in the blood. The polymer and its derivative were used to form a 3D composite rod with layer-by-layer structure using *in situ* precipitation method. The physical characteristic features like investigating CHF surface using SEM demonstrated sufficient rough surfaces and edges which could enhance the mechanical combining stress between fibre (CHF) and matrix (CS).The Colony biofilm assay was used to check biofilm forming ability of the catheter implant isolated *S. epidermidis* on the polymer rod. The biocompatibility test done by implanting the rod in 9 days old embryonated egg's chorio-allantoic membrane formed no adverse effects on the blood vessel formation. The data acquired in this study proved the biofilm retarding ability of the polymeric rods can be suggested as a better implantable material for long-term implantation in human body

Reducing Antioxidant Power of Methanolic Extracts of Plants Containing Phenolic Compounds

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Abstract

Medicinal plants have been found as important contributors to the pharmaceutical, agriculture and food industries. With the onset of the synthetic era, pharmaceutical industries are producing a lot of synthetic drugs that help to alleviate diseases. This paper reports the reducing antioxidant power of methanolic extracts of some plant namely *Aerva lanata, Biophytum sensitivum, Cynodon dactylon, Vernonia cinera.* The parameters analysed were total phenolic content and Reducing antioxidant power. The reducing property of the plants may be due to the phenolic content of the plants. Hence the level of phenolic contents and the reducing property of individual plants were analysed. The yield of the extract was found to be maximum for *Vernonia cinerea* and the phenolic content also was found to be maximum for the same plant. The reducing power analysed also found to be related with the total phenolic content in the present study. It can be concluded that the medicinal property of plants are somehow related with the antioxidant property of the plants. The antioxidant property has a direct relation with the total phenolic content also.

Introduction:

All plants produce an amazing diversity of secondary metabolites. One of the most important groups of these metabolites are phenolic compounds. Plant phenolics are commonly found in both edible and non-edible plants, and have been reported to have multiple biological effects, including antioxidant activity. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, the hydrogen donators etc. The phenolic compounds are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. Recently there is

an emerging trend in researches to support the biological activities of medicinal plant.

The aim of the study was to analyze the total phenolic content and reducing antioxidant power of the methanolic extract of four medicinal plants.

Materials and methods:

Sample preparation

- Plants selected for the present study were *Aerva lanata, Biophytum sensitivum, Cynodon dactylon* and *Vernonia cinera*
- Plants were air dried and powdered in a grinder which were used for the study

Preparation of methanolic extract

- The samples were weighed and soaked with methanol [in ratio methanol : plant (6:1)] for seven days.
- The samples were then filtered and methanol was completely removed by vacuum evaporator.
- The crude extracts were weighed and stored at 4^0C

Determination of total phenolic contents

- The amount of total phenolics in extract was determined with Folin–Ciocalteu reagent according to the method of Singleton and Rossi (1965) with slight modification using tannic acid as a standard.
- The total phenolic content was determined as mg of tannic acid equivalent (TAE) using an equation obtained from the standard tannic acid calibration graph.

Determination of Reducing antioxidant power

- The reducing antioxidant power of plant methanolic extracts was determined by the method of Oyaizu (1986) using potassium ferricyanide and trichloroacetic acid.
- The absorbance was measured at 700 nm against a blank using UV-Vis spectrophotometer. Increased absorbance of the reaction mixture indicates increase in reducing power.

Results and Discussions

Total yield of the methanolic extracts in different plants analyzed

Sl No	**Plant**	**Yield of extract (weight in g)**	**Yield of extract (%)**
1	*Aerva lanata*	4.52	0.94
2	*Biophytum sensitivum*	6.48	0.81
3	*Cynodon dactylon*	3.06	0.62
4	*Vernonia cinerea*	7.9	0.98

Total phenolic content of the methanolic extracts of the plants

Sl No	**Plant**	**Total phenolic content (gallic acid equivalent/100g dry weight)**
1	*Aerva lanata*	6.70
2	*Biophytum sensitivum*	6.90
3	*Cynodon dactylon*	6.50
4	*Vernonia cinerea*	7.20

The yield of methanolic extract and phenolic content of the extract

The yield of extracts was found to be maximum for *Vernonia cinerea* in the present study. It was previously observed that the percentage of extraction yield will increase with the particle size of the sample, extraction

temperature and ratio of the solvent and sample extraction. In the present study the lowest yield of extraction was for *Cynodon dactylon.* The maximum phenolic content was shown by *Vernonia cinerea*.

Reducing power

The results obtained for the reducing power in the present study are given in the form of graphs. The reducing power was found to be comparatively lower in *Cynodon dactylon.* It was also observed that the phenolic content of the same plant is lower when compared to other plants. In all the plants analysed, the antioxidant property was found to be directly related with the phenolic content of the plant. It can be concluded that the medicinal property of plants are mainly due to the antioxidant property of the plants and the antioxidant property is attributed to the total phenolic content.

Figure 1 – reducing power of methanolic extract of *Aerva lanata*

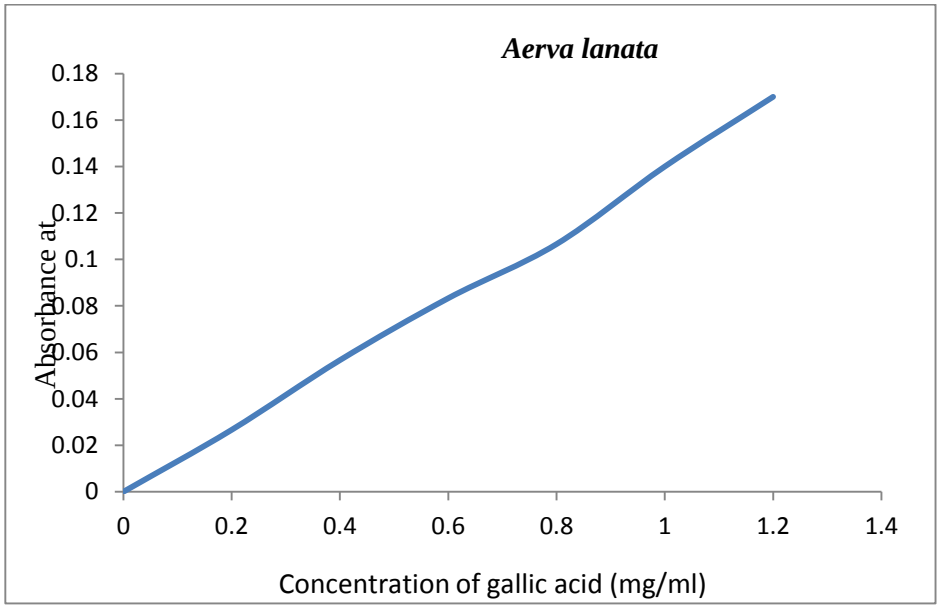


Figure 2 – Reducing power of methanolic extract of *Vernonia cinerea*

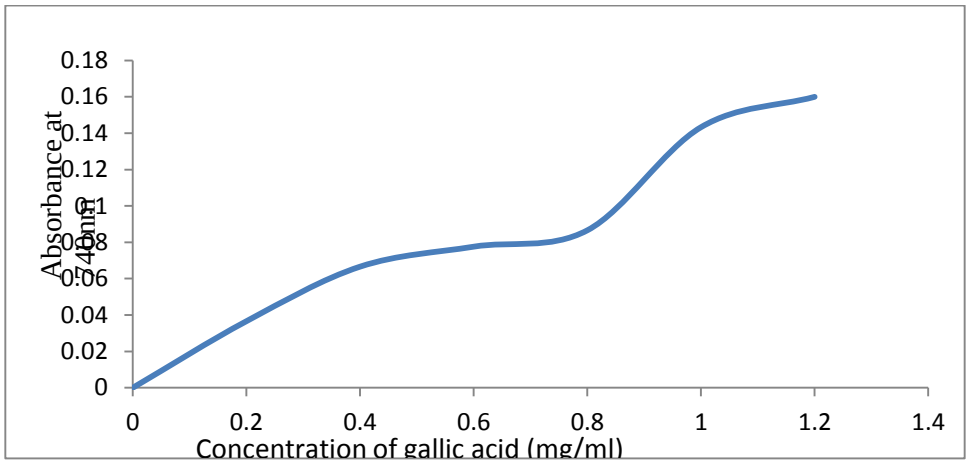


Figure 3 – Reducing power of methanolic extract of *Biophytum sensitivum*

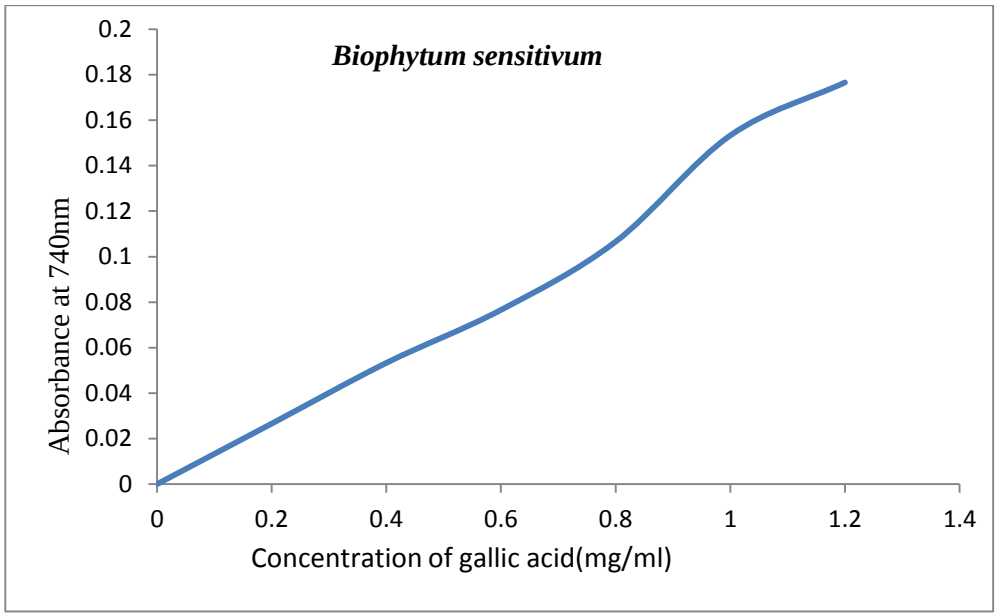
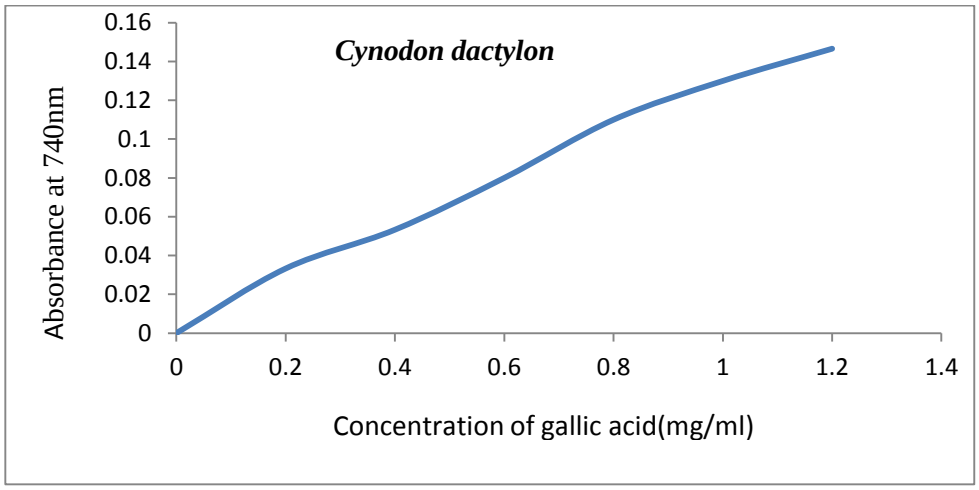


Figure 4– Reducing power of methanolic extract of *Cynodon dactylon*



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Antimicrobial activity of six well known spices

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Abstract

Spices have been shown to possess medicinal value including antimicrobial activity. This study compared the antimicrobial activity of ethanolic extract of various spices against four different microorganisms. The spices used were cinnamon, cumin, clove, fenugreek, coriander, and mustard. Staphylococcus aureus, E. coli, Klebsiella and Bacillus are the four different microorganisms used in this study. Of the different spices tested only cumin, cinnamon, and clove were found to possess antimicrobial activity out of which cinnamon showed significant activity. Spices might have a great potential to be used as antimicrobial agents.

Introduction

Use of antibiotics and other chemotherapeutic agents has led to the emergence of drug resistant microbes and other several disadvantages. Herbs and spices are generally considered safe and proved to be effective against certain ailments. The word spice comes from Latin *species*, meaning a commodity of value and distinction. A spice is a dried seed, fruit , root, bark, or vegetative substance primarily used for flavoring, coloring or preserving food. Spices have always been believed to have healing and magical qualities.

The study was aimed to analyze and compare the antimicrobial properties of six well known spices – Cinnamon, Clove, Fenugreek, Cumin, Mustard and Coriander- against four test organisms (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus* and *Klebsiella*).

Methods

Ethanolic extracts of the spices were prepared at a final concentration of 100 mg/ml. The antimicrobial activity of the spices was assayed by disc diffusion method.

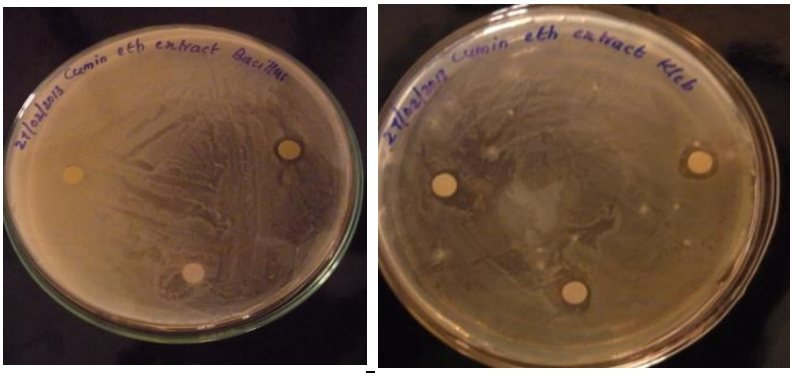
Paper discs impregnated with specific spice extracts are placed on the surface of nutrient agar medium inoculated with the test organisms. The plates are incubated and the zones of inhibition around each disc were measured. Paper disc with ethanol was taken as control.

Results

CUMIN

Microorganism	**Diameter of the zone of inhibition (mm)**
Bacillus	7.7±1.5
Klebsiella	9.7 ± 1.2

The antimicrobial activity of cumin was slightly higher against *Klebsiella* when compared to *Bacillus*. Cumin extract did not show any antimicrobial activity against *E.coli* and *S. aureus*.

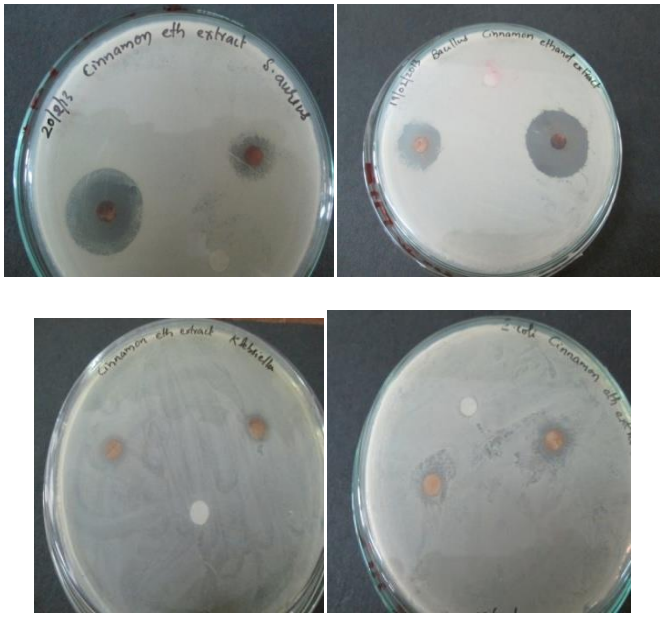


CINNAMON

Microorganism	Diameter of the zone of inhibition (mm)
Staphylococcus aureus	12.7 ±1.5
Bacillus	15.7 ±0.58
E.coli	11.3 ± 0.58

Klebsiella	7.7±0.58

Cinnamon extract was found to be effective against all the test organisms. The activity was highest against Bacillus, as indicated by the diameter of inhibition zone. The zone was almost similar for S.aureus and E.coli while the lowest activity was against Klebsiella. The results showed that cinnamon extract was equally effective against gram negative as well as gram positive bacteria.

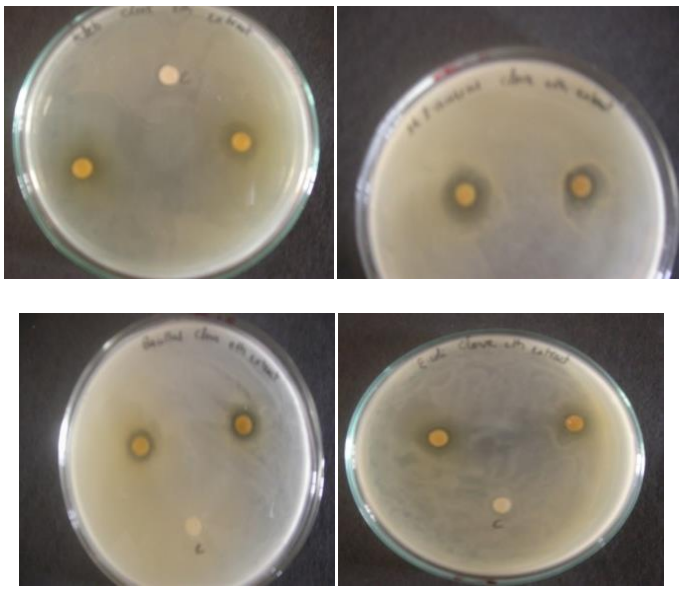


CLOVE

Microorganism	**Diameter of the zone of inhibition (mm)**
Staphylococcus aureus	11.7 ±1.2
Bacillus	10.3 ± 0.58
E.coli	9 ± 1
Klebsiella	10.3 ± 1.5

Ethanolic extract of clove showed antimicrobial activity against all the test organisms. The activity was slightly higher against S. aureus. The inhibition

zone was similar against all the other three microorganisms. Like cinnamon extract, clove extract was found to be equally effective against both gram positive and gram negative bacteria,

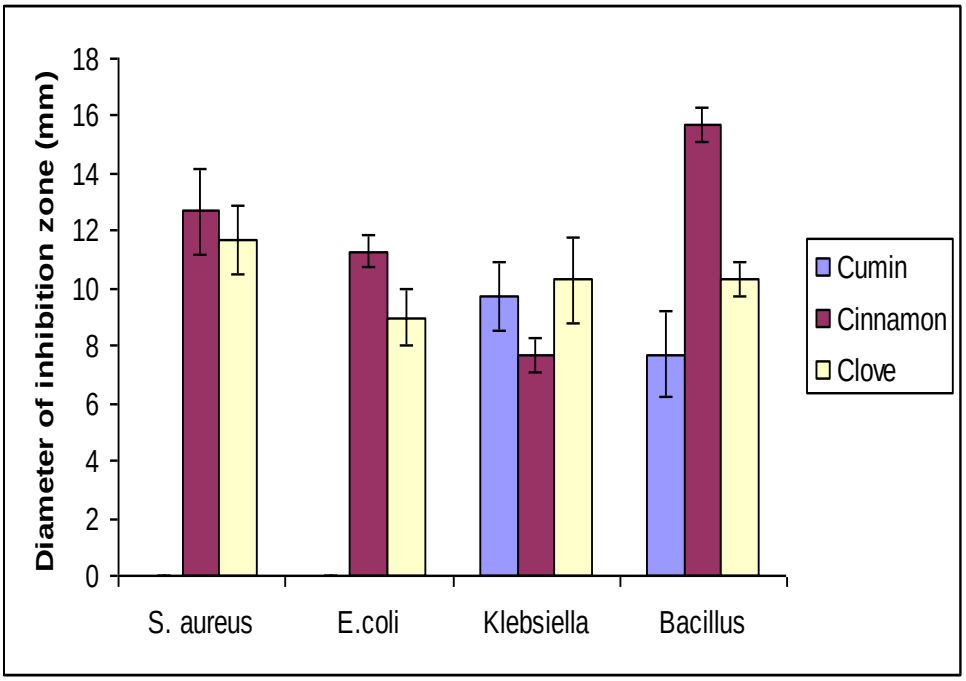


FENUGREEK, MUSTARD, CORIANDER

These spice extracts did not show any significant zone of inhibition against any of the test organisms

COMPARATIVE ANALYSIS OF SPICE EXTRACTS

The analysis revealed that both cinnamon and clove extract was equally effective against *S.aureus* while against *E.coli* cinnamon was slightly more effective. Sensitivity of *Klebsiella* towards cumin and clove extract was similar. The activity of cinnamon was comparatively low against Klebsiella. Against Bacillus, cinnamon extract was much effective compared to cumin and clove.



The results and comparison revealed that each spice extract vary in their antagonistic effect towards different microorganism. In other words, each microorganism can be effective controlled by a combination of active ingredients from different spices.

Conclusion

Use of antibiotics leads to several side effects and emergence of multidrug resistant strains. Natural therapy based on herbal and spice extracts will be effective. In this study, antimicrobial activity of six Indian spices against four test organisms were investigated. The diameter of inhibition zone was measured and antimicrobial activity was calculated. Antimicrobial activity of each spice extract varied with respect to the microbes used. Extracts of cumin, cinnamon and clove showed antimicrobial activities. Mustard, coriander and fenugreek didn't show any antimicrobial activity. Combination of spice extracts should be used for an effective removal of microorganisms. The particular antimicrobial ingredient present in spices should be isolated and further studies must be done.