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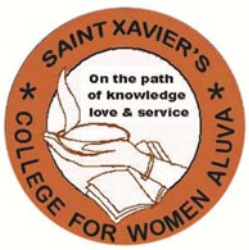

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EDITORIAL

This special issue of Discourse is the collection of research papers presented in the KSCSTE sponsored International Conference on Immune Response in Health and Disease, conducted by Department of Microbiology. It includes the original research work of various researchers based on the theme of the conference and also papers of general nature. Of the diversity of the articles published in this issue, we are sure that the readers will obtain several vast knowledge and information which benefit to your research and career.

Dr. Aneymol V.S.

Contents

A STUDY ON THE RADIOPROTECTIVE ACTION OF CARUM COPTICUM LINN

Revathy Babu[1] and **Jose Padikkala**[2]
[1]Assistant Professor, Department of Zoology, Sree Sankara College, Kalady
[2]Associate Professor, Amala Cancer Research Centre, Trissur.

Introduction

Cancer is a disorder in which several molecular changes are involved to initiate normal cells to form cancerous cells. Cancer is an imprecise term used to describe an estimated 200 different malignant tumors, marked by uncontrolled growth and spread of abnormal cells. When the control over cell division in some cells is lost, they start dividing indiscriminately to form a mass cells. This new growth of abnormal cells is called neoplasm or tumor which may be benign or malignant (Sanghvi, 1994).

The three important modalities for treating cancer are: Surgery, radiation and chemotherapy. The most commonly used modalities of cancer are chemotherapy and radiotherapy. But these therapies are not devoid of disturbing side effects. Hence, the search is still on to find novel drugs that can act as radioprotectors and chemoprotectors which will serve as powerful immune as well as antioxidant enhancing drugs. Since different types of neoplasms have their own responses to the various modalities of therapy, a histological diagnosis is imperative in planning the appropriate management of malignant disease. Because of their different strategies each of these treatment is associated with specific risks and side effects (Benham *et al*, 1983).

One of the new areas of current interest in cancer research is the development of less toxic anti cancer drugs. In order to obtain better tumor control, the normal cells and tissues should be protected against the radiation

injury. Thus the role of radio -protective compounds is important in clinical cancer therapy.

Unlike in the advanced countries, one of the major challenges in cancer research in the developing countries is to develop cost effective and easily available drugs. So investigations into the natural products and traditional medicine to explore the possibility of developing plant drugs from local resources should be given priority. Plants have always been a common medicament either in the form of traditional preparations or pure active principles in India.

Carum copticum is a herb belonging to the family Apiaceae is cultivated throughout India, Baluchistan etc. It is commonly called as Bishop's weed and in Malayalam it is called Ajwain. It has several beneficial effects on our body and in traditional Indian medicine. The plant is used for various diseases including ulcer, tumors etc.

The seeds are considered to be powerful detoxifying agents. The seeds are bitter and hot, carminative, diuretic, galactogogue, tonic, expectorant, cure weakness of limbs, and paralysis, chest pains, improves speech and eyesight, stimulate the intestine, good for ear boils, liver, spleen, vomiting, dyspepsia, kidney troubles, inflammations.

The essential oil of *Carum copticum* contains not less than 40-50% of Thymol brown as Ajwain-Ka-phol (Crude thymol) which is antihelmintic. Ajwain oil is shown to be toxic at different dilutions to pathogenic bacteria and is shown inhibitory to various microorganisms. It is also applied to retrieve rheumatoid and neuralgic pain. The seeds are also immune enhancing.

With all these wide spectrum of medicinal properties we propose to study its radio-protective activities in this work.

Materials & Methods

9% of seed extract, of *Carum copticum* (collected from Amala Ayurveda Hospital) was obtained by using 70% methonal and further evaporated, dried and dissolved in distilled water.

Test animals were the male Swiss Albino Mice between 22-27 g produced from the animal breeding station of College of Veterinary and Animal Science-Mannuthy, Thrissur. Four groups of six animals each were formed for the radioprotective experiment. The first group was not subjected to any sort of treatment in the experiment.

The second group served as control and the animals were exposed to radiation at a dose of 600 rad/ animal using a cobalt-60 gamma source. The third and fourth group were in addition treated with *C. copticum* seed extract at a dose of 50 mg/kg body wt. and 100mg / kg body wt. respectively. Every fifth day the haemoglobin and haematological parameters were checked. At the 20^{th} day the animals were sacrificed and the blood tissue parameters and bone marrow cellularity were checked.

Standard chemicals of analytical reagent grade were used. Estimation of super oxide dismutase activity was carried out by the method Mc Cord Beauchamp and Fridovich, 1969. Estimation of catalase was done by the method of Aebi,1974, Glutathione and glutathione peroxidase activities were estimated using the method of Moron *et.al*,1979. Estimation of tissue lipid peroxidase was carried out by the reaction described by Ohkava *et al.*, 1969. Creatinine was estimated using the method of Broa and Siroto 1980.

Total WBC count was carried out by Haemocytometer method, differential count and haemoglobin level was estimated using cyanmet haemoglobin method,

by collecting blood at fifth day interval from the caudal vein into heparinised tubes. Bone marrow cellularity at the end of 20^{th} day was carried out by flushing the bone marrow cells from both femur, into phosphate buffer saline containing 2% bovine calf serum. The number of bone marrow cells were determined using a haemocytometer and expressed as total live cells of ($X10^6$) femur.

For histopathological study, the tissue of intestine were excised and permanent slides were prepared according to the standard methods. The results were at the end subjected to students test for statistical analysis of the data to determine the statistical significance between two values in the control and treated group.

Result and Discussion

The Present study reports for the first time the radioprotective activity of *C. copticum* seeds against γ- radiation induced damage. The administration of 70 % methanolic extract (50mg/kg body weight and 100mg/kg body weight) of *C. copticum* seeds significantly increased the Total WBC count and Haemoglobin levels compared with the control group (radiation alone). On the 20^{th} day the animals were sacrificed the liver, kidney, blood and bone marrow were taken. The liver kidney and blood samples were subjected to biochemical analysis which revealed a significant increase in antioxidant enzyme levels in these groups (*C. copticum* treated groups) compared to control group. The bone marrow cellularity were also checked in which it was found to be coming closer to the normal levels in extract treated groups, compared with the control group. The liver and kidney marker enzyme levels are also coming closer to the normal levels in treated groups of animals. The histopathological studies also revealed the cytoprotective activity of *C. copticum* seeds against Radiation induced toxicities.

The ability of ionizing radiations to kill cancer cells through the induction of cell damage makes this an important modality in the therapeutic approach against cancer in humans. But normal human tissues are not immune to the damaging effects of ionizing radiations. The degree of cell damage induced by radiations depends on numerous factors, including the radiation dose, its scheduled administration, the stage of the cell within the cell cycle, the levels of cellular antioxidant defense system and the availability of oxygen in the tissues (Weichselbaum *et al.*, 1997).

The interaction of ionizing radiation with biological system results in the generation of many highly reactive short-lived reactive oxygen species (ROS) mainly due to the hydrolysis of water. These ROS attack cellular macromolecules like DNA, RNA, proteins, membranes etc, causes its dysfunction and damage. ROS increased the lipid peroxidation which in turn can alter the integrity of membrane structure leading to inactivation of membrane bound enzymes, loss of permeability of the membrane and decrease in membrane fluidity. Whole body irradiation increased the levels of lipid peroxidation both in serum and tissue. *C.copticum* treated animals showed a very low level of lipid peroxide levels comparable to normal levels. The in vivo antioxidant enzyme levels such as SOD Catalase, GSH and GPx levels are also coming closer to the normal value in *C.copticum* treated groups. One of the most important side effects of ionizing radiation is myelosuppression. In extract treated groups of animals an increase in total count and bone marrow cellularity were also observed which indicate the protective effect of *C.copticum* on radiation induced damage.

C. copticum contains several active ingredients. Of these terpenes and polyphenols are important. Many of these compounds show excellent antioxidant properties and they are good inhibitors of lipid peroxidation.

Although the exact mechanism of action of *C. coptocum* is not clear, the combined action of above components makes it a good radio protector.

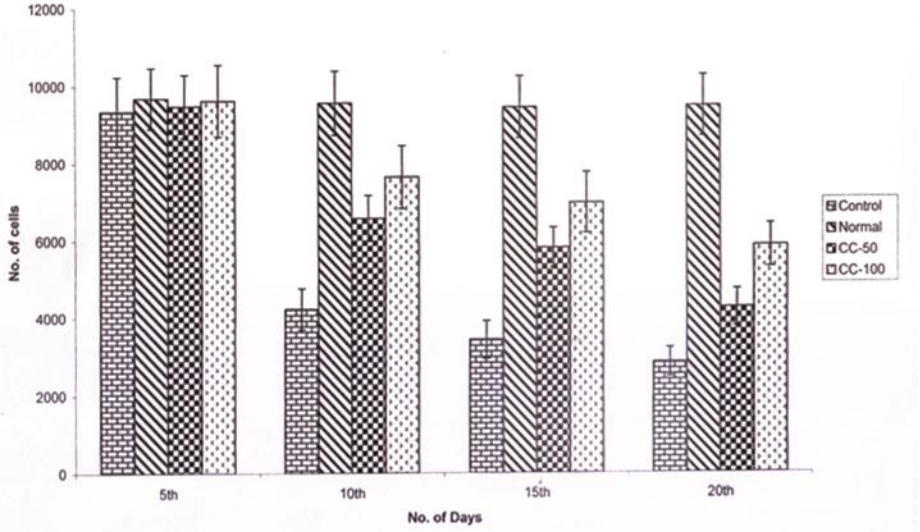

Figure 1: Effect of *C. copticum* on haematological parameters in gamma-radiation induced toxicity[WBC count (per µl)]

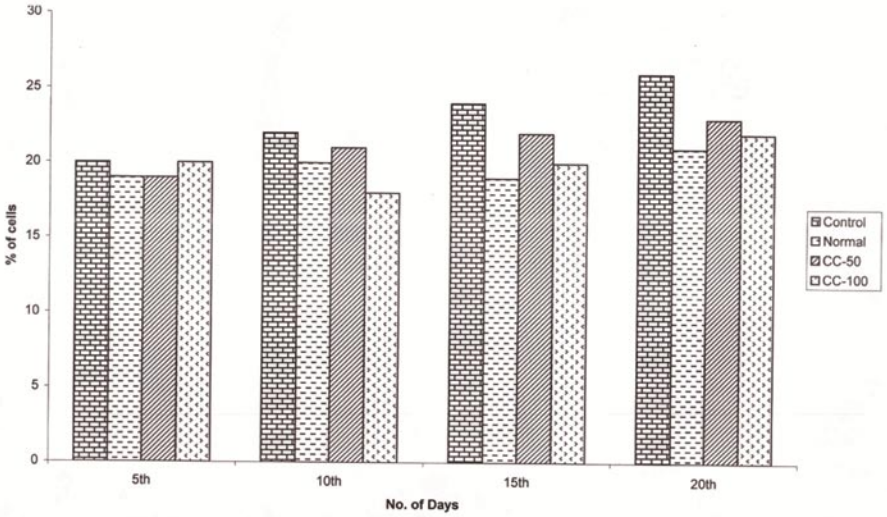

Figure 2: Effect of *C. copticum* on Gamma radiation induced toxicity-% of Polymorphonuclear lymphocytes

Table 1: Effect of *C. copticum* on blood antioxidant levels in Radiation treated mice.

	SOD U/mg Hb	CATALASE K/gm. Hb	GSH (nmol/ml)	GPx U/l of Haemolysate
Control	523.2±45.26	224.76±36.2	24.18±5.28	982.58±138.51
Normal	1352.46±106.73**	330.41±75.58	32.51±8.49*	2435.21±389.29**
CC50 mg/Kg	1026.35±156.48*	422.82±84.36*	29.53±6.89**	1324.54±98.72**
CC100 mg/Kg	1574.37±220.46*	629.57±92.81**	34.84±5.64**	1921.68±235.56

P<*0.05, **0.01. Values are mean ±S.D. of 6 animals in each group.

Table 2: Effect of *C. copticum* on Liver antioxidant levels in Radiation treated mice

	SOD U/mg protein	CAT U/mg protein	GSH nmol/mg	GPx nmol/mg
Control	281.25±82.51	183.76±32.55	8.41±1.83	28.31±6.56
Normal	392.54±55.28**	363.25±83.2*	13.25±3.52	39.84±7.25*
CC50 mg/Kg	582.63±102.95**	324.51±52.86**	12.31±2.34*	33.86±7.19**
CC100 mg/Kg	853.94±95.86**	582.47±72.64**	15.48±1.98**	42.61±8.84**

P<*0.05, **0.01. Values are mean ± S.D. of 6 animals in each group.

Table 3: Effect of *C. copticum* on Kidney antioxidant levels in Radiation treated mice

	SOD U/mg protein	CAT U/mg protein	GSH nmol/mg	GPx nmol/mg
Control	217.23±35.48	164.29±40.27	9.28±0.63	24.53±4.28
Normal	428.63±86.10*	489.61±56.20**	15.61±2.68	38.61±6.74*
CC50 mg/Kg	516.28±66.89**	325.47±62.5	11.31±1.19	29.82±3.64*
CC100 mg/Kg	796.82±113.64	427.34±81.35	16.82±1.28**	37.61±5.28

P<*0.05, **0.01. Values are mean ± S.D. of 6 animals in each group.

Table 4: Effect of *C. copticum* on Lipid peroxidation levels in Radiation treated mice

	SERUM nmol/ml	LIVER nmol/ml	KIDNEY nmol/ml
Control	4.28±0.82	5.56±0.63	4.64±0.58
Normal	0.86±0.07	1.04±0.12*	0.97±0.14
CC50 mg/Kg	2.53±0.28**	3.51±0.69	3.18±0.79
CC100 mg/Kg	1.35±0.21	1.81±0.35	1.52±0.81*

P<*0.05, **0.01. Values are mean ± S.D. of 6 animals in each group.

Table 5: Effect of *C. copticum* on Haemoglobin levels in Radiation treated mice

	5th Day	10th Day	15th Day	20th Day
Control	15.25±1.62	13.48±0.89	12.61±0.91	7.82±0.52
Normal	17.19±0.94**	18.21±0.78**	17.63±1.26*	17.94±0.61**
CC50 mg/Kg	16.48±0.96**	14.21±1.36	13.68±1.42	10.11±0.87*
CC100 mg/Kg	17.11±0.88**	15.86±1.21	14.14±0.82**	11.43±0.76**

P<*0.05, **0.01. Values are mean ± S.D. of 6 animals in each group

Table 6: Effect of *C. copticum* on Alkaline phosphatase and Creainine levels (serum) in Radiation treated mice

	Creatinine (mg/dl)	Alkaline phosphatase(U/L)
Control	0.96±0.12	92.47±8.51
Normal	0.64±0.1	38.1±5.61
CC50 mg/Kg	0.89±0.16	80.09±4.15**
CC100 mg/Kg	0.72±0.15	58.4±8.26

P<*0.05, **0.01. Values are mean ± S.D. of 6 animals in each group.

Table 7: Effect of *C. copticum* on bone marrow cellularity in Radiation treated mice

Control	Normal	CC50	CC100
5.28±0.4	13.42±0.51	7.21±0.64	8.58±0.62

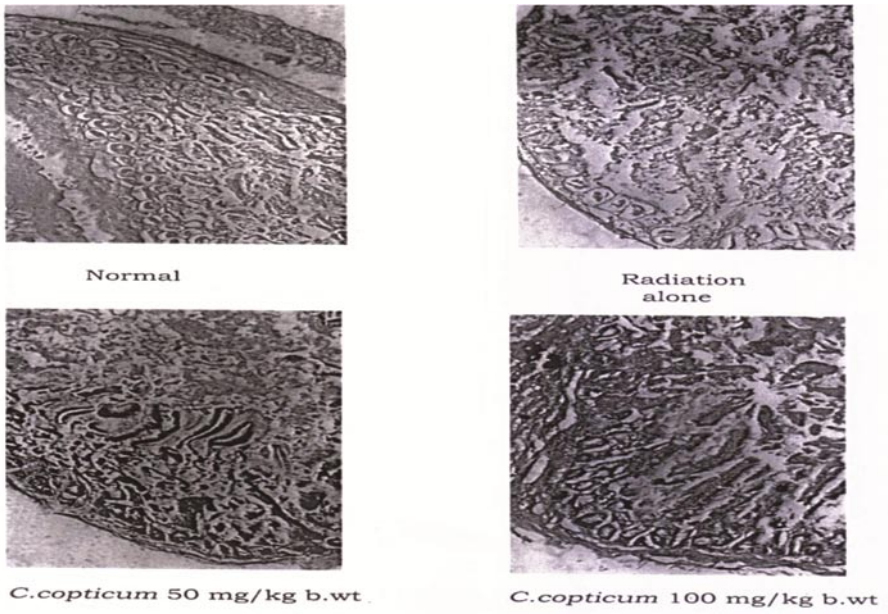

Figure 3: Inhibitory effect of *C.copticum* on gamma radiation induced intestinal damage

Conclusion

The present study reports for the first time the radioprotective activity of 70 % methanolic extract of *C. copticum* seeds γ-irradiation induced radio protective animal models. The administration of *C. copticum* seeds extract (50mg/k b.wt. and 100mg/kg b. wt.) significantly reduced the γ-radiation induced damage in a dose dependent manner. The phytochemical screening of the plant showed the presence of terpenes and polyphenols. So in the present study it can be concluded that the radioprotective activity of *C. copticum* is due to the presence of these compounds and its antioxidant effects.

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RELATIVE STUDY ON EVOLUTION OF DIFFERENT HAEMOGLOBIN CHAINS IN *HOMO SAPIENS*

Sinoy Johnson[1] and **Vincent Terrence Rebello**[2*]
[1]Final year BSc Zoology, St. Albert's College, Ernakulam.
E-mail: sinoyjohnson0022@gmail.com
[2]Associate Professor, St. Albert's College, Ernakulam-682 018, Kerala, S. India.

Abstract

In most vertebrates, haemoglobin (Hb), the iron-containing oxygen-transport metalloprotein, is a hetero-tetramer composed of two dissimilar globin chains, the alpha and beta chains. In addition, haemoglobin is found to exist in other forms by substituting these typical globin subunits with modified residues. In the present investigation, the peptide sequences of ten different globin chains present in human beings (*Homo sapiens*), namely, the Alpha, Beta, Gamma 1, Gamma 2, Delta, Epsilon, Mu, theta, Zeta and Myoglobin chains were considered for analysis. The multiple amino acid sequences obtained from NCBI and UniProt databases were analysed using the ALIGN tool to determine similar and variant regions. A phylogenetic tree was illustrated which depicts the relation among variants. The study reveals that the alpha and beta chains, being the most stable forms, have experienced divergent evolution to form the other six variants, which allow them to take up different functions producing other haemoglobin molecules. The ancestral haemoglobin chain has undergone functional divergence to form two broad groups of globin chains – the alpha and beta like chains. It can also be inferred that, these globins share a common ancestry with molecules like myoglobin. Advanced studies in this area may open possibilities in the diagnosis and treatment of haemoglobin associated diseases like Thalassemia and Sickle cell anaemia at the molecular level. Furthermore, the sequence of ancestral globin could be deduced by the method of maximum likelihood. This creates the possibility of resurrecting the ancestral globin molecule by molecular modelling, spreading the exposure for studies on evolutionary past.

Key words: *Homo sapiens*, Haemoglobin, Globin subunits, divergent evolution, NCBI, UniProt, ALIGN, Phylogenetic tree.

* **Corresponding author:**Associate Professor, St. Albert's College, Ernakulam-682 018, Kerala, S. India.Correspondence e-mail: drterencerebello2012@gmail.com

Introduction

Evolution can be defined as any change in the heritable characteristics of biological populations over successive generations. Evolutionary processes give rise to biodiversity at every level of biological organisation, including the levels of species, individual organisms, and molecules. The process of evolution at the molecular levels such as in nucleic acids, proteins etc. leads to molecular evolution.

The field of molecular evolution uses principles of evolutionary biology and population genetics to explain patterns in the changes taking place. Molecular evolution techniques can be very helpful in tracing the phylogenetic relationship among organisms. The branch of phylogeny that analyses hereditary molecular differences, mainly in DNA and protein sequences, to gain information on an organism's evolutionary relationships is called as molecular phylogenetics. To trace phylogenetic relationship among organisms, molecular comparisons can be carried out at the level of DNA, whole genome sequence and amino acid sequences in individual proteins. Differences in amino acid sequences in individual proteins provide information about the evolution of individual genes and organisms. Not all regions within a protein mutate at the same rate; functionally important areas mutate more slowly and amino acid substitutions involving similar amino acids occur more often than dissimilar substitutions (Jeffrey, 2000).

Protein or gene sequences are called homologous if they share a common ancestry. Homology among proteins or DNA is typically inferred from their sequence similarity. A significant similarity in the sequences is strong evidence that two sequences are related by divergent evolution from a common ancestor. Alignments of multiple sequences are used to indicate the homologous regions in each sequence. (Russel, 2010).

Haemoglobin is the iron-containing oxygen-transport metalloprotein in the red blood cells of all vertebrates as well as the tissues of some invertebrates. Haemoglobin in the blood carries oxygen from the respiratory organs (lungs or gills) to the rest of the body (i.e. the tissues).

Within a species, different variants of haemoglobin always exist, although one sequence is usually a "most common". Mutations in the genes for the haemoglobin protein in a species result in haemoglobin variants. Many of these mutant forms of haemoglobin cause no disease. Some of these mutant forms of haemoglobin, however, can cause a group of hereditary diseases, a common example of this being Sickle cell anaemia.

Haemoglobin consists of protein subunits (the "globin" molecules), and these proteins, in turn, are folded chains of a large number of different amino acids called polypeptides. The amino acid sequence of any polypeptide created by a cell is in turn determined by the respective genes. It is the amino acid sequence that determines the protein's chemical properties and function. In most vertebrates, the haemoglobin molecule is an assembly of four globular protein subunits.

In adult humans, the most common haemoglobin type is a tetramer called haemoglobin A (HbA), consisting of two α and two β subunits non-covalently bound, each made of 141 and 147 amino acid residues, respectively. This is denoted as $\alpha_2\beta_2$. The subunits are structurally similar and about the same size. Haemoglobin A is the most intensively studied of the haemoglobin molecules. These four polypeptide chains are bound to each other by salt bridges, hydrogen bonds and hydrophobic effect. (Ross, 1999)

Phylogenetic studies suggest that the ancestral chain of haemoglobin is related to the myoglobin chain, indicating a common ancestry. Scientists agree that the event that separated myoglobin from haemoglobin occurred after

Lampreys diverged from jawed vertebrates (Veer Bala Rastogi, 2014). This separation of myoglobin and haemoglobin allowed the two molecules to arise and develop differently; myoglobin has more to do with oxygen storage while haemoglobin is tasked with oxygen transport. The development of α and β chains created the potential for haemoglobin's ability to transport oxygen. Having multiple subunits contributes to haemoglobin's ability to bind oxygen cooperatively as well as be regulated allosterically. Subsequently, the alpha gene and beta genes also underwent a duplication event to form various forms. These further duplications and divergences have created a diverse range of α- and β-like globin chains that are regulated so that certain forms occur at different stages of development.The various globin chains found in human beings and their respective roles are as follows:

1. Alpha chain (HBA)
    - Involved in oxygen transport from the lung to the various peripheral tissues.
    - Forms the haemoglobin tetramer structure along with two beta chains
2. Beta chain (HBB)
    - Involved in oxygen transport from the lung to the various peripheral tissues.
    - Forms the haemoglobin tetramer structure along with two alpha chains
3. Gamma chains (HBG1 & HBG2)
    - Found in two different forms viz. Gamma 1 and Gamma 2
    - In human infants, the haemoglobin molecule is made up of 2 α chains and 2 γ chains.
    - The gamma chains are gradually replaced by β chains as the infant grows

4. Delta chain (HBD)
    - Two alpha chains plus two delta chains constitute HbA2
    - HbA2 is found at low levels in normal human blood.
    - The HbA2 levels may be increased in situations like beta thalassemia and sickle cell anaemia
5. Epsilon (HBE)
    - Two epsilon chains together with two zeta chains (an alpha-like globin) constitute the embryonic haemoglobin Hb Gower I
    - Two epsilon chains together with two alpha chains form the embryonic haemoglobin Hb Gower II
    - Both of these embryonic haemoglobins are normally replaced by foetal, and later by adult haemoglobin
6. Mu chain (HBM)
    - Alpha like globin
    - This protein has not been detected yet.
7. Theta chain (HBAT)
    - Alpha like globin
    - Found in human foetal erythroid tissue
8. Zeta chain (HBAZ)
    - Alpha like globin
    - Synthesized in the yolk sac of the early embryo
9. Myoglobin (MYG)
    - Serves as a reserve supply of oxygen and facilitates the movement of oxygen within muscles.

Materials and Methods

Present day technologies of molecular biology have facilitated to sequence various proteins and nucleic acids. The sequence of amino acids that codes for various proteins has been stored in databanks. Databanks in molecular biology contain nucleic acid and protein sequences, macromolecular structure and functions, expression patterns and networks of metabolic pathways. The archive of amino acid sequences of proteins, now determined almost exclusively from translation of gene sequences, is maintained by *The United Protein Database (UniProt),* a merger of the databases *SWISS-PROT*, *The Protein Identification Resource (PIR)* and *Translated EMBL (TrEMBL)*. Searching in databases for homologues of known proteins is a central theme of bioinformatics. For this, Scientists in the field of bioinformatics have developed technological tools like BLAST and ALIGN easing the study of molecular phylogeny (David, 2001).

A sequence alignment is a way of arranging the sequences of DNA, RNA, or protein to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships between the sequences. Aligned sequences of nucleotide or amino acid residues are typically represented as rows within a matrix. Gaps are inserted between the residues so that identical or similar characters are aligned in successive columns (Arthur, 2002).

The basic principle behind these studies and development of such comparison tools is that the origin of similarity is common ancestry. They can be used to derive a conclusion about the evolutionary patterns. The results of phylogenetic studies are usually presented in the form of an evolutionary tree. Such a tree illustrates all the descendants of a single original ancestor. These trees are often based on inferences from the patterns of similarity. We generally assume that the more similar the characters, the more closely related the species are (Paul, 2005).

In the present study, the amino acid sequences of various globin chains were obtained from the UniProt database. The different forms of globin chains present in Human beings (*Homo sapiens*) are the Alpha, Beta, Gamma 1, Gamma 2, Delta, Epsilon, Mu, theta and Zeta chains. In addition to these chains, the oxygen storing myoglobin was also considered to infer about their phylogenetic relation with haemoglobin molecule and its ancestor.

The various polypeptide sequences in FASTA format are as follows:

1. Alpha chain (HBA) : >sp|P69905|HBA_HUMAN Haemoglobin subunit alpha OS=Homo sapiens GN=HBA1 PE=1 SV=2
   MVLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKT
   YFPHFDLSHGSAQVKGHGKKVADALTNAVAHVDDMPNALSAL
   SDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTPAVHASLDK
   FLASVSTVLTSKYR

2. Beta chain (HBB) : >sp|P68871|HBB_HUMAN Haemoglobin subunit beta OS=Homo sapiens GN=HBB PE=1 SV=2
   MVHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRF
   FESFGDLSTPDAVMGNPKVKAHGKKVLGAFSDGLAHLDNLKGT
   FATLSELHCDKLHVDPENFRLLGNVLVCVLAHHFGKEFTPPVQA
   AYQKVVAGVANALAHKYH

3. Gamma 1 chain (HBG1) : >sp|P69891|HBG1_HUMAN Haemoglobin subunit gamma-1 OS=Homo sapiens GN=HBG1 PE=1 SV=2
   MGHFTEEDKATITSLWGKVNVEDAGGETLGRLLVVYPWTQRFF
   DSFGNLSSASAIMGNPKVKAHGKKVLTSLGDAIKHLDDLKGTFA
   QLSELHCDKLHVDPENFKLLGNVLVTVLAIHFGKEFTPEVQASW
   QKMVTAVASALSSRYH

4. Gamma 2 chain (HBG2) : >sp|P69892|HBG2_HUMAN Haemoglobin subunit gamma-2 OS=Homo sapiens GN=HBG2 PE=1 SV=2
   MGHFTEEDKATITSLWGKVNVEDAGGETLGRLLVVYPWTQRFF
   DSFGNLSSASAIMGNPKVKAHGKKVLTSLGDAIKHLDDLKGTFA
   QLSELHCDKLHVDPENFKLLGNVLVTVLAIHFGKEFTPEVQASW
   QKMVTGVASALSSRYH

5. Delta chain (HBD) : >sp|P02042|HBD_HUMAN Haemoglobin subunit delta OS=Homo sapiens GN=HBD PE=1 SV=2
   MVHLTPEEKTAVNALWGKVNVDAVGGEALGRLLVVYPWTQRF
   FESFGDLSSPDAVMGNPKVKAHGKKVLGAFSDGLAHLDNLKGT
   FSQLSELHCDKLHVDPENFRLLGNVLVCVLARNFGKEFTPQMQ
   AAYQKVVAGVANALAHKYH

6. Epsilon chain (HBE) : >sp|P02100|HBE_HUMAN Haemoglobin subunit epsilon OS=Homo sapiens GN=HBE1 PE=1 SV=2
   MVHFTAEEKAAVTSLWSKMNVEEAGGEALGRLLVVYPWTQRF
   FDSFGNLSSPSAILGNPKVKAHGKKVLTSFGDAIKNMDNLKPAF
   AKLSELHCDKLHVDPENFKLLGNVMVIILATHFGKEFTPEVQAA
   WQKLVSAVAIALAHKYH

7. Mu chain (HBM) : >sp|Q6B0K9|HBM_HUMAN Haemoglobin subunit mu OS=Homo sapiens GN=HBM PE=2 SV=1
   MLSAQERAQIAQVWDLIAGHEAQFGAELLLRLFTVYPSTKVYFP
   HLSACQDATQLLSHGQRMLAAVGAAVQHVDNLRAALSPLADL
   HALVLRVDPANFPLLIQCFHVVLASHLQDEFTVQMQAAWDKFL
   TGVAVVLTEKYR

8. Theta chain (HBAT) : >sp|P09105|HBAT_HUMAN Haemoglobin subunit theta-1 OS=Homo sapiens GN=HBQ1 PE=1 SV=2
   MALSAEDRALVRALWKKLGSNVGVYTTEALERTFLAFPATKTY
   FSHLDLSPGSSQVRAHGQKVADALSLAVERLDDLPHALSALSHL
   HACQLRVDPASFQLLGHCLLVTLARHYPGDFSPALQASLDKFLS
   HVISALVSEYR

9. Zeta chain (HBAZ) : >sp|P02008|HBAZ_HUMAN Haemoglobin subunit zeta OS=Homo sapiens GN=HBZ PE=1 SV=2
   MSLTKTERTIIVSMWAKISTQADTIGTETLERLFLSHPQTKTYFPH
   FDLHPGSAQLRAHGSKVVAAVGDAVKSIDDIGGALSKLSELHAY
   ILRVDPVNFKLLSHCLLVTLAARFPADFTAEAHAAWDKFLSVVS
   SVLTEKYR

10. Myoglobin (MYG) : >sp|P02144|MYG_HUMAN Myoglobin OS=Homo sapiens GN=MB PE=1 SV=2
    MGLSDGEWQLVLNVWGKVEADIPGHGQEVLIRLFKGHPETLEK
    FDKFKHLKSEDEMKASEDLKKHGATVLTALGGILKKKGHHEAE
    IKPLAQSHATKHKIPVKYLEFISECIIQVLQSKHPGDFGADAQGA
    MNKALELFRKDMASNYKELGFQG

The required multiple sequences were selected from the search results and aligned using the ALIGN (FASTA) tool. In sequence comparison, the multiple sequences are aligned parallel, one below the other, in such a way that that their similarity and differences are visible. However, the alignment is improved by placing a gap, by inserting the symbol of hyphen '-' and realigning the residues, such that the identical residues are placed in the same column. The alphabets that are same indicate common origin and thereby indicate that these positions have been conserved during the course of evolution. The variation in the

residues indicates that the two might be derived from an ancestral residue and the variation is deemed to be due to random mutation that occurs during replication of DNA. If many of the alphabets are same along the length of the sequence, then it is presumed that they have a common evolutionary origin. A phylogenetic tree was obtained centred on the identity values of aligned sequences.

The chains were analysed to evaluate the number of sites with variant and similar residues. The identity values obtained are tabulated for each alignment. These results were related with the functions of different globin in human beings to investigate how the globin chains have undergone functional divergence.

Result

The outcomes portrays that the various chains show wavering degree of similarity and variations. Moreover, the results disclose that all the chains have a common ancestor and that it is similar to the oxygen storing globin in human beings, the myoglobin. In the course of evolution the ancestral haemoglobin has mainly branched into two lineages. On the basis of the analysis, the phylogenetic tree has been depicted as follows:

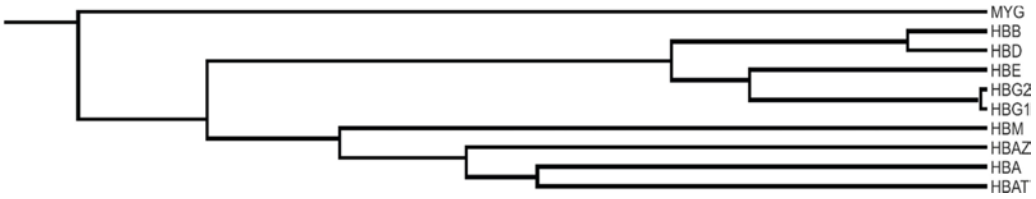

Figure 1: Phylogenetic tree

In view of the observations from the phylogenetic tree, the proximal sequences were aligned independently and progressively to analyse how they differ, the similar and dissimilar sites and the identity value was calculated to

portray the percentage of similarity. The alignment of various chains is as follows:

1. The various haemoglobin chains and myoglobin – Fig. 2
2. The Beta and Delta chains – Fig. 3
3. The Beta and Gamma 1, 2 chains – Fig. 4
4. The Beta and Epsilon chains – Fig. 5
5. The Beta, Gamma, Delta, Epsilon chains – Fig. 6
6. The Alpha and Zeta chains – Fig. 7
7. The Alpha and Theta chains – Fig. 8
8. The Alpha and Mu chains – Fig. 9
9. The Alpha, Mu, Theta and Zeta chains – Fig. 10
10. The Alpha, Beta, Gamma 1& 2, Delta, Epsilon, Mu, Theta and Zeta chains – Fig. 11

The results of the alignment are tabulated (Table 1) for easy comparison. The alignment depicts varying degree of similarity among the globin sequences which can be inferred from their respective identity values.

Discussion

The identity values of various chain comparisons describe the relationship among the descendants. Comparing the proximal chains and the respective functions of the descendants, it can clearly be concluded that the chains show similarity in accordance with their functions.

The normal haemoglobin is found to be a tetramer of the alpha and beta chains. Being common, it is inferred that the most stable forms among the various globin chains in human beings are the alpha and beta chains.

In human infants, the haemoglobin molecule (HbF) is made up of two alpha chains and two gamma chains. Thus, in foetal haemoglobin molecule, the

role of beta chains is taken up by the gamma chains. The alignment results of the beta and gamma chains make up an identity value of 72.79 % (Table 1).

The embryonic haemoglobin molecule of humans exists in two different forms *viz.* the HBE Gower - 1 and HBE Gower - 2. The HBE Gower 1 molecule is the primary embryonic haemoglobin molecule and is made up of two zeta chains (alpha like chains) and two epsilon chains. It is relatively unstable. The HBE Gower 2 molecule is made up of two alpha chains and two epsilon chains. It is relatively stable compared to the HBE Gower 1. Thus, in comparison with the normal haemoglobin molecule, the role of beta chains is taken up by the epsilon chains in the embryonic haemoglobin molecule. Both of these embryonic haemoglobins are normally replaced by foetal and later by adult haemoglobin. These chains, on alignment with the beta chain, show an identity value of 75.51 % (Table 1).

Another form of haemoglobin found in human being is the HbA2, which is made up of two alpha chains and two delta chains. This type of haemoglobin is found at low levels in normal human blood and their levels may be increased in conditions like Thalassemia, Sickle cell anaemia. Hence, in this form, the role of beta chains is carried out by the delta chains. The alignment results of the beta and delta chains make up an identity value of 93.20 % (Table 1).

Thus the above mentioned chains, the gamma1, gamma 2, delta and epsilon chains act as a substituent for the beta chains under different conditions and hence fall under the same group. These chains show an identity value of 63.95 % (Table 1). The phylogenetic tree illustrate that these chains share a common ancestry. Hence, it can be inferred that these beta like chains have been descended from the ancestral sequence to take up similar structure and function.

The alpha chain is the other globin subunit present in normal haemoglobin molecule. The other alpha like forms recognized in human beings are theta, zeta and Mu chains. In embryonic haemoglobin (HBE Gower 1), the alpha chains are substituted by the zeta chains. The alignment results of alpha and zeta chains make up an identity value of 59.86 % (Table 1).

Theta chains are found as a substituent for alpha chains in human foetal erythroid tissue, giving an identity value of 61.97 % (Table 1). Also, the Mu chain, whose protein has not yet been discovered, is also an alpha like chain found in human beings. It shows an identity value of 45.07 % (Table 1) with the alpha chain.

Hence, these identity values express that the theta, Mu and zeta chains act as the substituent for the alpha chains. It is also clear from the phylogenetic tree that these chains share a common ancestry. Therefore these chains, with an identity value of 33.80 % (Table 1), belong to same group. Thus the alpha like chains have been formed from the ancestral sequence to perform similar functions.

Also, the phylogenetic tree analysis unveils that the ancestral chain of haemoglobin is similar to the myoglobin molecule in human beings. The haemoglobin and myoglobin molecules have descended from a common ancestor a long time ago. This is indicated by the primary branching in the phylogenetic tree. Experiments in the past have already established that the event that separated myoglobin from haemoglobin occurred after Lampreys diverged from jawed vertebrates. Thus, the results obtained are in accordance with the earlier theories (Veer Bala Rastogi, 2014).

Therefore the investigation indicates that the ancestral haemoglobin shares a common origin with the myoglobin molecule. The haemoglobin and myoglobin separated when a species diverged to form two different species.

Hence they are said to be orthologous chains. The ancestral sequence of the haemoglobin molecule has diverged particularly into two broad divisions – the alpha like and the beta like chains. The first group consists of the Alpha, Mu, Theta and Zeta chains. Of these, the Alpha chain is the most stable and common. The other three descendants have evolved to form the alpha like globin molecules in structure and functions. The second group consists of the Beta, Gamma 1, Gamma 2, Delta and Epsilon chains with the Beta chain being the most stable and common. The other chains have descended to form Beta like globin molecules in structure as well as functions. One member, each from the two groups, together constitutes the tetramer structure of various haemoglobin molecules observed in Human beings. The alpha and beta chains, along with their descendants are said to homologous, as they share a common ancestry.

Conclusion and Suggestions

The present investigation explains how the haemoglobin molecule underwent evolution to form different globin chains. The ancestral chain diverged to form two broad groups of alpha like and beta like chains. The members of the two groups form different tetramers of haemoglobin molecule in various stages of human beings. Advanced studies in this may create possibilities of treatment of haemoglobin associated diseases like Sickle cell anaemia and Thalassemia at the molecular level. Also, the ancestral sequence of haemoglobin can be deduced by analysing the chains by maximum likelihood method. This sequence with the desired ancestral characters could be resynthesized by molecular modelling.

Acknowledgement

We would like to express our deepest gratitude for providing a chance to submit this work. Thanks to Almighty for its successful completion. Most importantly we would also like to thank our colleagues who have provided us their support and encouragement throughout this work.

Tables and Figures

The various values and sequence alignments were obtained from the online database of UniProt (http://www.uniprot.org). The alignment of multiple sequence is prepared using the available ALIGN tool.

Table 1: Alignment results of different chains

Chains	Length	Identical positions	Similar positions	Identity
Gamma 1 Gamma 2	147	146	1	99.32 %
Beta Delta	147	137	7	93.20 %
Beta Gamma 1 & 2	147	107	30	72.79 %
Beta Epsilon	147	111	28	75.51 %
Beta, Gamma 1, Gamma 2, Epsilon, Delta	147	94	40	63.95 %
Alpha Zeta	142	85	36	59.86 %
Alpha Theta	142	88	37	61.97 %
Alpha Mu	142 141	64	47	45.07 %
Alpha, Theta, Zeta Mu	142 141	48	42	33.80 %
Beta, Gamma, Delta, Epsilon Theta, Zeta, Alpha Mu	147 142 141	28	49	18.79 %
Beta, Gamma, Delta, Epsilon Theta, Zeta, Alpha Mu Myoglobin	147 142 141 154	15	38	9.68 %

An alignment will display the following symbols denoting the degree of conservation observed in each column:

An * (asterisk) indicates positions which have a single, fully conserved residue.

A : (colon) indicates conservation between groups of strongly similar properties

A . (period) indicates conservation between groups of weakly similar properties

```
P68871 HBB_HUMAN       1   MVHLTPEEKSAVTALWGKVN--VDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDAVMGN     58
P69905 HBA_HUMAN       1   -MVLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFD------LSHGS     53
P69892 HBG2_HUMAN      1   MGHFTEEDKATITSLWGKVN--VEDAGGETLGRLLVVYPWTQRFFDSFGNLSSASAIMGN     58
P02042 HBD_HUMAN       1   MVHLTPEEKTAVNALWGKVN--VDAVGGEALGRLLVVYPWTQRFFESFGDLSSPDAVMGN     58
P69891 HBG1_HUMAN      1   MGHFTEEDKATITSLWGKVN--VEDAGGETLGRLLVVYPWTQRFFDSFGNLSSASAIMGN     58
P02008 HBAZ_HUMAN      1   -MSLTKTERTIIVSMWAKISTQADTIGTETLERLFLSHPQTKTYFPHFD------LHPGS     53
P02100 HBE_HUMAN       1   MVHFTAEEKAAVTSLWSKMN--VEEAGGEALGRLLVVYPWTQRFFDSFGNLSSPSAILGN     58
P09105 HBAT_HUMAN      1   -MALSAEDRALVRALWKKLGSNVGVYTTEALERTFLAFPATKTYFSHLD------LSPGS     53
Q6B0K9 HBM_HUMAN       1   --MLSAQERAQIAQVWDLIAGHEAQFGAELLLRLFTVYPSTKVYFPHLS------ACQDA     52
P02144 MYG_HUMAN       1   -MGLSDGEWQLVLNVWGKVEADIPGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKAS     59
                             ::  :   :   *  :        * * * :  .* *   *  :

P68871 HBB_HUMAN      59   PKVKAHGKKVLGAFSDGLAHLDNLKGTFATLSELHCDKLHVDPENFRLLGNVLVCVLAHH    118
P69905 HBA_HUMAN      54   AQVKGHGKKVADALTNAVAHVDDMPNALSALSDLHAHKLRVDPVNFKLLSHCLLVTLAAH    113
P69892 HBG2_HUMAN     59   PKVKAHGKKVLTSLGDAIKHLDDLKGTFAQLSELHCDKLHVDPENFKLLGNVLVTVLAIH    118
P02042 HBD_HUMAN      59   PKVKAHGKKVLGAFSDGLAHLDNLKGTFSQLSELHCDKLHVDPENFRLLGNVLVCVLARN    118
P69891 HBG1_HUMAN     59   PKVKAHGKKVLTSLGDAIKHLDDLKGTFAQLSELHCDKLHVDPENFKLLGNVLVTVLAIH    118
P02008 HBAZ_HUMAN     54   AQLRAHGSKVVAAVGDAVKSIDDIGGALSKLSELHAYILRVDPVNFKLLSHCLLVTLAAR    113
P02100 HBE_HUMAN      59   PKVKAHGKKVLTSFGDAIKNMDNLKPAFAKLSELHCDKLHVDPENFKLLGNVMVIILATH    118
P09105 HBAT_HUMAN     54   SQVRAHGQKVADALSLAVERLDDLPHALSALSHLHACQLRVDPASFQLLGHCLLVTLARH    113
Q6B0K9 HBM_HUMAN      53   TQLLSHGQRMLAAVGAAVQHVDNLRAALSPLADLHALVLRVDPANFPLLIQCFHVVLASH    112
P02144 MYG_HUMAN      60   EDLKKHGATVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLEFISECIIQVLQSK    119
                            .:  **  :  :.   :   ..    :  *:. *.   ::    : :: . :   *  .

P68871 HBB_HUMAN     119   FGKEFTPPVQAAYQKVVAGVANALAHKYH------                            147
P69905 HBA_HUMAN     114   LPAEFTPAVHASLDKFLASVSTVLTSKYR------                            142
P69892 HBG2_HUMAN    119   FGKEFTPEVQASWQKMVTGVASALSSRYH------                            147
P02042 HBD_HUMAN     119   FGKEFTPQMQAAYQKVVAGVANALAHKYH------                            147
P69891 HBG1_HUMAN    119   FGKEFTPEVQASWQKMVTAVASALSSRYH------                            147
P02008 HBAZ_HUMAN    114   FPADFTAEAHAAWDKFLSVVSSVLTEKYR------                            142
P02100 HBE_HUMAN     119   FGKEFTPEVQAAWQKLVSAVAIALAHKYH------                            147
P09105 HBAT_HUMAN    114   YPGDFSPALQASLDKFLSHVISALVSEYR------                            142
Q6B0K9 HBM_HUMAN     113   LQDEFTVQMQAAWDKFLTGVAVVLTEKYR------                            141
P02144 MYG_HUMAN     120   HPGDFGADAQGAMNKALELFRKDMASNYKELGFQG                            154
                             :*    :.: :* :  .   :  .*:
```

Figure 2: Alignment of various haemoglobin chains and Myoglobin

```
P68871 HBB_HUMAN      1   MVHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDAVMGNPK    60
P02042 HBD_HUMAN      1   MVHLTPEEKTAVNALWGKVNVDAVGGEALGRLLVVYPWTQRFFESFGDLSSPDAVMGNPK    60
                          *********:**.********* **************************:*********

P68871 HBB_HUMAN     61   VKAHGKKVLGAFSDGLAHLDNLKGTFATLSELHCDKLHVDPENFRLLGNVLVCVLAHHFG    120
P02042 HBD_HUMAN     61   VKAHGKKVLGAFSDGLAHLDNLKGTFSQLSELHCDKLHVDPENFRLLGNVLVCVLARNFG    120
                          *************************: ****************************::**

P68871 HBB_HUMAN    121   KEFTPPVQAAYQKVVAGVANALAHKYH                                147
P02042 HBD_HUMAN    121   KEFTPQMQAAYQKVVAGVANALAHKYH                                147
                          ***** :********************
```

Figure 3: Alignment of Beta and Delta chains

```
P68871 HBB_HUMAN      1   MVHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDAVMGNPK    60
P69892 HBG2_HUMAN     1   MGHFTEEDKATITSLWGKVNVEDAGGETLGRLLVVYPWTQRFFDSFGNLSSASAIMGNPK    60
P69891 HBG1_HUMAN     1   MGHFTEEDKATITSLWGKVNVEDAGGETLGRLLVVYPWTQRFFDSFGNLSSASAIMGNPK    60
                          * *:* *:*:::*:*******::.***:***************:***:**: .*:*****

P68871 HBB_HUMAN     61   VKAHGKKVLGAFSDGLAHLDNLKGTFATLSELHCDKLHVDPENFRLLGNVLVCVLAHHFG    120
P69892 HBG2_HUMAN    61   VKAHGKKVLTSLGDAIKHLDDLKGTFAQLSELHCDKLHVDPENFKLLGNVLVTVLAIHFG    120
P69891 HBG1_HUMAN    61   VKAHGKKVLTSLGDAIKHLDDLKGTFAQLSELHCDKLHVDPENFKLLGNVLVTVLAIHFG    120
                          ********* ::.*.: ***:****** ****************:******* *** ***

P68871 HBB_HUMAN    121   KEFTPPVQAAYQKVVAGVANALAHKYH                                147
P69892 HBG2_HUMAN   121   KEFTPEVQASWQKMVTGVASALSSRYH                                147
P69891 HBG1_HUMAN   121   KEFTPEVQASWQKMVTAVASALSSRYH                                147
                          ***** ***::**:*:.**.**: :**
```

Figure 4: Alignment of Beta and Gamma chains

```
P68871 HBB_HUMAN      1   MVHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDAVMGNPK    60
P02100 HBE_HUMAN      1   MVHFTAEEKAAVTSLWSKMNVEEAGGEALGRLLVVYPWTQRFFDSFGNLSSPSAILGNPK    60
                          ***:* ***:***:**.*:**:*.******************:***:**:*.*::****

P68871 HBB_HUMAN     61   VKAHGKKVLGAFSDGLAHLDNLKGTFATLSELHCDKLHVDPENFRLLGNVLVCVLAHHFG    120
P02100 HBE_HUMAN     61   VKAHGKKVLTSFGDAIKNMDNLKPAFAKLSELHCDKLHVDPENFKLLGNVMVIILATHFG    120
                          ********* :*.*.: ::**** :**.****************:*****:* :** ***

P68871 HBB_HUMAN    121   KEFTPPVQAAYQKVVAGVANALAHKYH                                147
P02100 HBE_HUMAN    121   KEFTPEVQAAWQKLVSAVAIALAHKYH                                147
                          ***** ****:**:*:.** *******
```

Figure 5: Alignment of Beta and Epsilon chains

```
P68871 HBB_HUMAN      1   MVHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDAVMGNPK    60
P69892 HBG2_HUMAN     1   MGHFTEEDKATITSLWGKVNVEDAGGETLGRLLVVYPWTQRFFDSFGNLSSASAIMGNPK    60
P02042 HBD_HUMAN      1   MVHLTPEEKTAVNALWGKVNVDAVGGEALGRLLVVYPWTQRFFESFGDLSSPDAVMGNPK    60
P69891 HBG1_HUMAN     1   MGHFTEEDKATITSLWGKVNVEDAGGETLGRLLVVYPWTQRFFDSFGNLSSASAIMGNPK    60
P02100 HBE_HUMAN      1   MVHFTAEEKAAVTSLWSKMNVEEAGGEALGRLLVVYPWTQRFFDSFGNLSSPSAILGNPK    60
                          * *:* *:*:::.:**.*:**: .***:***************:***:**: .*::****

P68871 HBB_HUMAN     61   VKAHGKKVLGAFSDGLAHLDNLKGTFATLSELHCDKLHVDPENFRLLGNVLVCVLAHHFG    120
P69892 HBG2_HUMAN    61   VKAHGKKVLTSLGDAIKHLDDLKGTFAQLSELHCDKLHVDPENFKLLGNVLVTVLAIHFG    120
P02042 HBD_HUMAN     61   VKAHGKKVLGAFSDGLAHLDNLKGTFSQLSELHCDKLHVDPENFRLLGNVLVCVLARNFG    120
P69891 HBG1_HUMAN    61   VKAHGKKVLTSLGDAIKHLDDLKGTFAQLSELHCDKLHVDPENFKLLGNVLVTVLAIHFG    120
P02100 HBE_HUMAN     61   VKAHGKKVLTSFGDAIKNMDNLKPAFAKLSELHCDKLHVDPENFKLLGNVMVIILATHFG    120
                          ********* ::.*.: ::*:** :*: ***************:*****:* :** :**

P68871 HBB_HUMAN    121   KEFTPPVQAAYQKVVAGVANALAHKYH                                147
P69892 HBG2_HUMAN   121   KEFTPEVQASWQKMVTGVASALSSRYH                                147
P02042 HBD_HUMAN    121   KEFTPQMQAAYQKVVAGVANALAHKYH                                147
P69891 HBG1_HUMAN   121   KEFTPEVQASWQKMVTAVASALSSRYH                                147
P02100 HBE_HUMAN    121   KEFTPEVQAAWQKLVSAVAIALAHKYH                                147
                          ***** :**::**:*:.** **: :**
```

Figure 6: Alignment of Beta like chains

```
P69905 HBA_HUMAN      1   MVLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG   60
P02008 HBAZ_HUMAN     1   MSLTKTERTIIVSMWAKISTQADTIGTETLERLFLSHPQTKTYFPHFDLHPGSAQLRAHG   60
                          * *: :::* : : *.*:.::*.  *:*:***:***.* **********  ****::.**

P69905 HBA_HUMAN     61   KKVADALTNAVAHVDDMPNALSALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTP  120
P02008 HBAZ_HUMAN    61   SKVVAAVGDAVKSIDDIGGALSKLSELHAYILRVDPVNFKLLSHCLLVTLAARFPADFTA  120
                          .**. *: :**  :**: .*** **:***: ********************::**:**

P69905 HBA_HUMAN    121   AVHASLDKFLASVSTVLTSKYR                                      142
P02008 HBAZ_HUMAN  121   EAHAAWDKFLSVVSSVLTEKYR                                      142
                           .**: ****: **:***.***
```

Figure 7: Alignment of Alpha and Zeta chains

```
P69905 HBA_HUMAN      1   MVLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG   60
P09105 HBAT_HUMAN     1   MALSAEDRALVRALWKKLGSNVGVYTTEALERTFLAFPATKTYFSHLDLSPGSSQVRAHG   60
                          *.**  *:: *:* * *:*::.* * :***** **:**:***** *:*** **:**:.**

P69905 HBA_HUMAN     61   KKVADALTNAVAHVDDMPNALSALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTP  120
P09105 HBAT_HUMAN    61   QKVADALSLAVERLDDLPHALSALSHLHACQLRVDPASFQLLGHCLLVTLARHYPGDFSP  120
                          :******: ** ::**:*:******.*** :*****..*:**.******** * *.:*:*

P69905 HBA_HUMAN    121   AVHASLDKFLASVSTVLTSKYR                                      142
P09105 HBAT_HUMAN  121   ALQASLDKFLSHVISALVSEYR                                      142
                          *::*******: * :.*.*:**
```

Figure 8: Alignment of Alpha and Theta chains

```
P69905 HBA_HUMAN      1   MVLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG   60
Q6B0K9 HBM_HUMAN      1   -MLSAQERAQIAQVWDLIAGHEAQFGAELLLRLFTVYPSTKVYFPHLSACQDATQLLSHG   59
                           :**  :::::  .*. :..* .::*** * *:*  :*:**.****:. .:.::*: .**

P69905 HBA_HUMAN     61   KKVADALTNAVAHVDDMPNALSALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTP  120
Q6B0K9 HBM_HUMAN     60   QRMLAAVGAAVQHVDNLRAALSPLADLHALVLRVDPANFPLLIQCFHVVLASHLQDEFTV  119
                          :::  *:  ** ***::  *** *:****  *****.** ** :*: *.**:**  ***

P69905 HBA_HUMAN    121   AVHASLDKFLASVSTVLTSKYR                                      142
Q6B0K9 HBM_HUMAN    120   QMQAAWDKFLTGVAVVLTEKYR                                      141
                           ::*: ****:.*:.***.***
```

Figure 9: Alignment of Alpha and Mu chains

```
P69905 HBA_HUMAN      1   MVLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG   60
P02008 HBAZ_HUMAN     1   MSLTKTERTIIVSMWAKISTQADTIGTETLERLFLSHPQTKTYFPHFDLHPGSAQLRAHG   60
P09105 HBAT_HUMAN     1   MALSAEDRALVRALWKKLGSNVGVYTTEALERTFLAFPATKTYFSHLDLSPGSSQVRAHG   60
Q6B0K9 HBM_HUMAN      1   -MLSAQERAQIAQVWDLIAGHEAQFGAELLLRLFTVYPSTKVYFPHLSACQDATQLLSHG   59
                            *:  ::: :   *  :. :     :* * * *  .* **.** *:.   .::*: .**

P69905 HBA_HUMAN     61   KKVADALTNAVAHVDDMPNALSALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTP  120
P02008 HBAZ_HUMAN    61   SKVVAAVGDAVKSIDDIGGALSKLSELHAYILRVDPVNFKLLSHCLLVTLAARFPADFTA  120
P09105 HBAT_HUMAN    61   QKVADALSLAVERLDDLPHALSALSHLHACQLRVDPASFQLLGHCLLVTLARHYPGDFSP  120
Q6B0K9 HBM_HUMAN     60   QRMLAAVGAAVQHVDNLRAALSPLADLHALVLRVDPANFPLLIQCFHVVLASHLQDEFTV  119
                          .::  *:  **  :*::  *** *:.***  *****..* ** :*: *.** :   :*:

P69905 HBA_HUMAN    121   AVHASLDKFLASVSTVLTSKYR                                      142
P02008 HBAZ_HUMAN   121   EAHAAWDKFLSVVSSVLTEKYR                                      142
P09105 HBAT_HUMAN   121   ALQASLDKFLSHVISALVSEYR                                      142
Q6B0K9 HBM_HUMAN    120   QMQAAWDKFLTGVAVVLTEKYR                                      141
                            :*: ****: *  .*..:**
```

Figure 10: Alignment of Alpha like chains

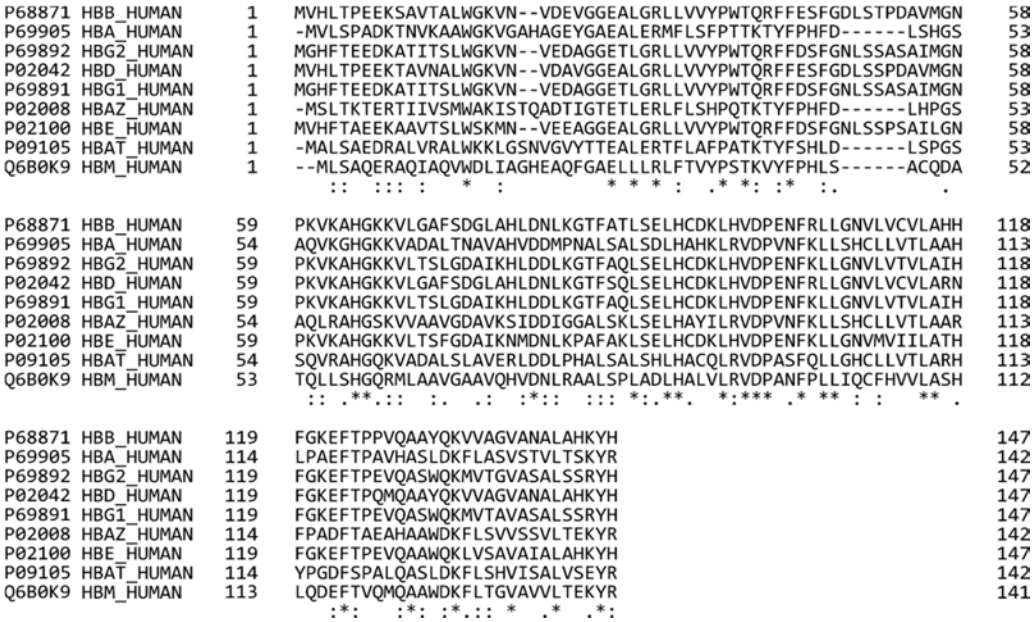

Figure 11: Alignment of various Haemoglobin subunits

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A STUDY ON ISOLATION OF FUNGI FROM SURFACE WATER

Manuel Thomas[1*] and **M. Thangavel**[2]

[1]Research and Development Centre, Bharathiar University, Coimbatore
[2] Department of Microbiology, Sree Narayana Guru College, K.G. Chavadi
Coimbatore, Tamil Nadu, India PIN 641 105

Abstract

The presence of fungi in water was least studied and mycological quality of water is still in infancy. Fungal diseases are emerging globally and waterborne transmission routes are well reported from several countries. The present study is an attempt to study the presence of fungi among selected well, wetland and pond water samples in Central Travancore region in Kerala. Fourteen species of fungi were isolated from the collected 30 water samples tested. Genus *Aspergillus* (3 species) was more diverse followed by *Fusarium* and *Mucor* (2 species each). The wetland water was more prone to fungal presence (9 species) followed by pond water (8 species) and well water (4 species). The results suggest that water has fungi holding and transmission potential which poses health hazards, as the population of individuals with immunomalignancies is on the rise in the society. Introduction of 'Mycological water quality' as a water quality parameter is urgently needed to address the issue, eventhough the relevance of waterborne fungi for water quality and human health is poorly understood. As fungi can influence the water quality in various ways, the mycobiota of water, especially drinking water should be dealt seriously with proper awareness and policy formations.

Key Words: Mycolcal Water Quality, Fungi, Surface Water, Kerala

* **Corresponding author:** Consortium & Training Academy for Biosciences (CTAB), Manarcadu P.O., Kottayam, Kerala, India PIN 686 019. Email: doctorfungus007@gmail.com

Introduction

Water, the elixir of life is affected badly by anthropogenic activities and is declining due to rapid urbanization, demographic explosion, climate change and several other factors. Surface water quality is an indispensable component of the natural environment and a matter of serious concern today (Liu *et al*., 2011). Among the water quality concern, microbial water quality is most significant, as several waterborne diseases and epidemics are recurrently reported across the world (Ashbolt, 2004). Bacteria are probably the most frequently studied group of microorganisms with respect to the quality of drinking water. The role of fungi in water quality has bobbed up in 1970s, when water with foul taste and odour involving health episodes are assessed (Bays *et al*., 1970). The past decade has witnessed several reports from many regions regarding the occurrence of fungi in drinking water (Anaissie *et al.,* 2001; Warris *et al.,* 2002; Hageskal *et al.,* 2006; Pires-Goncalves *et al*., 2008). However, relatively little attention has been paid to the occurrence of fugal species in water in the State of Kerala. The present study is an attempt in this line.

Materials and Methods

Surface water sample were aseptically collected in pre sterilized bottles from well, wetland and pond water samples in Central Travancore region in Kerala during 2015 (ten samples each). 100 ml of the collected sample were membrane filtered (0.45µm), inoculated on to Sabouraud Dextrose Agar (SDA) with antibiotics and incubated for 5-10 days at room temperature (Hageskal *et al.,* 2006). The developed colonies were counted and identified up to species level by using the macroscopic (colony texture, topography, exudates production and pigmentation) and microscopic (hyphal characteristics, conidia ornamentation, presence or absence of micro conidia and macro

conidia) characteristics (Howard, 2002; Watanabe, 2002) by performing scotch tape method (Davey *et al*., 1996).

Results

Fourteen species of fungi were isolated from the collected 30 water samples tested. Genus *Aspergillus* (3 species) was more diverse followed by *Fusarium* and *Mucor* (2 species each) (Table 1 and Fig 1). The wetland water was more prone to fungal presence (9 species) followed by pond water (8 species) and well water (4 species). *C. geniculata* is the only representation from dematiaceous fungal group along with few *Mycelia sterilia (*dematiaceous*)* isolates. *Chrysosporium* is the only keratinophilic fungi isolated which is only present in wetland water samples. A detail of colony count was given in Table 2 and Fig 2.

Table 1: List of fungi isolated (frequency of occurrence %)

Sl. No.	Fungi isolated	Well water	Wetland water	Pond water
1.	*Aspergillus flavus*	10	50	--
2.	*A. fumigatus*	--	20	10
3.	*A. niger*	30	70	40
4.	*Chrysosporium* sp.	--	60	--
5.	*Curvularia geniculata*	--	10	20
6.	*Fusarium* sp.	--	10	--
7.	*F. semitectum*	--	--	20
8.	*Mucor* sp.	30	20	--
9.	*M. circinelloides*	--	30	--
10.	*Mycelia sterilia*	--	--	30
11.	*Mycelia sterilia (*dematiaceous*)*	--	30	--
12.	*Paecilomyces lilacinus*	--	--	10
13.	*Penicillium* sp.	10	-	20
14.	*Scopulariopsis brevicaulis*	--	--	10

Table 2: Colony count of fungi from surface water samples

Sample No.	Well water	Wetland water	Pond water
1.	3	6	2
2.	1	10	8
3.	--	3	11
4.	1	9	3
5.	--	4	15
6.	1	12	18
7.	5	9	11
8.	--	6	7
9.	1	15	16
10	1	20	13

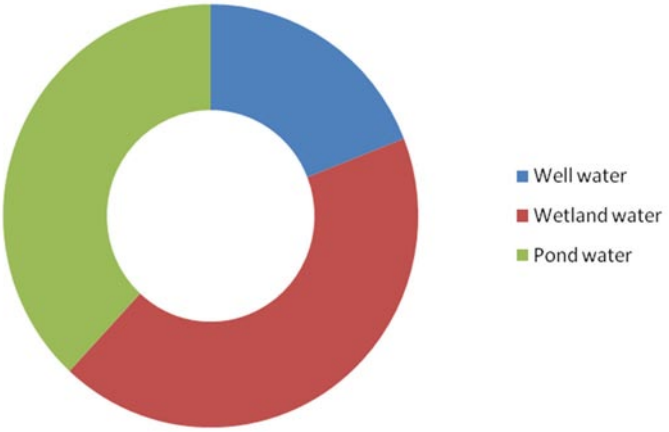

Figure 1: Fungal isolation among tested samples

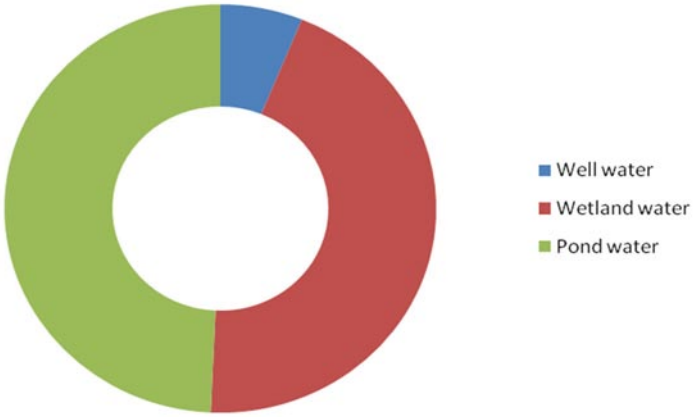

Figure 2: Fungal distribution (CFU) among tested samples

Discussion

Fungi are ubiquitous and cosmopolitan as they are able to colonize, multiply and survive in diversified habitats irrespective of geographic realms. Geographic location, climatic conditions, microhabitat, substrate type, distribution of fauna and flora are the significant factors contributing to fungal distribution and diversity (Manoharachary *et al*., 2005). The main portal of entry of fungi is inhalation, several studies have indicated that exposure from water can also occur (Warris *et al.,* 2001). The role of water distribution systems in spreading of potentially allergenic, toxigenic, and opportunistic fungal species in hospitals and private homes are well established (Kelley *et al*., 2003). Investigations from Sweden and Finland have pointed about the allergic and respiratory health effects of fungi in water (Muittari*et al*., 1980).

In the present study, a total of fourteen species of fungi were isolated from the collected 30 water samples. Genus *Aspergillus* (3 species) was more diverse followed by *Fusarium* and *Mucor* (2 species each). The wetland water was more prone to fungal presence (9 species) followed by pond water (8 species) and well water (4 species). Various studies also previously demonstrated the presence of fungi in both groundwater and surface water (Hageskal *et al*., 2006 and Hageskal *et al*., 2009). Pereira *et al*. (2009) found notably higher levels of fungi in surface and spring water than in groundwater. Thus the present results are in tune with the available reports worldwide.

The density and diversity of the fungal communities in waterbodies are governed by physicochemical factors like temperature, hydrogen-ion concentration, oxygen content, dissolved organic and inorganic matter, concentration of ions like phosphate, sulphate etc. (Jan *et al*., 2014) which reflects local environmental conditions. It can be concluded that the seasonal

differences in water chemistry is the reason for the differences in colony count and number of isolates. Abbott *et al*. (2006) reported that the periodicity and intensity of rainfall have much influence on microbial contamination in rain water entering tanks. Sewage intrusion, runoff from waste sites to streams, lakes and wetlands are also the contributors.

Studies of fungi in water have demonstrated that fungi are relatively common in water system. Moreover, species of pathogenic, allergenic, and toxigenic concern are isolated from water, sometimes in high concentrations. Fungi in water may be aerosolized into air, and introduced to immunocompromised patients and results in much complicated clinical manifestations. The potential health implications of fungi in water are still contradictory and needs further studies and elimination steps. In future, monitoring of water systems for the present of fungi may be required along with installation of mycological water quality.

Conclusion

The study disclosed the evidence of fungi in water (well water, wetland water and pond water) in Kerala, India. However, more detailed investigations are needed to identify the role of fungi in the current paradigm of fungal emergence in the microbial loop. Environmental conditions vary at each aquatic system which has a major effect on number of fungal individuals, even at the microclimate level. The ecology and transmission dynamics of fungi associated with water are complex and multifaceted. The typical ecology and climatic conditions prevailing in small holder ecosystem in Kerala favors easier transmission of fungal pathogens which needs special attention.

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ASSESSMENT OF MICROBIAL QUALITY OF READY TO DRINK FOODS AND WATER FROM A PUBLIC HEALTH PERSPECTIVE

Grace Baby*, Simi P, Chinchu V. R, Saritha K.R, Ria Elsa Roy and **Dr. Mukundan M.K**

The Food Quality Monitoring Laboratory under The Council For Food Research and Development, Government of Kerala, Perinjottakkal P.O, Pathanamthitta Dist, Kerala-689692

Abstract

Nowadays with Food Safety Standards Act & its Rules & Regulations, 2016, food safety and quality are important health, social and economic issue. Presence of certain pathogens in foods is a key factor for assessing the quality and safety of any food. A study was carried out to evaluate the quality and safety of food items collected from 14 districts in Kerala from 24/02/2014 to 29/09/2016. The food categories selected for this study are Ready to drink foods and Drinking water. To assess the microbial quality of Ready to Drink foods the parameters analysed are *Salmonella,* Total Plate Count, *E.coli* and *Staphylococcus aureus.* Total Plate count, Coliforms and *E.coli* were tested for Drinking water. Total 167 Ready to Drink foods and 41 water samples were analysed. Among these 50 (30 %) Ready to Drink foods and 35 Nos (85.37%) Drinking water samples were found to be defective due to the presence of *Salmonella* or exceeding the tolerance limits of *E.coli, Coliforms, Staphylococcus aureus* and Total Plate Count. In the light of this data the role of each parameter in food safety and the remedial measures are discussed.

Keywords: Ready to drink food, microbiological quality, drinking water, food safety.

***Corresponding author:** E mail : cacfrd@gmail.com

Introduction

Fruit juices are well recognized for their nutritive value, mineral and vitamin content. In many tropical countries they are common man's beverages and are sold at all public places and roadside shops. However in view of their ready consumption, quick methods of cleaning, handling and extraction they could often prove to be a public health threat. There are reports of food borne illness associated with the consumption of fruit juices at several places in India and elsewhere (Health Canada, 2000; Parish, (2013). Traditionally, fruit products have been regarded as microbiologically safer than other unprocessed foods. However, many outbreaks of human infections have been associated with the consumption of contaminated fruit juices (Poonam U Sharma., 2013). The objective of this study was to evaluate the microbiological safety and quality of fruit juices and drinking water being served in different districts of Kerala. A study aimed at examining the quality and safety of ready to drink beverages and drinking water based on standard techniques showed that most these remained hygienically poor due to high bacterial loads and presence of pathogens . The occurrence of pathogenic *E. coli, S. aureus* and *Salmonella* is alarming enough for an immediate action by the suitable agency. It is suggested that regular monitoring of the quality of ready to drink beverages and drinking water for human consumption must be introduced to avoid any future disease outbreaks. (Sandeep et al., 2001)

An adequate supply of safe drinking water is one of the major pre requisites for a healthy life. Forhumanbeings,thecriticalissuewhenusing drinking waterishygiene.Morethan4million people die of illnesses contacted through microorganisms, and most cases are causedby water contaminated by microorganisms (YuheiInamori NaoshiFujimoto). Drinking water is derived from two basic sources: surface waters, such as rivers and reservoirs, and

groundwater. In general, groundwater is less vulnerable to pollution than surface waters. Contaminated water has always been an important agent in the spread of certain disease. Ingestion may cause gastrointestinal diseases, and skin diseases may be caused by immersion (Warrington P.D, 2013). Contaminated drinking water is a major contributor to the problem of diarrheal disease, which continues to plague in children worldwide. . To address the problem of unsafe drinking water, methods are needed to assess quality of drinking water. Rather than directly assessing presence of pathogens in water, indicator organisms characteristic of fecal contamination are used as a proxy measure of a recent fecal contamination (Karen Levy, et al., 2002). Microbial contamination of drinking water at the water source, plumbing lines and household storage is the prime reason for the waterborne infections in the developing counties (Vaithiyanathan Lavanya and Seetharaman Ravichandran., 2013).

The contamination of drinking water by pathogens is in a growing concern of public health authorities. The problem arises as a consequence of contamination of water by faecal matter, particularly human faecal matter, often containing pathogenic organisms. Drinking water is not, however, sterile and bacteria can be found in the distribution system and at the tap. Most of these organisms are harmless, but some opportunist pathogens such as Pseudomonasaeruginosa and Aeromonasspp. may multiply during distribution given suitable conditions. Microbial contamination of drinking water thus remains a significant threat and constant vigilance is essential, even in the most developed countries (John Fawell, Mark J Nieuwenhuijsen, 2002).

Bacteriological analysis of water help us to estimate the numbers of bacteria present and, if needed, to find out what sort of bacteria are present. It

represents one aspect of water quality. Indicator micro-organisms have been used to suggest the possible presence of pathogens (Berg., 1978) Microbiological criteria are presently undergoing re-evaluation throughout the world, and the historical dependence upon total and fecal coliforms is being supplanted by more specific, epidemiologically-derived indicators of water quality. Enterococci are better indicators than fecal coliforms and most closely approach the ideal characteristics of an indicator for gastrointestinal diseases (Warrington P.D, 2013)

E. coli is a type of fecal coliform bacteria commonly found in the intestines of animals and humans. . The presence of E. coli in water is a strong indication of recent contamination from sewage or animal waste as E. coli comes from gastrointestinal tract of warm blooded animals including man. One of the hundreds of strains of the Escherichia coli, viz E. coli O157:H7 is an emerging cause of food borne and waterborne illnesses. Microbial contamination especially faecal contamination drinking water thus remains a significant threat and constant vigilance is essential. (www.ct.gov/dph/lib/dph/drinking_water/pdf/E_coli.pdf).

As per Drinking water specification *E.coli* shall not be detectable in any 100ml sample for all water intended for drinking and no sample should contain more than 10 coliform organisms per 100ml sample. (IS 10500:1991)

Materials and Methods

Sample Collection

Ready to drink products like Mango drink, Non alcoholic sweet beer, Grape juice, Lemon juice, Curd, Soda, Cool drink etc were sampled from 14 districts in Kerala State. The samples were purchased and brought to lab in insulated chilled boxes (0-4^0 C) and maintained in same condition until analysis. These samples were analysed for various microbiological parameters

to evaluate the suitability of these food items for human consumption. Sampling and analysis are done as per AFNOR (Association of France for Normalization) and BAM (Bacteriological Analytical Methods) procedure.

Water samples are collected aseptically in sterile bottles from hotels, restaurants, bakeries, public distribution system etc and transported to the lab in insulated chilled boxes (0-4^0 C). The samples are retained in same condition until analysis.

Analysis of TPC, *E.coli&Staphylococcus aureus* in Ready to Drink samples

Microbiological parameters tested are Total Plate Count, E.coli, Staphylococcus aureus and Salmonella. A fully automated instrument, TEMPO is used for the enumeration of Total Plate Count (TPC), E.coli and Staphylococcus aureus. This equipment is able to give performance levels similar to the standards EN ISO 4833(1), (AOAC official method 966.23)

The TEMPO test system consists of a vial of culture medium and a card which are specific to the selected test (TPC/*E.coli*/*Staphylococcus*). The inoculated medium is transferred by the TEMPO filler into the card containing of 48 wells of 3 different volumes. The card is designed to simulate the Most Probable Number method. Depending on the number and type of the positive wells, the TEMPO system calculates the number of microorganisms present in the original sample according to a calculation based on the MPN method. Aseptically add 10ml of sample to 90ml Butter fields Phosphate buffer and homogenized in the TEMPO bag. Reconstituted the appropriate culture medium for TPC/*E.coli*/*Stahylococcusaureus* by dispensing 3ml of secondary diluent (Distilled water) per vial using the dispenser. Using a sterile pipette, transferred 1ml from the filtered compartment of the TEMPO bag into the vial containing the reconstituted culture medium. Homogenized for approximately 3 seconds

using a vortex type mixer. The inoculated vial was introduced into the TEMPO Filler; the medium was filled into a TEMPO card and sealed. The sealed cards were incubated. (Temperature: 37ºC, Time: 40 to 48 hours for TVC, 22 to 27 hours for *E.coli*, 24 to 27 hours for *Staphylococcus aureus*). The incubated cards were introduced into the TEMPO reader, which scans the bar code of each card and interprets the results.

Salmonella

Salmonella detection was done by fully automated equipment namely VIDAS (Vitek Immuno Diagnostic Assay System).Salmonella detection using the conventional time consuming protocol, can take up to 5 days to confirm a sample is negative. Instead of classical method, the study used enzyme immuno assay (EIA) based technique to simplify and accelerate the detection. This method is certified by AFNOR (Bio 12/10-09/02) for human and animal food products. For pre enrichment, 25 ml of sample aseptically added to 225 ml of sterilized Buffered peptone water and mixed in stomacher type bag and incubated for 16-20 hours at 37±1ºC.After incubation, 0.1ml of suspension was transferred to 10ml of Rappaport Vassiliadis Soya Broth (RVS) and incubated for 6-8 hours at 41.5±1ºC for enrichment. For post enrichment, after incubation, 1ml of RVS broth to M Broth was transferred and incubated for 16-20 hours at 41.5±1ºC.After incubation, the assay of Salmonella was performed in VIDAS. All positive results have been confirmed by biochemical and serological methods as per VIDAS and US/FDA BAM, 2016.

Analysis of TPC, *E.coli* &*Staphylococcus aureus* of drinking water.

Analysed as per the procedure IS 1622:1981(RA 2003): Methods of sampling and microbiological examination of water.

a. **TOTAL PLATE COUNT (TPC):** The bacterial population in different samples was estimated by pour plate method on Plate Count Agar for Total Plate Count (TPC). Plates are incubated at 35- 37^0C for 48 h and all colonies were noted and counted after incubation.

b. **COLIFORMS & *E.COLI* :** Method as per IS 1622:1981 is followed. Shake the water samples thoroughly before making dilutions or before inoculation.

Results

Outof 167 Ready to drink samples tested 50 samples (30%) were defective The details are given Table 4. Microbiological quality of Ready to drink products sampled from 14 districts of Kerala were analysed in this study. The parameters analysed were *Salmonella*, Total Viable Count (TVC), *E.coli, and Staphylococcus aureus.* Total 167 various Ready to drink products were analysed (Table 1). Among 167 samples, *Salmonella* was detected in 6.59% samples (11Nos out of 167). Out of 82 samples studied for *E.coli* 2.43% samples were contaminated with *E.coli*(2 Nos).Out of 75 samples, 58.67% samples shown Total Viable Count above the test limit (44 Nos).79 samples examined for *Staphylococcus aureus* showed 2.53% samples to be above test limit (2 Nos).Highest Total Viable count detected was $49x10^9$ cfu/ml in mixed fruit juice. Highest *E.coli* count of 1200 cfu/ml, was detected in sugar cane juice. Two samples were defective due to *Staphylococcus aureus* and the same were in curd samples .The details of defective samples are given in Table 2.

Microbiological quality of drinking water sampled from different parts of Kerala were analysed in this study. Out of 41 samples, 35 samples (85.37%) were defective. The parameters analysed were Total Plate Count (TPC), Coliforms and *E.coli*. Total 41 samples were analysed. (Table 3) Among the 41

samples, all the three parameters (TPC, Coliforms &*E.coli*) analysed for 28 samples, for 10 samples two parameters (*E.c*oli and Coliforms) were tested, for 2 samples *E.coli* and TPC were analysed and for one sample only *E.coli* were analysed. Test results showed that among the 41 samples analysed for *E.coli*, it was detected in 24 samples. That is *E.coli* was present in 58.54% samples. Out of 38 samples tested for Coliforms as per Drinking water specification 79% sample (30 Nos) showed coliforms above tolerance. Among the 30 samples analysed, Total plate count was higher in 24 samples. That is TPC was higher in 80.0% samples. Highest Coliform count is 1600MPN/100ml detected in 23 samples. Highest E.coli count is also 1600MPN/100ml and it was found in 4 samples. TPC was also higher in those samples which show higher coliform count. Out of 28 samples analysed for Coliforms, *E.coli*& TPC, 15 were defective.

Discussion

Out of 167 Ready to drink samples analysed, 11 samples were found to be contaminated with *Salmonella*.*Salmonella* is a potential pathogen .It usually causes food poisoning. The most common symptoms include diarrhea, abdominal cramps and fever. The presence of Salmonella in the food can be due to poor hygiene and sanitation and Good Manufacturing Practices. Processing conditions, improper handling, prevalence of unhygienic conditions contribute substantially to the entry of bacterial pathogens in juices prepared from these fruits or vegetables (Oliveira *et al.*, 2006; Nicolas *et al.*, 2007; Durgesh *et al.*, 2008; Odu and Adeniji, 2013). In this study, *Salmonella* was detected mostly in milk shakes and mango drinks. *Salmonellaspp*. still remains the main cause of many food-borne infections. Many outbreaks of *Salmonella spp.* have been linked with the consumption of unpasteurized juices (Harris *et al.,* 2003). This result shows there is a gross neglect on the part of the food processor as well as regulatory agencies. So there is need for more vigilance.

Out of 75 Ready to drink samples tested, 41 samples were found TVC value above tolerance limit. The presence of microbial contaminants in all the products could be a reflection of the quality of the raw materials, processing equipments, environment, packaging materials and the personnel's in the production process. The presence of these organisms needs to be controlled to prevent spoilage and food borne illness (Mudgil *et al*., 2004; Oranusi *et al.*, 2007).

More than 50 % of the sample is defective due the high count of Mesophilic aerobic bacteria. The enumeration of Total plate count is used to determine the quality of the product and can express its state of freshness or deterioration. Studies have shown that increase of TPC to a level below 1 lakhwill retain the acceptability of food (Mukundan. M.K. et al[4]). Conversely TPC values above 1 lakh for a ready to eat/drink food are an indication it is not suitable for human consumption due to spoilage.

Out of 82 samples 2 samples were found to be contaminated with *E.coli*. *Escherichia coli* (abbreviated as *E. coli*) are bacteria found in the environment, foods, and intestines of people and animals.Most *E.coli* are harmless and actually are an important part of a healthy human intestinal tract. However, some *E. coli* can cause diarrhea, urinary tract infections, respiratory illness, bloodstream infections, and other illnesses. The types of *E. coli* that can cause illness can be transmitted through contaminated water or food, or through contact with animals or people. *E.coli* is a faecal indicator organism. The presence of *E.coli* in food generally indicates direct or indirect contamination with fecal matter from human or animal origin. The tolerance limit for *E.coli* is < 10 cfu/g for ready to eat/drink food. (Mukundan. M.K[4]. et al). The presence of *E.coli* above the tolerance limit is a sure indication poor hygiene of food handlers. It may also arise from cross contamination by insects and rodents.

In this study, total 79 Ready to drink samples tested for *Staphylococcus aureus* and 2 samples were found to be contaminated with *Staphylococcusaureus*. *Staphylococcus aureus* is simultaneously an indicator organism as well as a food poisoning organism (Mukundan. M.Ketal). Their presence above tolerance limit (<1000cfu/g) in food suggest poor hygiene of workers, which can lead to the occurrence of food poisoning as well as spoilage of the food materials. Once contamination and toxin production occurs removal of toxicity is not possible as the toxin is heat stable.

In this study total 41 water samples were tested. *E.coli* and Coliforms were detected in most of the samples analysed. *E.coli* was present in 58.54% samples analysed. It indicates the water is contaminated with fecal matter. Water pollution caused by fecal contamination is a serious problem due to the potential for contracting diseases from pathogens. The contamination of drinking water by pathogens causing diarrheal disease is the most important aspect of drinking water quality. *E.coli* and their presence indicate the workers do not observe hygiene and sanitation .It can also come from contamination arising from toilets. So the water used for food processing in most of these cases need correction and if it is ground water suppose there is *E.coli*, the best correction is either chlorination or disinfection with hydrogen peroxide before water is used for processing.

Coliforms are also is indicator of contamination. 79% samples were contaminated with coliforms. This contamination has happened may be from workers or from animals. Obviously there is a need for purify the water which is used for food processing.

TPC was also higher in those samples which show higher coliform count.TPC was higher in 80.0% of the Drinking water samples shows above

tolerance limit as per IS 4251-1967. Microorganisms will normally grow in water and on surfaces in contact with water as biofilms. TPC tests were employed as indicators of the proper functioning of purification processes and thereby as indirect indicators of water safety.

Conclusion

Total 167 Ready to Drink foods and 41 water samples were analysed. Among these 50 (30 %) Ready to Drink foods, and for Drinking water samples 35 Nos (85.37%) were found to be defective due to the high count of *E.coli, Coliforms*, *Salmonella* or Total plate count. This is a real alarming situation with respect to food safety. The study indicated that most ready to drink food and drinking water were contaminated. These results clearly indicate deviation from minimum procedures for processing like observation of Hygiene and Sanitation, and Good Manufacturing practices. Hence to protect the public health relevant regulatory authorities shall look into this problem for urgent remedial measures.

Table 1: Percentage of defective Ready to drink samples

Sl.No	Test parameter	Total samples	No. of defective samples	% of defective samples
1	Salmonella	167	11	6.59%
2	*E.coli*	82	2	2.43%
3	Total viable count	75	44	58.67%
4	*Staphylococcus aureus*	79	2	2.53%

Table 2: Details of defective Ready to drink samples

Sl.No	Name of Sample	Test parameter	Result
1	Mango juice	TVC	1000000 cfu/ml
2	Mixed fruit juice	TVC	>49x10^9 cfu/ml
3	Lime juice	TVC	490000000 cfu/ml
4	Lassy	TVC	52000000 cfu/ml
5	Packaged drinking water	TVC	21000000 cfu/ml
6	Sugar cane Juice	TVC	4900000000 cfu/ml
		E.coli	21 cfu/ml
7	Curd	TVC	5500000000 cfu/ml
8	Lime	Salmonella	Present in 25ml
		TVC	370x10^6 cfu/ml
9	Mango drink	Salmonella	Present in 25ml
		TVC	1500000 cfu/ml
10	Mango drink	TVC	>490000000 cfu/ml
11	Elaneer - bottled	TVC	>490000000 cfu/ml
12	Fermented coconut drink	TVC	930000 cfu/ml
		Salmonella	Present in 25 ml
13	Refreshing drink	TVC	6800000 cfu/ml
14	Lemon juice	TVC	>49000000 cfu/ml
		Salmonella	Present in 25 ml
15	Mango juice	TVC	>49000000 cfu/ml
16	Mango drink	TVC	13x10^4 cfu/ml
17	Sugar cane juice	TVC	82x10^6 cfu/ml
		E.coli	1200 cfu/ml
18	curd	Staphylococcus aureus	49000 0cfu/ml
19	Milk shake vanilla	Salmonella	Present in 25ml
20	Pineapple juice	Salmonella	Present in 25ml
21	Refresh mango	TVC	>4900000 cfu/ml
22	soya milk vanilla flavor	Salmonella	Present in 25ml
23	Pomegranate	TVC	180000 cfu/ml
24	Guava	TVC	900000 cfu/ml
25	Mango drink	TVC	30000 0cfu/ml
26	Mango drink	TVC	10000 00cfu/ml

27	Mango drink	TVC	6700 00cfu/ml
28	Vanilla milk shake	TVC	370000 cfu/ml
29	Chocolate milk shake	TVC	>4900000 cfu/ml
30	Strawberry milk shake	TVC	510000 cfu/ml
31	Butter skotch milk shake	TVC	>4900000 cfu/ml
32	Mango drink	Salmonella	Present in 25ml
		TVC	350000 cfu/ml
33	Vanilla milk shake	TVC	490000 cfu/ml
34	Soda water	TVC	49000 cfu/ml
35	Mango drink	TVC	4900000000 cfu/ml
36	Chocolate milk shake	TVC	>4900000 cfu/ml
		Salmonella	Present in 25ml
37	Mango drink	TVC	>4900000 cfu/ml
38	Mango Juice	TVC	490000000 cfu/ml
		Salmonella	Present in 25ml
39	Carbonated beverage	Salmonella	Present in 25ml
40	Orange flavor	TVC	860000 cfu/ml
41	Soda cool drink	TVC	>150000000 cfu/ml
42	Orange juice	TVC	5600000000 cfu/ml
43	Butter milk	TVC	91000000 cfu/ml
44	Mango drink	TVC	>490000 cfu/ml
45	Curd	TVC	21000000 cfu/ml
46	Orange flavored drink	TVC	330000 cfu/ml
47	curd	TVC	3300000 cfu/ml
48	curd	Staphylococcus aureus	49000 cfu/ml
49	Lemon juice	TVC	370000000 cfu/ml
50	Mango drink	TVC	7100000 cfu/ml

Table 3: Details of water samples all over Kerala

Sl No	Sample No	Coliforms(MPN/100ml)	E.coli(MPN/100ml)	TPC(cfu/ml)
	1.	220	9	-
	2.	500	2	-
	3.	1600	8	-
1	4.	900	<2	-
	5.	23	8	-
	6.	14	14	-
	7.	350	34	-
2	8.	1600	13	48000
	9.	1600	80	33000
	10.	1600	1600	23000
3	11.	<2	<2	<10
	12.	<2	<2	<10
	13.	1600	<2	-
4	14.	1600	<2	$<1 \times 10^6$
	15.	-	<2	$>49 \times 10^5$
	16.	1600	34	-
	17.	<2	<2	<10
	18.	<2	<2	<10
	19.	<2	<2	<10
5	20.	2	<2	18
	21.	7	<2	1300
6	22.	39	<2	4.2×10^4
	23.	1600	140	26×10^4
	24.	1600	1600	3.2×10^7
7	25.	-	<2	12×10^6
	26.	<2	<2	600
	27.	1600	<2	1×10^6
	28.	1600	31	26×10^7
8	29.	1600	1600	3.8×10^4
	30.	1600	140	1.8×10^6
	31.	1600	90	5.2×10^4
	32.	-	1600	-
	33.	1600	175	-
9	34.	1600	14	3.8×10^7
	35.	1600	90	1.9×10^7
	36.	1600	350	4.2×10^6
	37.	1600	26	2.9×10^6
10	38.	1600	<2	$>1 \times 10^6$
	39.	1600	33	33×10^6
	40.	1600	<2	1×10^6
	41.	1600	75	49×10^6

- Sample No 1 – Well water
- Sample No. 2 & 4 – Tap water from Public Distribution systems
- Sample No. 5 & 9 – drinking water from Govt. Hospitals
- Sample No.3,6,10,12,14-17,20-22,26,28,32–34,36-39,41 – From Hotels & Restaurants
- Sample No. 7,8,11,13,18,19,23-25,27,29,30,31,35,40 – From Bakeries & Coolbars

Table 4: Details of the test samples

Sl. No.	Name of Sample	Sample Code	Test Parameter	Results
01.	Mango juice	FQML/2015FS/0052	Salmonella	Absent in 25ml
02.	Butter Milk	FQML/2015FS/0053	Salmonella	Absent in 25ml
03.	Milk Shake Powder (Chocolate Flavor)	FQML/2015FS/0054	Salmonella	Absent in 25ml
04.	Mixed Fruit Juice	FQML/2015FS/0055	Salmonella	Absent in 25ml
05.	Guava Juice	FQML/2015FS/0056	Salmonella	Absent in 25ml
06.	Mango drink	FQML/2015FS/0062	Salmonella	Absent in 25ml
07.	Milk shake	FQML/2015FS/0063	Salmonella	Absent in 25ml
08.	Fruit Juice	FQML/2015FS/0064	Salmonella	Absent in 25ml
09.	Milk shake	FQML/2015FS/0065	Salmonella	Absent in 25ml
10.	Milk shake	FQML/2015FS/0071	Salmonella	Absent in 25ml
11.	Soyamilk Vanilla Flavor	FQML/2015FS/0072	Salmonella	**Present in 25ml**
12.	Lassi	FQML/2015FS/0073	Salmonella	Absent in 25ml
13.	Mango drink (Bottle)	FQML/2015FS/0103	Salmonella E Coli S. aureus	Absent in 25ml <10cfu/ml <10cfu/ml
14.	Fruit Drink	FQML/2015FS/0104	Salmonella E Coli S. aureus	Absent in 25ml <10cfu/ml <10cfu/ml
15.	Grape juice	FQML/2015FS/0105	Salmonella S. aureus	Absent in 25ml <10cfu/ml
16.	Butter Skotch Milk Shake	FQML/2015FS/0106	Salmonella	Absent in 25ml
17.	Mango drink (Packet)	FQML/2015FS/0107	Salmonella	Absent in 25ml
18	Strawberry Milk Shake	FQML/2015FS/0108	Salmonella	Absent in 25ml
19.	Chocolate Milk Shake	FQML/2015FS/0109	Salmonella S.aureus	Absent in 25ml <10cfu/ml
20.	Vanilla Milk Shake	FQML/2015FS/0110	Salmonella	Absent in 25ml
21.	Mango drink	FQML/2015FS/0111	Salmonella E Coli S. aureus	Absent in 25ml <10cfu/ml <10cfu/ml

22.	Sambharam	FQML/2015FS/0112	Salmonella	Absent in 25ml
23.	Pomegranate Juice	FQML/2015FS/0203	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml 18000cfu/ml
24.	Guava Juice	FQML/2015FS/0204	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml 90000cfu/ml
25.	Mango drink	FQML/2015FS/0205	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml 30000cfu/ml
26.	Mango drink	FQML/2015FS/0206	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml 10000cfu/ml
27.	Mango drink	FQML/2015FS/0207	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml 6700cfu/ml
28.	Apple juice	FQML/2015FS/0251	Salmonella	Absent in 25ml
29.	Packaged drinking water	FQML/2015FS/0254	Salmonella	Absent in 25ml
30.	Vanilla milk shake	FQML/2015FS/0258	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml **370000cfu/ml**
31	Chocolate Milk Shake	FQML/2015FS/0259	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml **>4900000cfu/ml**
32.	Strawberry Milk Shake	FQML/2015FS/0260	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml **510000cfu/ml**
33.	Butter Skotch Milk Shake	FQML/2015FS/0261	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml **>4900000cfu/ml**

34.	Mango drink	FQML/2015FS/0266	Salmonella S.aureus TPC	**Present in 25ml** <10cfu/ml **350000cfu/ml**
35.	Packaged Drinking water	FQML/2015FS/0292	Salmonella	Absent in 25ml
36.	Milkshake Vanilla	FQML/2015FS/0328	Salmonella E coli S.aureus TPC	**Present in 25ml** <10cfu/ml <10cfu/ml 69000cfu/ml
37.	Strawberry Milkshake	FQML/2015FS/0329	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml <100cfu/ml
38.	Chocolate Milkshake	FQML/2015FS/0330	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml 100cfu/ml
39.	Butter skotch Milkshake	FQML/2015FS/0331	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml <100cfu/ml
40..	Mango drink	FQML/2015FS/0332	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml >4900000cfu/ml
41..	Mango Drink	FQML/2015FS/0333	Salmonella	Absent in 25ml
42.	Fruit drink	FQML/2015FS/0334	Salmonella	Absent in 25ml
43.	Mango Drink	FQML/2015FS/0335	Salmonella	Absent in 25ml
44..	Packaged drinking water	FQML/2015FS/0336	Salmonella	Absent in 25ml
45.	Mango drink	FQML/2015FS/0339	salmonella	Absent in 25g
46.	Fermented coconut drink	FQML/2015FS/0365	Salmonella	Absent in 25g
47.	Curd	FQML/2015FS/0405	Salmonella	Absent in 25ml
48.	Curd	FQML/2015FS/0406	Salmonella	Absent in 25ml
49.	Mango flavoured milk	FQML/2015FS/0408	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml 100cfu/ml

50	Pineapple flavoured milk	FQML/2015FS/0409	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml <100cfu/ml
51	Mango refresh drink	FQML/2015FS/0410	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml 1000000cfu/ml
52	Strawberry flavoured milk	FQML/2015FS/0411	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml <100cfu/ml
53.	Curd	FQML/2015FS/0416	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml **>49000cfu/ml** 46000cfu/ml
54.	Mango Drink	FQML/2015FS/0490	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml **490000cfu/ml**
55.	Mango Drink	FQML/2015FS/0491	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml **>4900000cfu/ml**
56.	Chocolate Drink	FQML/2015FS/0495	E coli S.aureus TPC	<10cfu/ml <10cfu/ml **>490000cfu/ml**
57.	Pineapple Drink	FQML/2015FS/0496	Salmonella E coli S.aureus	**Present in 25ml** <10cfu/ml <10cfu/ml
58.	Soda	FQML/2015FS/0502	Salmonella	Absent in 25 ml
59.	Lime juice	FQML/2015FS/0503	Salmonella	Absent in 25 ml
60.	Lemon juice	FQML/2015FS/0504	Salmonella	Absent in 25 ml
61.	Green orange juice	FQML/2015FS/0505	Salmonella	Absent in 25 ml
62.	Lime juice	FQML/2015FS/0507	Salmonella	Absent in 25 ml
63.	Curd	FQML/2015FS/0619	Salmonella E coli TPC	Absent in 25ml <10cfu/ml 63000cfu/ml

64.	Curd	FQML/2015FS/0620	Salmonella E coli TPC	Absent in 25ml <10cfu/ml **3300000cfu/ml**
65.	Curd	FQML/2015FS/0621	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml **490000cfu/ml** 3000cfu/ml
66.	Lime juice	FQML/2015FS/0632	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml 2500cfu/ml
67.	Vanilla Milkshake	FQML/2015FS/0685	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml **490000cfu/ml**
68.	Soda water	FQML/2015FS/0686	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml **490000cfu/ml**
69.	Mango drink	FQML/2015FS/0687	Salmonella E coli S.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml **4900000000cfu/ml**
70.	Chocolate milkshake	FQML/2015FS/0688	Salmonella E coli TPC	**Present in 25ml** <10cfu/ml **>490000cfu/ml**
71.	Strawberry milkshake	FQML/2015FS/0689	Salmonella E coli S.aureus	Absent in 25ml <10cfu/ml <10 cfu/ml
72.	Mango Drink	FQML/2015FS/0690	Salmonella E coli TPC	Absent in 25ml <10cfu/ml **>4900000 cfu/ml**
73.	Mango Juice	FQML/2015FS/0691	Salmonella Staphylococcus aureus TPC	**Present in 25ml** <10cfu/ml **490000000cfu/ml**
74.	Packaged Drinking water	FQML/2015FS/0692	Salmonella Staphylococcus aureus E.coli	Absent in 25ml <10cfu/ml <10cfu/ml

75.	Carbonated drink	FQML/2015FS/0693	Salmonella	**Present in 25ml**
			Staphylococcus aureus	<10cfu/ml
			E coli	<10cfu/ml
76.	Lime juice	FQML/2015FS/0696	Salmonella	Absent in 25ml
77.	Non Alcoholic sweet beer	FQML/2015FS/00723	Salmonella	Absent in 25ml
			E coli	<10cfu/ml
			S.aureus	<10cfu/ml
			TPC	<10cfu/ml
78.	Mango drink	FQML/2015FS/00724	Salmonella	Absent in 25ml
			E coli	<10cfu/ml
			S.aureus	<10cfu/ml
			TPC	**670000cfu/ml**
79.	Grape juice	FQML/2015FS/00728	Salmonella	Absent in 25ml
			E coli	<10cfu/ml
			S.aureus	<10cfu/ml
80.	Lemon juice	FQML/2015FS/00732	Salmonella	Absent in 25ml
			E coli	<10cfu/ml
			S.aureus	10cfu/ml
			TPC	**370000000cfu/ml**
81.	Curd	FQML/2015FS/00745	Salmonella	Absent in 25ml
82.	Mango drink	FQML/2015FS/00751	Salmonella	Absent in 25ml
			S.aureus	<10cfu/ml
			TPC	**7100000cfu/ml**
83.	Lemon drink	FQML/2015FS/00752	Salmonella	Absent in 25ml
84.	Orange drink	FQML/2015FS/00753	Salmonella	Absent in 25ml
85.	Mango drink	FQML/2015FS/00754	Salmonella	Absent in 25ml
86.	Lemon flavoured drink	FQML/2015FS/0809	Salmonella	Absent in 25ml
			S.aureus	<10cfu/ml
			TPC	<10000cfu/ ml
87.	Orange flavoured drink	FQML/2015FS/0810	Salmonella	Absent in 25 ml
			E coli	<10cfu/ ml
			S.aureus	<10cfu/ ml
			TPC	**860000cfu/ml**
88.	Soda cool drink	FQML/2015FS/0822	Salmonella	Absent in 25 ml
			E coli	<10cfu/ ml
			S.aureus	<10cfu/ ml
			TPC	**>150000000cfu/ ml**
89.	Orange juice	FQML/2015FS/0848	Salmonella	Absent in 25 ml
			E coli	<10cfu/ ml
			S.aureus	<10cfu/ ml
			TPC	**5600000000cfu/ml**

90.	Butter milk	FQML/2015FS/0854	Salmonella E coli S.aureus TPC	Absent in 25 ml <10cfu/ ml 450cfu/ ml **91000000cfu/ ml**
91.	Lemon juice	FQML/2015FS/0859	Salmonella S.aureus TPC	Absent in 25 ml <10cfu/ ml **320000cfu/ ml**
92.	Mango Drink	FQML/2016FS/0919	Salmonella E-coli S. Aureus TPC	Absent in 25 ml <10cfu/ ml <10cfu/ ml 64000cfu/ ml
93.	Mango Drink	FQML/2016FS/0920	Salmonella E-coli S. Aureus TPC	Absent in 25 ml <10cfu/ ml 10cfu/ ml **>490000cfu/ ml**
94.	Mango Drink	FQML/2016FS/0921	Salmonella E-coli S. Aureus TPC	Absent in 25 ml <10cfu/ ml <10cfu/ ml 1200cfu/ ml
95.	Curd	FQML/2016FS/0926	Salmonella TPC S.aureus	Absent in 25 ml **21000000cfu/ ml** <10cfu/ ml
96.	Orange Flavoured drink	FQML/2016FS/0936	Salmonella TPC S.aureus	Absent in 25 ml **330000cfu/ ml** <10cfu/ ml
97.	Curd	FQML/2016FS/0990	Salmonella	Absent in 25 ml
98.	Apple Blueberry Juice	FQML/2016FS/0993	Salmonella E-coli S.aureaus TPC	Absent in 25 ml <10cfu/ ml <10cfu/ ml 1800cfu/ ml
99.	Apple Blue berry Juice	FQML/2016FS/0994	Salmonella	Absent in 25 ml
100	Lime Juice	FQML/2016FS/0995	Salmonella E-coli S.aureaus TPC	Absent in 25 ml <10cfu/ ml <10cfu/g ml **490000000cfu/ ml**
101	Lemon Juice	FQML/2016FS/1002	Salmonella	Absent in 25 ml.
102	Lassy	FQML/2016FS/1010	Salmonella E-coli S.aureaus TPC	Absent in 25 ml. 10cfu/ ml <10cfu/ ml**52000000cfu/ ml**

103	Milk Shake	FQML/2016FS/1011	Salmonella	Absent in 25ml
104	Packaged drinking water	FQML/2016FS/1012	Salmonella E-coli S.aureaus TPC	Absent in 25 ml <10cfu/ml <10cfu/ml **21000000cfu/ml**
105.	Pinapple Juice	FQML/2016FS/1017	Salmonella	Absent in 25 ml
106	Sugar cane Juice	FQML/2016FS/1019	Salmonella E-coli S.aureaus TPC	Absent in 25 ml **21cfu/ml** 460cfu/ ml **4900000000cfu/ ml**
107.	Curd	FQML/2016FS/1047	Salmonella E-coli S.Aureus TPC	Absent in 25 ml <10cfu/ ml <10cfu/ ml **5500000000cfu/ ml**
108.	Mango Juice	FQML/2016FS/1096	E-coli S.aureus TPC	<10cfu/ ml <10cfu/ ml **1000000cfu/ ml**
109.	Lime juice	FQML/2016FS/1101	Salmonella E-coli S.aureus TPC	Absent in 25 ml <10cfu/ ml <10cfu/ ml 1×10^{3} cfu/ml
110.	Mixed Fruit Juice	FQML/2016FS/1102	Salmonella S.aureus E.coli TPC	Absent in 25 ml <10cfu/ ml <10cfu/ ml **$>49\times10^{9}$ cfu/ ml**
111.	Lime juice	FQML/2016FS/1145	Salmonella E-coli s.aureus TPC	Absent in 25ml <10cfu/ml <10cfu/ml <10,0000 cfu/ml
112.	Lime juice	FQML/2016FS/1148	S. aureus Salmonella E-coli TPC	<10cfu/ml Absent in 25ml <10cfu/ml 10,000cfu/ml
113.	Curd	FQML/2016FS/1168	Salmonella	Absent in 25ml
114.	Apple Drink	FQML/2016FS/1171	Salmonella	Absent in 25ml
115.	Apple Drink	FQML/2016FS/1172	Salmonella	Absent in 25ml
116.	Lemon Juice	FQML/2016FS/1173	Salmonella	Absent in 25ml
117.	Lime Juice	FQML/2016FS/1205	Salmonella	Absent in 25ml
118.	Lemon flavoured Drink	FQML/2016FS/1208	Salmonella	Absent in 25ml

119.	Lime Juice	FQML/2016FS/1232	Salmonella TPC	**Present in 25ml** **370 ×10^6 cfu/ ml**
120.	Mango Drink	FQML/2016FS/1242	Salmonella TPC S.aureus E-coli	**Present in 25 ml** **1500000cfu/ ml** <10cfu/ ml <10cfu/ ml
121.	Mango Drink	FQML/2016FS/1252	Salmonella TPC	Absent in 25ml **>490000000cfu/ml**
122.	Elaneer Drink	FQML/2016FS/1259	Salmonella E-coli S. aureus TPC	Absent in 25ml <10cfu/ ml <10cfu/ ml **>490000000cfu/ ml**
123.	Fermented Coconut Drink	FQML/2016FS/1260	Salmonella E-coli TPC	**Present in 25ml** <10cfu/ml **930000cfu/ml**
124.	Lychee Juice	FQML/2016FS/1261	Salmonella E-coli TPC S. aureus	Absent in 25ml <10cfu/ml **6800000cfu/ml** <10cfu/ml
125.	Orange Juice	FQML/2016FS/1267	Salmonella E-coli TPC S. aureus	Absent in 25 ml <10cfu/ ml **210000cfu/ ml** <10cfu/ ml
126.	Lemon Juice	FQML/2016FS/1268	Salmonella E-coli TPC S. aureus	**Present in 25 ml** <10cfu/ml **>49000000 cfu/ml** <10cfu/ml
127.	Mango Juice	FQML/2016FS/1269	Salmonella E-coli TPC S. aureus	Absent in 25 ml <10cfu/ ml **>49000000cfu/ ml** <10cfu/ ml
128.	Curd	FQML/2016FS/1285	Salmonella	Absent in 25ml
129.	Grape Juice	FQML/2016FS/1291	Salmonella	Absent in 25ml
130.	Lime Juice	FQML/2016FS/1292	Salmonella	Absent in 25ml
131.	Lemon Juice	FQML/2016FS/1356	Salmonella	Absent in 25 ml
132.	Lemon Drink	FQML/2016FS/1364	Salmonella	Absent in 25 ml
133.	Mango Drink	FQML/2016FS/1365	Salmonella	Absent in 25 ml
134.	Mango Drink	FQML/2016FS/1385	Salmonella S.aureus	Absent in 25 ml 10cfu/ml

135.	Mango Drink	FQML/2016FS/1386	Salmonella	Absent in 25 ml
136.	Lemon Juice	FQML/2016FS/1389	Salmonella	Absent in 25 ml
137.	Curd	FQML/2016FS/1391	Salmonella	Absent in 25 ml
138.	Packaged Drinking water	FQML/2016FS/1394	Salmonella	Absent in 25 ml
139.	Orange Drink	FQML/2016FS/1402	Salmonella	Absent in 25 ml
140.	Mango Drink	FQML/2016FS/1403	Salmonella E-coli TPC S.aureus	Absent in 25 ml <10cfu/ ml **13×10^4 cfu/ml** <10cfu/ml
141.	Orange Juice	FQML/2016FS/1404	Salmonella E-coli TPC S.aureus	Absent in 25ml <10cfu/ml 23000cfu/ml <10cfu/ml
142.	Fruit Drink	FQML/2016FS/1425	Salmonella E-coli TPC S.aureus	Absent in 25ml <10cfu/ml **510000cfu/ml** <10cfu/ml
143.	Sugarcane Juice	FQML/2016FS/1427	Salmonella E-coli TPC S.aureus	Absent in 25ml **1200cfu/ml** **82×10^6 cfu/ml** 170cfu/ml
144.	Lychee Juice	FQML/2016FS/1444	Salmonella	Absent in 25ml
145.	Pineapple Soft Drink	FQML/2016FS/1463	Salmonella	Absent in 25ml
146.	Orange Juice	FQML/2016FS/1464	Salmonella	Absent in 25ml
147.	Apple Drink	FQML/2016FS/1471	Salmonella	Absent in 25ml
148.	Orange Drink	FQML/2016FS/1472	Salmonella	Absent in 25ml
149.	Mango Drink	FQML/2016FS/1473	Salmonella	Absent in 25ml
150.	Lemon Juice	FQML/2016FS/1474	Salmonella	Absent in 25ml
151.	Packaged Drinking water	FQML/2016FS/1523	E.coli	<10 cfu/ml
152.	Mango drink	FQML/2016FS/1526	Salmonella	Absent in 25ml
153.	Lemon Juice	FQML/2016FS/1527	Salmonella	Absent in 25ml
154.	Orange Drink	FQML/2016FS/1528	Salmonella	Absent in 25ml
155.	Curd	FQML/2016FS/1529	Salmonella	Absent in 25ml
156.	Water Melon Juice	FQML/2016FS/1530	Salmonella	Absent in 25ml
157.	Mango Drink	FQML/2016FS/1542	Salmonella	Absent in 25ml

158.	Apple Flavor Drink	FQML/2016FS/1543	Salmonella	Absent in 25ml
159	Lemon Flavor Drink	FQML/2016FS/1544	Salmonella	Absent in 25ml
160	Lemon Juice	FQML/2016FS/1546	Salmonella	Absent in 25ml
161	Grape Juice	FQML/2016FS/1549	Salmonella	Absent in 25ml
162	Guaava Juice	FQML/2016FS/1550	Salmonella	Absent in 25ml
163	Pineapple Juice	FQML/2016FS/1551	Salmonella	Absent in 25ml
164	Sip-up	FQML/2016FS/1552	Salmonella	Absent in 25ml
165	Vanilla Sip-Up	FQML/2016FS/1553	Salmonella	Absent in 25ml
166	Packaged drinking water	FQML/2016FS/1556	E.coli	<10 cfu/ml
167	Juice	FQML/2016FS/1557	Salmonella	Absent in 25ml

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INSECTICIDAL EFFECT OF CRUDE EXTRACTS OF *MANIHOT ESCULENTA* AND *ADENOCALYMMA ALLIACEUM* ON THE THIRD INSTAR LARVAE OF *ORYCTES RHINOCEROS*

Uma Surendran*, Aswathi Anilkumar and **Parvathy Syam**
Department of Zoology, Baselius College, Kottayam
Email: achu.aswathy15@gmail.com

Abstract

*Oryctes rhinoceros***,** known as rhinoceros beetle is one of the major pests of coconut, *Cocos nucifera*, severely damaging the central spindle, spathe and tender shoot. Due to its wide distribution, the species *Oryctes rhinoceros* is the most important and studied pest of coconut. In the present study, an attempt has been made to compare the effect of crude extracts of *Manihot esculenta* and *Adenocalymma alliaceum* in the management of *Oryctes rhinoceros*. 30%, 50%, 75% and 100% solutions of both *Manihot esculenta* and *Adenocalymma alliaceum* were prepared. Among the two extracts *Manihot esculenta* produced maximum death in its 100% concentration compared with 90% mortality caused by *Adenocalymma alliaceum* at its 100% extract concentrations. The use of plants and plant parts as insecticides will benefit our agricultural sector. They are not only of low cost, but have no environmental impact in terms of insecticidal hazard. Therefore, the findings of the current experiments strongly support the use and exploration of plants and plant parts in pest management practices.

Key words: *Manihot esculenta, Adenocalymma alliaceum, Oryctes rhinoceros,* bio pesticide

***Curresponding author** :Assistant Professor, Dept of Zoology,Baselius College, Kottayam
Email : umasurendran88@gmail.com

Introduction

Palms are the most important crops in the tropics. Coconut palm, *Cocos nucifera* is an important plantation crop that produces nuts. The rhinoceros beetle, *Oryctes rhinoceros*, (Family: Scarabaeidae, Sub Famly: Dynastinae) is the most important pest of palm, throughout Indian and South East Asia. The adults are the destructive stage. They damage palms by boring into the centre of the crown, resulting in wedge shaped or "V" cuts in the fronds that unfurl. They damage the meristematic tissues in the crown of the palm. The beetle feeds on exuding tissue juices. As they bore into the crown, they cut through the developing leaves. This causes a decrease in the nut set (Vargo, 2000). Male beetles produce an aggregation pheromone, ethyl-4-methyl octanoate (Halleet *et al*., 1995) that attracts both the male and female beetles, which results in a patchy distribution of beetles within a stand of palms. In palm, *O. rhinoceros* bores into the base of cluster of spears, causing wedge shaped cuts in the unfolded fronds. In younger palms the effect of damage can be much more severe (Wood 1968; 1976). They may provide entry points for the lethal secondary attacks by other pests or pathogens (Bedford, 1980). Trees killed by the adult beetles provide breeding sites for future larval generations (Moore, 2007, Zelazny and Alfiler, 1987).In India, the damage of inflorescence is also reported in severely infested areas which cause reduction in yield up to 10%. From artificially pruned leaf damage stimulation studies, it was observed that damage to 50% fronds corresponds to leaf area reduction of 13% and decrease in nut yield by 23%. Several control measures both mechanical and chemical methods have been devised from time immemorial to control this beetle. But, insecticide has important disadvantages. Insecticide resistance, pest resurgence, and pest replacement can occur following repetitive applications of these compounds. Therefore, we may see decreasing effectiveness with their use.

Moreover, insecticides may have a negative impact on non-target species. Furthermore insecticide present risks to the user. Many insecticide compounds are highly toxic to humans and can injure or kill when applied improperly or when accidents occur (Larry. P. Pedigo, 1996). All these harmful effects of the chemical insecticide are due to its high persistence.

Plants virtually are "nature's chemical factories", providing practically unlimited natural sources of botanical pesticides. Plants contain complex mixture of compounds which then plant sequestered and stored over the years and have formed part of their survival strategy (Yang and Tang, 1987), These compounds may out synergistically (Berenbanm,1955) and become more lethal to pests than the individual components of the mixture (Berenbanm *et al*., 1991; Chen *et al*., 1995). However due to geographical differences in the distribution of biologically active compounds (Errnel *et al*., 1087), field activities of extracts from some plant species on pests of crops are varied (Raymundo and Alcazar, 1983; Olaifa and Adenuga, 1988; Willian, and Mansingh, 1993).

Cassava (*Manihot esulenta*), is a woody shrub of the Euphorbiaceae (spurge family) native to South America, is extensively cultivated as an annual crop in tropical and subtropical regions. It has been known for over 150 years that a wide variety of plants are potentially toxic because they contain a glycoside which releases HCN upon hydrolysis. The glycoside itself is not toxic, but the HCN which is released upon hydrolysis is by an endogenous enzyme may be toxic because its principal site of action is cytochrome oxidase, a key enzyme necessary for the survival of aerobic organisms.The principal cyanogenetic glycoside present in cassava is linamarin, which is a b-glucoside of acetone cyanohydrin: the liberation of HCN is affected by an endogenous enzyme called linamariase. Cassava contained prussic acid (from cynanide chemical group) which have shown lethal effect on some mammals.

Pseudocalymma alliaceum (Bignoniaceae), commonly called as Garlic vine,) is an evergreen tropical shrubby vine that is native to the Amazon rainforest which is reported to contains several of the main sulfur compounds that garlic does.It is these compounds which are responsible for the garlic-like odor and taste of *P. alliaceum*.The leaves and/or flowers of this plant contain the known anti-inflammatory and antibacterial plant steroids beta sitosterol, stigmasterol, daucosterol, and fucosterol. Chemicals reported in ajos sacha thus far include: 24-ethyl-cholest-7-en-3-beta-ol, 3-beta-hydroxy-urs-18-en-27-oic acid, alliin, allyl sulfides, alpha 4-hydroxy-9-methoxy-lapachone, alpha 9-methoxylapachone, apigenins, aspartic acid, beta-sitosterol, beta amyrin, beta-peltoboykinolic acid, cosmosiin, cyanidin-3-o-beta-d-rutinoside, daucosterol, diallyl sulfides, 1-2: 3-vinyl-dithi-4-ene, 1-2: 3-vinyl-dithi-5-ene, dithiacyclopentene, dotriacontan-1-ol, fucosterol, glutamic acid, glycyrrhetol, hentriacontanes, hexacosan-1-ol, hexatriacontans, leucine, luteolin, n-nonacosane, oct-1-en-3-ol, octacosan-1-ol, pentatriacont-1-en-17-ol, scutellarein-7-o-beta-d-glucuronide, stigmasterol, triacontan-1-ol, triallyl sulfides, trithiacyclohexene, n-tritriacontane, and ursolic acid (Devang Pandya *et.al,* 2012)

In the present study an attempt has been made to find the effect of crude extracts of *M. esculenta* and *P. alliaceum* in the management to *O. rhinoceros* by studying its effects on the third instar larva

Materials and Methods

Third instar grubs of *O. rhinoceros* were collected from local manure pits. They were reared in the laboratory and used for the present study. Tender stem and leaves of *M. esculenta* and *P. alliaceum* were collected locally. The crude extracts for the study was prepared using the leaves of *M. esculenta* and *P. alliaceum* and mixed with sun dried cow dung in three different proportions.

Cow dung with 30ml water served as control. 30 ml crude extracts of *M. esculenta* [30%, 50% , 75% and 100% solutions] was thoroughly mixed with cow dung and another set was mixed with 30 ml crude extract of *P. alliaceum* [30%, 50%, 75% and 100% solutions]. Later ten third instar grubs were introduced into small containers with 1kg cow dung plant mixture. The containers were covered with plastic lids with holes for proper aeration. Ten grubs were used for a treatment with each dose of leaf cow dung medium. Daily observations were made on the development of grubs. Larval mortality was recorded for 10 days.

Observation and Results

Daily observation shows that after 5-6 days of inoculation, the larvae treated with *P. alliaceum* become sluggish and often come upon the surface of the feeding and ultimately they died with 7-10 days. After 3 days of of inoculation, the larvae treated with, the larvae treated with *M. esculenta* become sluggish and often come upon the surface of the feeding and ultimately they died with 4-7 days. Finally, the cadavers became brownish black and mummified.

In third instar larvae treated with *M. esculenta* 100% (maximum) mortality of grubs was recorded by the bottle containing 100% solution of crude extract of *M. esculenta* .40%, 60% and 80% rates of mortality occurred in 30, 50 and 75 percent extract treated bottles respectively. (Table. 1)Among the grubs treated with selected concentrations *of* extracts *P. alliaceum*, 90% of grubs were dead in the 100% extract treated bottle. Interestingly, maximum deaths occurred between the 7^{th} and 9th day of incubation. 20%, 40%, 60% rates of mortality occurred in 30, 50 and 75 percent extract treated bottles respectively. (Table 2) (Figure 1).

Table 1: Mortality of *P. esculenta* treatedgrubs in a period of 10 days,expressed as number of deaths.

Days	Percentage solution of extract			
	30%	50%	75%	100%
1				
2				
3				
4	2	1	1	4
5	2	4	2	3
6		1	4	2
7			1	1
8				
9				
10				

Table 2: Mortality of *P. alliaceum* treatedgrubs in a period of 10 days,expressed as number of deaths.

Days	Percentage solution of extract			
	30%	50%	75%	100%
1				
2				
3				
4				
5				
6				
7			2	2
8		2	1	4
9	2	1	2	3
10		1	1	

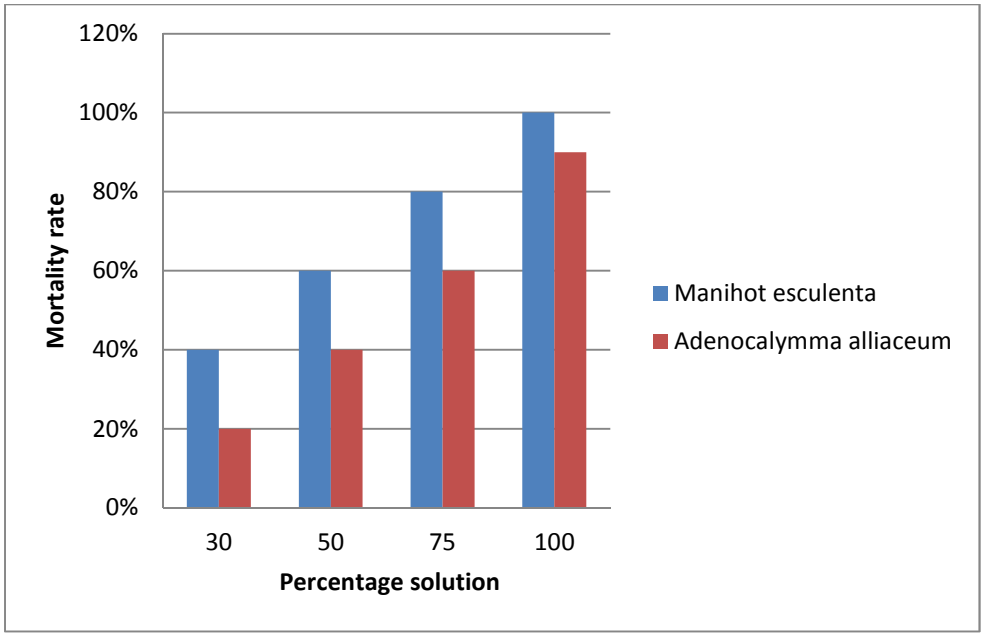

Figure 1: Multiple bar diagram showing mortality rate at various concentration of plant extracts

Discussion and Conclusion

There are numbers of naturally occurring compounds that possess plant protection properties. Already 10000 secondary metabolites have been chemically identified. In nature, many plants have unpalatable substances like high content of phenols, alkaloids, flavanoids, terpenes, quinone, coumarin etc., which play a defensive role against insect pests. These substances possess wide range of biological activities including antifeedant, insecticidal, and insect growth regulators. Identifying sources with useful biological activity is only the starting point in the long process of development of a botanical pest management product. Success of botanical in the field depends on number of factors such as, ongoing availability of the natural resources, adequate biomass to justify extraction, the feasibility of extraction near the harvest site and the stability of the extract in storage after preparation.

In the present study, *M.esculenta* produced maximum death, 100% in its 100% concentrations compared with 90% mortality caused by *P.alliaceum* at its 100% extract concentrations.

Fastest mortality was shown by 100% extract of *M.esculenta*, which produced 100% mortality within 4-7 days of treatment. The main toxic principle which occurs in varying amounts in all parts of the *M.esculenta* is a chemical compound called linamarin (Nartey, 1981). It often coexists with its methyl homologue called methyl-linamarin or lotaustralin. Linamarin is a cyanogenic glycoside which is converted to toxic hydrocyanic acid or prussic acid when it comes into contact with linamarase, an enzyme that is released when the cells of *M.esculenta* are ruptured. Ingested linamarin can liberate cyanide in the gut during digestion. *M.esculenta* also contains hydrocyanic acid or HCN. The normal range of cyanogen content of *M.esculenta* falls between 15 and 400 mg HCN/kg fresh weight (Coursey, 1973). The concentration varies greatly between varieties and also with environmental and cultural conditions. C. A. Jayaprakas reported larvicidal activity of *M.esculenta* on the fourth instar larvae of Spilarctia (Spilosoma) obliqua (Bihar hairy caterpillar)

In the third instar larvae treated with 100% *P.alliaceum* extract, 90% mortality was observed between 7^{th} and 9^{th} day .Shrankhla et al. reported that larvicidal activity of the hexane extract of *P. alliaceum* exhibited potential larvicidal against *Culex quinquefasciatus* and *Anopheles stephensi*. Alagarmalai Jeyasankar et al. reported antifeedant, insecticidal activity against *Spodoptera litura* and *Helicoverpa armigera*. The plant species have chemical compounds with various active ingredients which make them useful, according with Harnafi and Amrani reported that organic extracts of *P. alliaceum* contain

alkanes, alkanols, triterpenes, flavonoids and derivatives of lapachol and mentioned that plants containing flavonoids showed platelets action in the prevention of thrombosis. Reports have indicated that higher percentage of insecticidal activity was observed in seeds extract of *P. alliaceum*. Similar works have already reported insecticidal activity of many plants and their compounds against different groups of insects (Hashim M.S 2003). Insect growth regulation properties of plant extracts are very interesting and unique in nature, since insect growth regulator works on juvenile hormone. The enzyme ecdysone plays a major role in shedding of old skin and the phenomenon is called ecdysis or moulting. When the active plant compounds enter into the body of the larvae, the activity of ecdysone is suppressed and the larva fails to molt, remaining in the larval stage and ultimately dying (Lajide L. *et al.* 1993)

Among the two plant extracts investigated, both of them showed potential as insecticide.Moreover, it can be observed that the rate of mortality increases with increasing concentration. Further studies are required to exact the insecticidal properties of the above mentioned plant extracts. Hence this work could be considered as a preliminary study on the potential of the four selected plant extracts as insecticides and could provide as excellent platform for future research on management of the rhinoceros beetle.

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INSECTICIDAL EFFECT OF CRUDE EXTRACTS OF *CHROMOLAENA ODORATA* AND *ALLIVUM SATIVUM* ON THE THIRD INSTAR LARVAE OF *ORYCTES RHINOCEROS*

Uma Surendran*, Parvathy Syam and **Aswathi Anilkumar**
Department of Zoology, Baselius College, Kottayam
Email: talk2parvathysyam@gmail.com

Abstract

The Asiatic rhinoceros beetle or coconut rhinoceros beetle (*Oryctes rhinoceros*) is a species belonging to the Scarabaeidae family. O. *rhinoceros* is one of the most damaging insects to palms in Asia and the Pacific Islands. It is one of the major pests of coconut, *Cocos nucifera*. Adults eat the leaves and burrow into the crown, stunting plant development. Due to its wide distribution, the species *O. rhinoceros* is the most important and studied pest of coconut. In the present study, an attempt has been made to compare the effect of crude extracts of *Chromolaena odorata (Eupatorium odorata*) and *Allivum sativum* in the management of *O. rhinoceros*. Crude extracts of *C. odorata* and *A. sativum* {30%, 50%, 75% and 100%] was thoroughly mixed with cow dung and fed to third instar larvae of *O. rhinoceros*.At 100 % a maximum mortality of 100% and 90% was observed for *C. odorata* and *A. sativum* respectively . Botanical products are useful tools in many pest management programmes because they are effective and specifically target plant's natural enemies. Therefore, the findings of the current experiments strongly support the use and exploration of plants and plant parts in pest management practices.

Key words: *Chromolaena odorata, Allivum sativum, Oryctes rhinoceros,* bio pesticide

***Currasponding author:**Assistant Professsor, Dept of Zoology, Baselius College, Kottayam
E mail: umasurendran88@gmail.com

Introduction

Oryctes rhinoceros (L.), the coconut rhinoceros beetle, is a pest species occurring throughout many tropical regions of the world. Adults can cause extensive damage to economically important wild and plantation palms. Adults eat the leaves and burrow into the crown, stunting plant development. Nut production decreases leading to low yield. Decreased nut production brings about great economic loss to the farmers. Coconut palms are grown in more than 90 countries of the world, with a total production of 61 million tonnes per year. Most of the world production is in tropical Asia, with Indonesia, the Philippines, and India accounting collectively for 73% of the world total (Nirula,1955).

Excessive use of synthetic fertilizers and pesticides has caused tremendous harm to the environment as well affects human population indirectly. Continues use also results in developing resistance of the pest, which become difficult to control by other means. The use of synthetic chemical fertilizers leads to imperfectly synthesized protein in leaves, which is responsible for poor crops and in turn for pathological conditions in humans and animals fed with such deficient food. An ecofriendly alternative to chemical pesticides is biopesticides, which encompasses a broad array of microbial pesticides, biochemicals derived from micro-organisms and other natural sources. The potential benefits to agriculture and public health programmes through the use of biopesticides are considerable. The interest in biopesticides is based on the disadvantages associated with chemical pesticides are discussed. . Biopesticides being target pest specific are presumed to be relatively safe to non-target organism including humans. More than 2000 plant species have been known to produce chemical factors and metabolites of value in pest control programme. (Sreelatha C,and Geetha P.R,2011)

The present investigation aims to find out the possibility of eco-friendly management of the pest *O. rhinoceros* by incorporating a commonly seen weed *C.odorata* and *A.sativum* in its culture medium. The leaves and stems of *C. odorata* revealed the presence of essential oils, steroids, triterpenes and flavonoids. It has been reported to have antispasmodic, antiprotozoal, antitrypanosomal, antibacterial and antihypersensitive activities. It has also been reported to possess anti-inflammatory, astringent, diuretic and hepatotropic activities .The chemical volatile compounds contained in the leaf extracts of *C. odorata*, such as phytol, caryophyllene oxide, germacrene D,dodecyl acetate,oleic acid methyl ester, β-caryophyllene, di-n-octyl phthalate, α-terpineol, α-cubebene and hexadecanoic acid methyl ester have been responsible for the insecticidal property. Plant extracts containing a large amount of phytol, n hexadecanoic acids and caryophyllene oxide were known to exhibit insecticidal activities against Coleopteran (Oladipupo *et al*., 2015). The biocidal properties of garlic and related plants are attributed to the volatile substances derived from sulfur amino acids (Auger *et al*., 2002). The primary compounds emitted are thiosulfinates and zwiebelanes are converted in *Allium* products (extracts) to disulfides. Available data have shown that sulfur compounds in *Allium* can be classified not only as insecticides, acaricides, nematicides, herbicides, fungicides and bactericides, but also repellents against arthropods. The insecticidal activity of *Allium* plants, plant extracts and *Allium* sulfur volatiles has been widely studied (Nasseh, 1981; Hori, 1996; Lundgren, 1975; Weissling *et al.*, 1997; Nasseh, 1992; Amonkar, 1970; Renapurkar, 1984; Bhatnagar-Thomas *et al*., 1974; Flint *et al*., 1996; Ho *et al*., 1996) and many studies assessed repellent effects of plant sulphur compounds (Propoky et al., 1983; Bhuyan et al., 1974; Trematerra et al., 1999). The study of the negative effects of Allium has included research on insect physiology (Nasseh, 1981; Suryakala *et al.*, 1984).

Research for assessing the insecticidal properties possessed by plants is going on all over the world. The current study was undertaken to test the insecticidal property of two common plants, *C. odorata* and *A. sativum* with a view to control the coconut pest, *O. rhinoceros* using these plants. Hence in the study the effects of feeding the third instar larvae of *O.rhinoceros* with the crude extracts of leaves of *Chromoleana odorata* and bulbs of *Allium sativum* were evaluated.

Materials and Methods

Third instar grubs of *O. rhinoceros* were collected from local manure pits. They were reared in the laboratory and used for the present study. Tender stem and leaves of *C. odorata* were collected from our college campus and cloves of *A. sativum* were obtained from local market. The crude extracts for the study was prepared using the leaves of *C. odorata* and clove of *A. sativum* and mixed with sun dried cow dung in three different proportions. Cow dung with 30ml water served as control. 30 ml crude extracts of *C. odorata* [30%, 50% , 75% and 100% solutions] was thoroughly mixed with cow dung and another set was mixed with 30 ml crude extract of *A. sativum* [30%, 50% , 75% and 100% solutions] . Later ten third instar grubs were introduced individually into small containers with 1kg cow dung plant mixture. The containers were covered with plastic lids with holes for proper aeration. Ten grubs were used for a treatment with each dose of leaf cow dung medium. Daily observations were made on the development of grubs. Larval mortality was recorded for 10 days.

Observation and Results

Daily observation shows that after 5-6 days of inoculation, the larvae treated with*A.sativum* become sluggish and often come upon the surface of the feeding and ultimately they died within 7-10 days.

After 3 days of inoculation, the larvae treated with *C. odoratum* become sluggish and come up to the surface of the feeding and ultimately died within 4-7 days. In third instars larvae treated with *A. sativum* 100% (maximum) mortality of grubs was recorded by the bottle containing 100% solution of crude extract of. *A. sativum* .20%, 40% and 60% rates of mortality occurred in 30, 50 and 75 percent extract treated bottles respectively (Table.1).

Table 1: Mortality of *Allium sativum* treatedgrubs in a period of 10 days, expressed as number of deaths.

Days	**Percentage solution of extract**			
	30%	**50%**	**75%**	**100%**
1				
2				
3				
4				
5				
6				
7				1
8			1	4
9		2	3	2
10	2	2	2	3

Among the grubs treated with selected concentrations *of* extracts *C odoratum* 80% of grubs were dead in the 100% extract treated bottle. Interestingly, maximum deaths occurred between the 4^{th} and 6^{th}day of incubation. 20%, 60%, 70% rates of mortality occurred in 30, 50 and 75 percent extract treated bottles respectively (Table 2).

Table 2: Mortality of *Eupatorium odoratum* treatedgrubs in a period of 10 days, expressed as number of deaths.

Days	Percentage solution of extract			
	30%	**50%**	**75%**	**100%**
1				
2				
3				
4			4	2
5		2	2	3
6		4		3
7	2		1	
8				
9				
10				

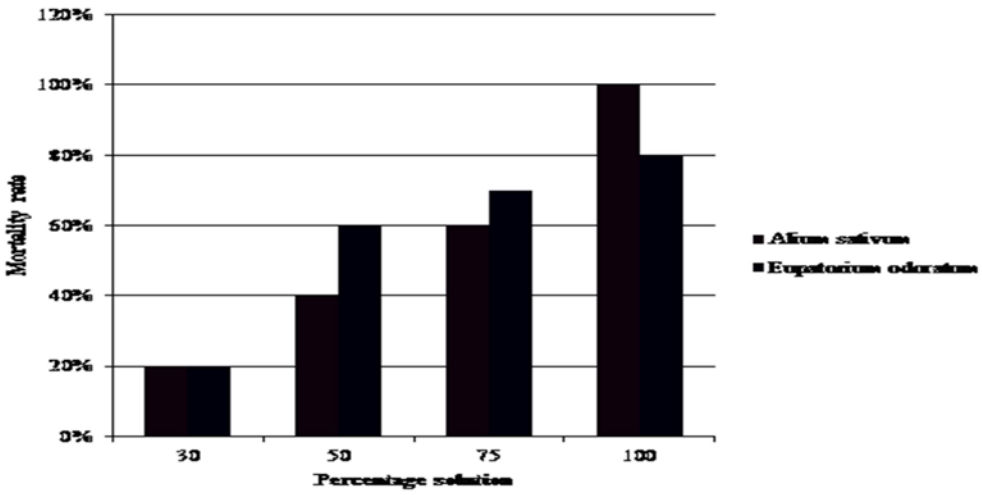

Figure 1: Multiple bar diagram showing mortality rate at various concentration of plant extracts

Discussion and Conclusion

Insect pests play a major role in damaging the agricultural crops and loss varies between 10% and 30% for major crops. India basically an agro –based country and more than 80% of population depends on agriculture and Indian economy is largely determined by agricultural productivity. The intensification of agriculture to fulfill food needs has increased the number of insect pest species attacking different crops, resulting in the annual production losses of the standing crops. In the past, synthetic pesticides have played a major role in crop protection programmes and have immensely benefited mankind. Nevertheless the indiscriminate use of synthetic pesticides has resulted in the development of resistance by pests (insects, weeds, etc.), resurgence and outbreak of new pests, toxicity to non- target organisms and hazardous effects on the environment endangering the sustainability of ecosystems. Among current alternative strategies aiming at decreasing or minimizing the use of chemical insecticides, eco-chemical control based on plant-insect relationships is one of the most promising methods. Plant derived chemicals offer a more natural and environmentally friendly approach to pest control than synthetic pesticides.

Chemical insecticides pose a great problem to the ecosystem due to its high persistence and residual action. Hence it is the need of the hour for a less persistent and safer alternative. A number of plant extracts has been reported which have notable insecticidal and antifeedant properties. Plants based extracts are generally less persistent and are hence easily decomposed. Thus the problem produced by the persistent insecticide like killing of non-target species, development of resistance against the insecticide by the pest, biomagnification etc. are minimal in the case of a biopesticide.

The present study investigated the insecticidal effects of two selected plant extracts on the third instar larvae of rhinoceros beetle, *O. rhinoceros.*

Among the two extracts, *A. sativum* produced maximum death, 100% in their 100% concentrations compared with 80% mortality caused by *C. odorata* at their 100% extract concentrations.In the present study, *A. sativum* caused a maximum mortality of 100% at 100% concentration between 7th and 10th day. The major active principles contained in the garlic bulb are mainly sulphur compounds such as Allicin, Di-allyl-disulphide, Allim (Khritchevsky, 1991) (29), thioacrolein, ajoene, 2-propene sulfenic acid, 2-propene thiol and propylene (Jain *et al*., 1993) (28). Plant lectins have been reported to affect survival and development of insect pest (Ferry *et al*., 2004) (20). The *A.sativum* leaf lectin (ASAL) has been reported to reduce pupal weight, pupal period, pupation and adult emergence of the pod borer, *Helicoverpa armigera* (Lepidoptera: Noctuidae) (Arora *et al*., 2005)(4). The insecticidal activity of carbohydrate-binding plant lectins against different insects belonging to the orders Coleoptera, Diptera, Lepidoptera and Homopterahave been well studied (Gatehouse *et al*., 1995(21); Schuler *et al*., 1998(49): Carlini *et al*., 2002(15)). The binding of ASAL to gut receptors may decrease the permeability of the membrane, thus affecting insects (Bandyopadhay *et al*., 2001). Denloye and Makanjuola and Denloye et al. have reported the insecticidal potency of the aqueous extracts of *A. sativum* against *Sitophilus zeamais* and *Anopheles* species. *A. sativum* like other plants with essential oils having ovicidal effects (I. Tunc *et al*., 2000)(57).

The chemical volatile compounds contained in the leaf extracts of *C. odorata* L, were. phytol,caryophyllene oxide,germacrene D,dodecyl acetate,oleic acid methyl ester, β-caryophyllene, di-n-octyl phthalate,α-terpineol,α-cubebene

and hexadecanoic acid methyl ester.The bioactivity of the plant extract could be due to the major compound or asynergy between the major and minor constituents. One or more compounds present in the extracts may have been responsible for the observed insecticidal property. Plant extracts containing a large amount of phytol, n hexadecanoicacids and caryophyllene oxide were known to exhibit insecticidal activities against Coleopteran (Oladipupo et al., 2015)(42).Aqueous extract of *C. odorata* exhibited the presence of polyphenol oxidase. Plant polyphenol oxidases (PPOs) are widely distributed and well-studied oxidative enzymes.Some mechanisms have been proposed by which PPO might affect insects. They are redox cycling of quinones which is generated by PPO may produce oxidative stress in the gut lumen of insects and phenolic oxidation products, such as quinones and reactive oxygen species (hydrogen peroxide) generated by quinone redox cycling, could be absorbed and have toxic effects on insects. The activity of ingested PPO is dependent on the chemical environment of the insect gut, such as oxygen and phenolic substrate levels, reductants, inhibitors and pH (Constabel et al.,2000)(17). It is known to cause decreased growth rate and eventual death of the insect.

Among the two plant extracts investigated, both of them showed potential as insecticides, especially *Allium sativum*. Moreover, it can be observed that the rate of mortality increases with increasing concentration. Further studies are required to exact the insecticidal properties of the above mentioned plant extracts. Hence this work could be considered as a preliminary study on the potential of the four selected plant extracts as insecticides and could provide as excellent platform for future research on management of the rhinoceros beetle.

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PRELIMINARY PHYTOCHEMICAL ANALYSIS OF SELECTED PLANTS OF THE FAMILY NYCTAGINACEAE

Minu Elizabeth Paul and **Nisha P.**
Department of Botany, St Xavier's College for Women Aluva

Abstract

Phytochemicals are naturally occurring bioactive compounds that are found in plants, including the medicinal plants, vegetables, fruits, flowers, leaves, roots and fibers. They act as a defense system against diseases or more accurately protect plants against diseases. Phytochemical techniques plays a significant role in searching resources and raw materials and for pharmaceutical industry. The medicinal values of the plants lie in bioactive phytochemical constituents that produce definite physiological actions on the human and animal body. Some of the most important bioactive phytochemical constituents are the glycosides, alkaloids, flavonoids, tannins, steroids, terpenoids, essential oils and phenolic compounds. Preliminary Phytochemical analysis are helpful in finding and locating chemical components which are source of pharmacologically active principles. Mostly herbal plants used in traditional medicine consist of wide range of bioactive compounds that can be used as alternative therapeutic tools for the prevention or treatment of many contagious diseases.

The aim of present study is to investigate the phytochemicals which are present in the leaves of *Boerhaavia diffusa,* which is perennial creeping weedand stem bark of *Bougainvillea spectabilis* which is a woody thorny shrub.Present study revealed the presence of alkaloids, phenols, tannins, phytosterols etc.

Keywords: Phytochemical, *Boerhaavia diffusa,Bougainvillea spectabilis*

Introduction

Medicinal plants have been of age long remedies for human diseases because they contain components of therapeutic value. Some of them are also used for prophylactic purposes. An increasing interest in herbal remedies has been observed in several parts of the world and many of the herbal remedies have been incorporated into orthodox medicinal plant practice. Diseases that have been managed traditionally using medicinal plants include malaria, epilepsy, infantile convulsion, diarrhea, dysentery, fungal and bacterial infections. Medicinal herb is considered to be a chemical factory as it contains multitude of chemical compounds like alkaloids, glycosides, saponins, resins, oleoresins, sesquiterpene, lactones and oils (essential and fixed) (Amrit pal Singh, 2005). India has one of the oldest, richest and most diverse cultural traditions associated with the use of medicinal plants.

According to an estimate, 120 or so plant based drugs prescribed for use through the world come from just 95 plant species. Natural antimicrobials can be derived from plants, animal tissues and microorganisms. Phytochemicals have been recognized as the basis for traditional herbal medicine practiced in the past and currently used in different parts of the world. In the search for phytochemicals that may be of benefit to the pharmaceutical industry, researchers sometimes follow leads provided by local healers in a region. Following such leads, plant parts are usually screened for phytochemicals that may be present. The presence of a phytochemical of interest may lead to its further isolation, purification and characterization. Then it can be used as the basis for a new pharmaceutical product.

Boerhaavia diffusa is a perennial creeping weed, prostrate or ascending herb, up to 1m long or more, having spreading branches. The roots are stout and

fusiform with a woody root stock. The stem is prostrate, woody or succulent, cylindrical, often purplish, hairy, and thickened at the nodes. Leaves are simple, thick, fleshy, and hairy, arranged in unequal pairs, green and glabrous above and usually white underneath. The shape of the leaves varies considerably – ovate-oblong, round, or subcordate at the base and smooth above. Leaves are up to 5.5x3.3 cm^2in area. Flowers are minute, subcapitate, present 4–10 together in small bracteolate umbels, forming axillary and terminal panicles.

Bougainvillea spectabilis grows as a shrub or thorny, woody vine reaching upwards of 12 meters tall and 7 meters wide. The leaves are simple and alternate, oval in shape, tapering to a point. Leaf size ranges from 4-13 cm long and 2-6 cm wide. These bracts vary in color from magenta and purple to orange, white and yellow. The plant is evergreen where rainfall occurs all year, and deciduous where a dry season occurs.

The aim of present study is to investigate the phytochemicals present in the leaves of *Boerhaavia diffusa* and stem bark of *Bougainvillea spectabilis.*

Materials and methods

Preparation of plant extract

The fresh leaves of *Boerhaavia diffusa* and stem bark of *Bougainvillea spectabilis* were collected and the materials were washed with distilled water and dried. The dried samples was milled into powder. The powder was stored in a sample tube and kept in refrigerator for further analysis plant extract was prepared out using deionized water and the filtered extract was subjected for phytochemical analysis. Phytochemical examinations were carried out for all the extracts as per the standard methods.

1 Detection of alkaloids

Dragendroff's Test: Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.

2 Detection of amino acids

Ninhydrin test: The extracts 0.25% ninhydrin reagent was added and boiled for a few minutes. Formation of the blue color indicates the presence of amino acid.

3 Detection of carbohydrates

Molisch's Test: Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. Formation of the violet ring at the junction indicates the presence of Carbohydrates.

4 Detection of flavanoids

A portion of crude powder was heated with 10 ml ethyl acetate over a steam bath for 3 minutes. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution (was observed a yellow coloration).

5 Detection of phytosterols

Salkowski's Test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

6 Detection of phenols

Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

7 Detection of proteins

Biurets Test: The extract was treated with 1 ml of 10% sodiunm hydroxide solution and heated drop of 0.7% copper sulphate solution to the above mixture was added.The formation of purpish violet color indicates the presence of proteins.

8 Detection of tannins

0.5g of the crude powder was stirred with 10 ml of distied water.This was filterd and ferric chloride reagent was added to the filterate a blue-black precipitate was taken as evidence for the presence of tannins.

9 Detection of terpenoids

The extract was mixed with 2 ml of chloroform and concentrated sulpuric acid (3 ml) is carefully added to form a layer. A reddish brown coloration of the interface is formed to show a positive result of the presence of terpenoids.

Results

Results obtained for qualitative screening of phytochemicals in leaves of *Boerhaavia diffusa* and stem bark of *Bougainvillea spectabilis*are presented in table 1.

Table 1

Sl:No	Phytochemical constituents	*Boerhaavia diffusa*	*Bougainvillea spectabilis*
1	Alkaloids	+	+
2	Amino acids	+	+
3	Carbohydrates	+	+
4	Flavanoids	+	-
5	Phytosterols	+	-
6	Phenols	-	+
7	Proteins	+	-
8	Tannins	-	-
9	Terpenoids	-	+

This study has revealed the presence of phytochemicals considered as active medicinal chemical constituents. Important medicinal phytochemicals such as terpenoids, reducing sugar, flavonoids, alkaloids were present in the samples.

The presence alkaloids,amino acids and carbohydrates were found in leaves of *Boerhaavia diffusa* and stem bark of *Bougainvillea spectabilis.* Presence offlavanoids, phytosterols, proteins were found only in leaves of *Boerhaavia diffusa*and these components were absent in stem bark of *Bougainvillea spectabilis.* Phenols and terepenoids were present in stem bark of *Bougainvillea spectabilis* and tannins were absent in both the plant sample.

Conclusion

The selected ten medicinal plants are the source of the secondary metabolites i.e., alkaloids, flavonoids, terpenoids and reducing sugars. Medicinal plants play a vital role in preventing various diseases. The anti-

diuretic, anti-inflammatory, anti-analgesic, anticancer, anti-viral, anti-malarial, anti-bacterial and anti-fungal activities of the medicinal plants are due to the presence of the above mentioned secondary metabolites. Medicinal plants are used for discovering and screening of the phytochemical constituents which are very helpful for the manufacturing of new drugs. The previous phytochemical analysis and present studies show nearly the similar results due to the presence of the phytochemical constituents. The phytochemical analysis of the medicinal plants are also important and have commercial interest in both research institutes and pharmaceuticals companies for the manufacturing of the new drugs for treatment of various diseases.

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HONEY AS A COMPLEMETARY THERAPEUTIC PRODUCT OF HONEY BEES

Leena Alexander[1] and **Thilagavathy Daniel**[2]
[1]Jyoti Nivas College Autonomous, Bengaluru, Karnataka
[2]Retired Professor, Gandhigram Rural Institute-Deemed University, Tamil Nadu

Introduction

Honey is a natural complex product of the hive produced by honey bees mostly from nectars extracted from the nectarines of flowers (Adebiyi *et al*., 2004). Freshly collected honey is an aromatic viscous liquid. Honey has a complex chemical composition and varies in colour from pale yellow to dark amber according to the season and the geographical conditions (Schmidt, 1996 and Adriana *et al*., 1999).

Honey is considered to be a good antioxidant, which forms a defense against free radicals and reactive oxygen species (ROS) such as hydroxyl radical, superoxide, nitric oxide, hydrogen peroxide and various lipid peroxides (Percival, 1996). Natural antioxidants can be phenolic compounds (tocopherol, flavonoids and phenolic acids) as reported by Al-Mamary *et al* (2002), Yao *et al* (2003b), Aljadi and Kamaruddin (2004) and Berette *et al* (2007); nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines) or carotenoids as well as ascorbic acid (Hudson, 1990 and Hall and Cuppet, 1997). Polyphenolic compounds and flavonoids are very efficient scavengers of free radicals because of their molecular structures, which include an aromatic ring with hydroxyl groups containing mobile hydrogens (Aruoma, 1994 and Atrooz *et al*., 2008. Research indicates that honey contains numerous phenolic and non-phenolic antioxidants, the amount and type of which depends largely upon the floral source of the honey (Yao *et al*., 2003 and Noor *et al*., 2014). The

darker honeys are generally higher in antioxidant content than the lighter honeys (Gheldof *et al*., 2003 and Schramm *et al*., 2003).

The medicinal properties of honey have been well documented since ancient times. Ayurveda describes honey as the nectar of life and recommends its use in various ailments and it has been used in treatment of surgical wounds, burns and ulcers (Subrahmanyam, 1991 and Zumla and Lulat, 1989) and its antibacterial and antifungal properties have been evaluated by Molan (1992), Cooper (2008), Gulfraz (2010) and Mandal and Mandal (2012).The use of honey in therapy is now enjoying a renaissance as an alternative or complementary therapy for many microbial infections that have become resistant to several antibiotics, however, the carefully selected honey has been used for treatment and as a prophylaxis in many disorders (Dixon, 2003). It is because it offers broad-spectrum antimicrobial properties that it promotes rapid wound healing (Cooper, 2008 and Gulfraz, 2010).

Methodology:

A) Estimation of Total Polyphenolic Content (TPC)

The method of Wolfe *et al*. (2003) was adopted to determine **total phenolic contents** of honey samples. A reaction mixture of 2.5 ml of 10% (v/v) Folin-Ciocalteu reagent and 2 mL of 7.5% (w/v) of sodium carbonate was added to 1 ml of the honey samples. The mixture was vortexed and incubated at 40°C for 30 min after which the absorbance was measured at 765 nm. The **total polyphenolic content** was calculated from the equation obtained from the calibration curve of Rutin and expressed as mg/ml RE (Rutin equivalent). Where ‘x’ is the absorbance and ‘y’ is the Rutin equivalent .

B) Estimation of Total Flavonoid Content (TFC)

Total flavonoids content of honey samples was determined by the modified method of Ordonez *et al*. (2006). One ml of the honey sample was mixed with 1 ml of 2% (w/v) Aluminium chloride prepared in ethanol and left in the dark at room temperature for 1 h. A yellow colour was observed which was measured spectrophotometrically at 420 nm. The total flavonoid content was calculated from the standard calibrated curve and was expressed as mg/ml RE (Rutin equivalent)

C) Determinationof Free Radical scavenging activity: Nitric oxide scavenging activity

Nitric oxide scavenging activity was spectrophotometrically analyzed using the method described by Marcocci *et al* (1994) to estimate the antioxidant property of all the 25 honey samples. To each tube containing 1 ml of honey samples 2.0 ml of sodium nitroprusside (10 mM) in phosphate buffer saline was added. The samples were incubated at room temperature for 150 minutes. The similar procedure was repeated with methanol as blank which served as control. After the incubation, 5 ml of Griess reagent was added to each tube including control. The absorbance of chromophore formed was measured at 546 nm on UV-visible spectrometer.

The percentage of inhibition was calculated using the given formula:

Percentage of inhibition= $(A_{control} - A_{sample}/A_{control}) \times 100$. Where $A_{control}$ = absorbance of the control and A_{sample} = the absorbance of honey sample.

D) Anti-microbial activity

Antimicrobial study was carried out against a Gram positive and a Gram negative bacterial species. Grampositive bacterium used was *Staphylococcus aureus* (ATCC 25923) and Gram negative bacterium used was *Escherichia coli* (ATCC 25922). Antimicrobial activity of all the twenty five honey samples was assessed using the standard antimicrobial procedures.

To determine the Minimum Inhibitory Concentration (MIC), different concentrations of honey (v/v) were prepared in a Muller-Hinton Broth (MHB) medium with pH 7 by serial dilution to give final concentrations of 40, 50, 60, 70, 80, 90 and 100 percent. The control used was the ampicilline antibiotic discs.

Results

Twenty five samples of unprocessed honey were collected for the present study. The place of honey collection, botanical source, type of honey and the source is given in Table 1. The 25 honey samples have been collected from 25 places of the 12 districts belonging to diverse eco-habitats, and agro-climatic regions of Karnataka State. The colour of the honey samples ranged from pale yellow, golden yellow, light brown, brown, reddish and dark brown as mentioned in the Table 1 and as seen in Plate 1. Eleven unifloral honey samples were collected, such as mango honey, eucalyptus honey, arecanut honey, rubber honey, coffee honey, litchi honey, konje honey, jamun honey, acacia honey, sunflower honey and soapnut honey. The colours are given in Table 1. Based on the source they are classified as unifloral honey and multifloral honey.

Table 1: Classification of the honey samples 1 to 25 collected from different eco-habitats

Sl. No	Type of honey	Code name of sample	Place of honey collection	Botanical source	Colour of the honey
01.	Multifloral honey from plain	MFP-S1	Bangalore urban (GKVK)	Several botanical source	Golden brown
02.	Multifloral honey-plain	MFP-S2	B'lore rural (Doddaballapur)	Several botanical source	Golden brown
03.	Eucalyptus honey-plain	UFP-S3	B'lore urban (Koramangala)	*Eucalyptus spp.*	Pale yellow
04.	Mango honey-plain	UFP-S4	Davanagere (Jagalur)	*Mangifera indica*	Pale yellow
05.	Arecanut honey-hills	UFM-S5	Uttara Kannada (Yellapur)	*Areca catechu.*	Reddish
06.	Multifloral Forest honey	MFF-S6	Chamrajnagar (BR hills)	Several botanical source	Dark brown
07.	Multifloral forest honey	MFF-S7	Kodagu (Virajpet)	Several botanical source	Dark brown
08.	Multifloral honey -hills	MFM-S8	Dakshina Kannada (Puttur)	Several botanical source	Brown
09.	Multifloral honey-hills	MFM-S9	Uttara Kannada (Sirsi)	Several botanical source	Reddish brown
10.	Multifloral honey-hills	MFM-S10	Uttara Kannada (Ankola)	Several botanical source	Golden yellow
11.	Rubber honey-hills	UFM-S11	Dakshina Kannada (Sullia)	*Hevea braziliensis*	Golden yellow
12.	Litchi honey-hills	UFM-S12	Dakshina Kannada (Puttur)	*Litchi chinensis*	Light brown
13.	Konje honey-hills	UFM-S13	Dakshina Kannada (Sullia)	*Casearia graveolens*	Golden yellow

14.	Multifloral Forest honey	MFF-S14	Kodagu (Bhagamandala)	Several botanical sources	Brown
15.	Naerale/jamun honey-hills	UFM-S15	Uttara Kannada (Sirsi)	*Syzygium* spp.	Golden
16.	Multifloral Forest honey	MFF-S16	Chikmagalur (Thathkola village)	Several botanical sources	Golden yellow
17.	Coffee honey-hills	UFM-S17	Chikmagalur (Mudigere)	*Coffea arabica*	Yellow
18.	Multifloral honey-hills	MFM-S18	Hassan (Sakleshpur)	Several botanical source	Brown
19.	Multifloral honey-plain	MFP-S19	Hassan(Arasikere)	Several botanical sources	Golden yellow
20.	Acacia honey-hills	UFM-S20	Shimoga (Sagar)	*Acacia* spp.	Pale yellow
21.	Multifloral honey-plain	MFP-S21	Kolar (Chintamani)	Several botanical sources	Pale yellow
22.	Multifloral honey-hills	MFM-S22	Shimoga (Soraba)	Several botanical sources	Light brown
23.	Sunflower honey-plain	UFP-S23	Chitradurga (Mettikurki)	*Helianthus annus*	Orangish yellow
24.	Soapnut honey-hills	UFM-S24	Uttara Kannada (Siddapura)	*Sapindus* spp.	Golden yellow
25.	Multifloral forest honey	MFF-S25	Dakshina Kannada (Belthangadi)	Several botanical sources	Brown

UFP=unifloral honey from plains UFM=unifloral honey from hills
MFP=multifloral honey from plains
MFM=multifloral honey from hills and MFF=multifloral honey from forests

Plate 1: Variously coloured honey samples from different eco-habitats of Karnataka

Polyphenolic and flavonoid content

The results of the comparison of the quantitative analyses for the total polyphenolic content and the total flavonoid content of the 25 honey samples collected from various agro-climatic zones and eco-habitats are furnished in Fig 1 for the samples 1 to 12 and Fig 2 for the honey samples. 13 to 25. It is elucidated form the investigation that all the 25 samples of honey contained polyphenolic compounds in varying amounts. However, flavonoids could be detected and quantified only in some of the honey samples and they were present in smaller quantities than the polyphenolic compounds

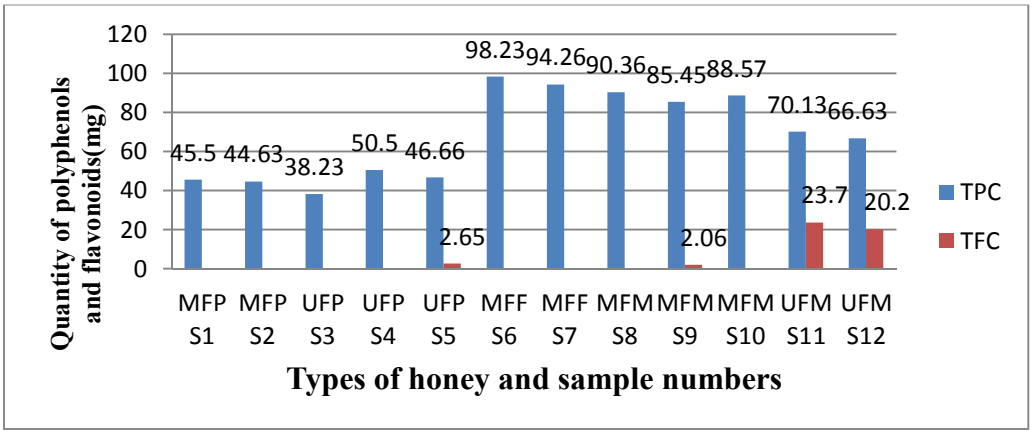

Figure 1: Comparison of the Total Polyphenolic and the Total Flavonoid contents of honey samples 1 to 12

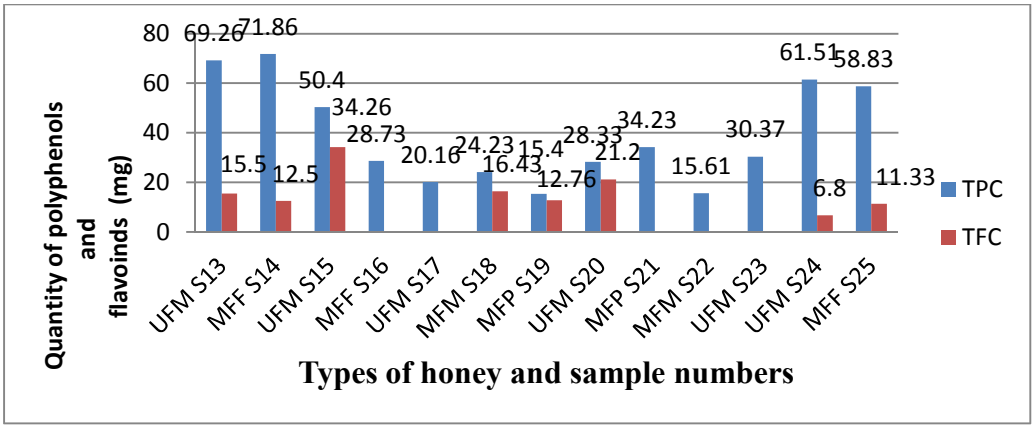

Figure 2: Comparison of the T otal Polyphenolic and the Total Flavonoid contents of honey samples 13 to 25

Table 2: The correlation between the Total polyphenolic content, total flavonoid content and the scavenging activities of nitric oxide free radicals by honey samples

Pearson Correlation	TPC	TFC	NOS
TPC	1	0.510**	0.927**
TFC		1	0.490*
NOS			1

Radical scavenging activity of honey samples

The results of the scavenging or quenching activity of the free radicals by the 25 honey samples were demonstrated by estimating their nitric oxide free radical scavenging activity. The results for nitric oxide radical scavenging activity are depicted in Fig 3 to Fig 7. The standard antioxidant used in the experiment was ascorbic acid. The results indicate that all the honey samples exhibited inhibition of nitric oxide at all concentrations, however, only a few showed the percentage of inhibition above the standard ascorbic acid. It is also clear that the percentage of free radical scavenging activity increases with increasing doses of honey samples. It is observed that the multifloral forest

honey samples MFF-S6 and MFF-S7 exhibited the highest percentage of nitric oxide inhibition and the lowest was shown by multifloral honey from the plains MFP-S19 at 100µl, 200µl, 400µl, 750µl and 1500µl dosage respectively.

The nitric oxide radical scavenging activity by the different honey types like the unifloral honeys from the plains (UFP), unifloral honeys from the hilly regions (UFM), multifloral honeys from the plains (MFP), multifloral honeys from the hilly regions (MFM) and multifloral honeys from the forest (MFF) are presented in Fig 3 to Fig 7.

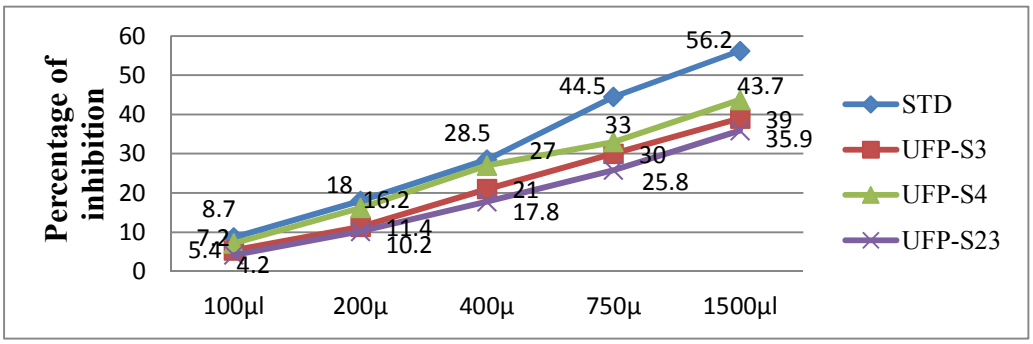

Figure 3: The percentage of Nitric oxide inhibition by the unifloral honey samples from the plains (UFP) at different concentrations

It is evident from these results that there is a linear increase in the nitric oxide radical scavenging activity by the honey samples from the different eco-habitats as the dosage increased from 100µl to 1500µl. It is also clear that all the three UFP showed a lower radical scavenging activity than the standard ascorbic acid. Among the unifloral honey from the hilly region the honey samples UFM-S11 and UFM-S13 showed a higher scavenging activity than the standard ascorbic acid as shown in Fig 4. All the multifloral honey samples from the plains showed a lower free radical activity than the standard ascorbic acid as seen in Fig 5. The quenching of nitric oxide by the five multifloral

honeys from the hilly regions (MFM) is shown in Fig 6 and it is clear that all of them exhibits a dosage dependent free radical scavenging activity and the

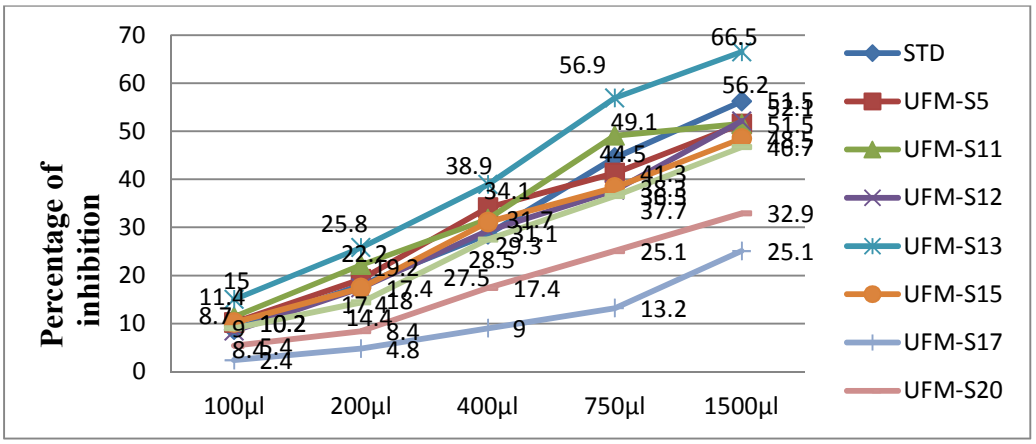

Figure 4: The percentage of Nitric oxide inhibition by the unifloral honey samples from the hilly regions (UFM) at different concentrations

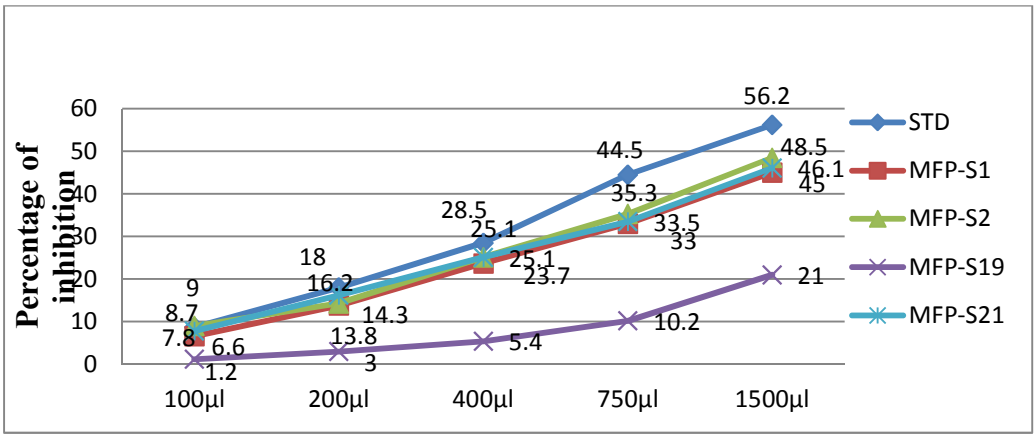

Figure 5: The percentage of Nitric oxide inhibition by multifloral honey samples from the plains (MFP) at different concentrations

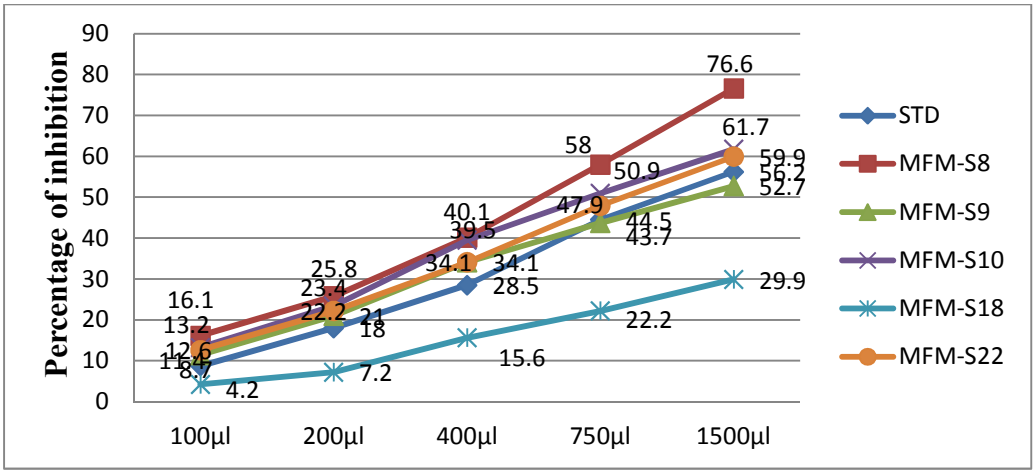

Figure 6: The percentage of Nitric oxide inhibition by multifloral honey samples from the hilly region (MFM) at different concentrations

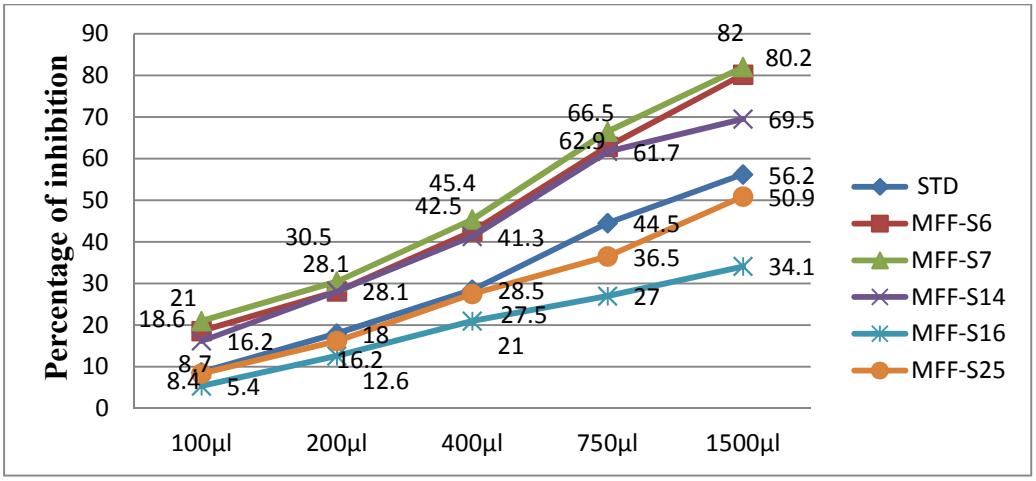

Figure 7: The percentage of Nitric oxide inhibition by multifloral honey samples from the forests (MFF) at different concentrations

honey samples MFM-S8, MFM-S9, MFM-S10 and MFM-S22 showed a higher scavenging activity of nitric oxide than the standard.The scavenging of nitric oxide by the five multifloral honey samples from the forests is depicted in Fig. 7

and it shows that all these honey samples had a higher free radical quenching activity except the honey sample MFF-S18 than the standard ascorbic acid. The study also revealed that there is close correlation between the polyphenolic and flavonoid contents of the honey samples and their free radical scavenging activity as shown in Table 2.

Antimicrobial activity of honey

The antimicrobial activity of the 25 honey samples collected for the different eco-habitats of Karnataka was tested against Gram positive and Gram negative bacterial strains. The control used was the Ampicillin drug. The minimum inhibition zones (MIC) were studied for these two bacterial species. Table 3 shows the zone of inhibition (ZI) and minimum inhibitory concentration (MIC) of the honey samples against the Gram positive bacteria, *Staphylococcus aureus* (ATCC 25923).The samples MFF-S6, MFF-S14 and UFM-S15 showed higher antibacterial activity then the control Ampicillin drug. The MIC for many of the honey samples was 70 percent, below which there was no zone of inhibition.

The zone of inhibition shown by the honey samples against the Gram negative bacterium *E. coli* (ATCC 25922) is shown in Table 4. The honey samples that showed a higher ZI than the ampicillin drug were UFP-S4,MFF-S6, MFF-S7, MFM-S9, MFF-S14 and UFM-S24 and the MIC was at 50% (v/v) for many of the honey against *E.coli*.

Table 3: Zone of inhibition of the Gram positive bacterium *Staphylococcus aureus* (ATCC 25923) by the honey samples 1 to 12 at different concentrations

Sl. No.	Honey samples	Zone of inhibition (in mm)						
		Concentration of the honey samples tested						
		100%	90%	80%	70%	60%	50%	40%
01.	MFP-S1	10.235± 0.073	10.125±0. 035	08.020± 0.071	06.205±0. 038	NZ	NZ	NZ
02.	MFP-S2	14.725± 0.082	13.615±0. 131	12.525± 0.057	10.335±0. 099	07.765± 0.045	NZ	NZ
03.	UFP-S3	07.955± 0.075	05.525±0. 065	NZ	NZ	NZ	NZ	NZ
04.	UFP-S4	04.725± 0.053	NZ	NZ	NZ	NZ	NZ	NZ
05.	UFM-S5	10.252± 0.073	08.520±0. 067	06.735± 0.087	NZ	NZ	NZ	NZ
06.	MFF-S6	28.525± 0.058	27.253±0. 131	25.562± 0.063	23.525±0. 034	25.765± 0.085	27.155± 0.067	13.755± 0.273
07.	MFF-S7	20.225± 0.042	17.735±0. 091	19.225± 0.016	21.355±0. 072	25.725± 0.062	18.925± 0.033	15.515± 0.044
08.	MFM-S8	14.505± 0.065	11.225±0. 079	07.715± 0.321	05.725±0. 521	NZ	NZ	NZ
09.	MFM-S9	11.735± 0.072	08.735±0. 045	05.325± 0.073	NZ	NZ	NZ	NZ
10.	MFM-S10	23.835± 0.051	21.635±0. 077	17.255± 0.053	13.785±0. 046	11.625± 0.073	09.770± 0.021	05.735± 0.091
11.	UFM-S11	13.725± 0.034	10.255±0. 082	08.325± 0.041	03.775±0. 022	NZ	NZ	NZ
12.	UFM-S12	08.725± 0.032	05.635±0. 041	NZ	NZ	NZ	NZ	NZ
13.	UFM-S13	13.027± 0.091	10.215±0. 031	07.615± 0.042	04.851±0. 023	NZ	NZ	NZ
14.	MFF-S14	23.252± 0.042	20.765±0. 031	21.705± 0.073	16.655±0. 023	12.550± 0.022	09.951± 0.013	07.755± 0.022
15.	UFM-S15	24.905± 0.071	21.225±0. 062	18.920± 0.031	15.752±0. 075	12.715± 0.091	08.420± 0.032	04.715± 0.012
16.	MFF-S16	12.810± 0.024	09.625±0. 036	05.515± 0.025	NZ	NZ	NZ	NZ

17.	UFM-S17	08.852± 0.029	04.515±0. 423	NZ	NZ	NZ	NZ	NZ
18.	MFM-S18	11.920± 0.037	07.525±0. 352	04.710± 0.022	NZ	NZ	NZ	NZ
19.	MFP-S19	09.753± 0.028	04.710±0. 025	NZ	NZ	NZ	NZ	NZ
20.	UFM-S20	09.912± 0.015	06.525±0. 017	NZ	NZ	NZ	NZ	NZ
21.	MFP-S21	06.880± 0.077	NZ	NZ	NZ	NZ	NZ	NZ
22.	MFM-S22	14.728± 0.017	09.920±0. 036	05.415± 0.027	NZ	NZ	NZ	NZ
23.	UFP-S23	06.011± 0.023	NZ	NZ	NZ	NZ	NZ	NZ
24.	UFM-S24	11.725± 0.016	09.518±0. 023	05.910± 0.015	NZ	NZ	NZ	NZ
25.	MFF-S25	19.751± 0.072	17.652±0. 039	14.985± 0.068	11.755±0. 025	09.825± 0.023	06.680± 0.041	NZ

UFP=Unifloral honey from plains

UFM=Unifloral honey from hills

MFP=Multifloral honey from plains

MFM=Multifloral honey from hills

MFF=Multifloral honey from forests

NZ=No zone of inhibition

Table 4: Zone of inhibition of the Gram negative bacterium *Escherichia coli* (ATCC 25922) by the honey samples 1 to 12 at different concentrations

Sl. No.	Honey samples	Zone of inhibition (in mm)						
		Concentration of the honey samples tested						
		100%	90%	80%	70%	60%	50%	40%
01.	MFP-S1	12.927±0.044	09.718±0.063	03.739±0.043	NZ	NZ	NZ	NZ
02.	MFP-S2	15.615±0.032	11.927±0.142	09.895±0.054	05.737±0.033	NZ	NZ	NZ
03.	UFP-S3	22.717±0.043	19.572±0.041	13.858±0.013	11.861±0.045	06.682±0.053	NZ	NZ
04.	UFP-S4	18.785±0.056	19.815±0.072	13.928±0.054	09.556±0.043	NZ	NZ	NZ
05.	UFM-S5	19.428±0.039	15.619±0.041	11.773±0.051	06.655±0.038	NZ	NZ	NZ
06.	MFF-S6	33.557±0.048	29.901±0.059	25.827±0.053	21.485±0.021	17.625±0.044	15.785±0.063	13.465±0.031
07.	MFF-S7	31.727±0.065	32.665±0.043	27.736±0.013	23.835±0.092	19.219±0.121	15.155±0.022	12.927±0.071
08.	MFM-S8	22.728±0.034	19.227±0.031	15.875±0.065	12.775±0.032	07.556±0.054	NZ	NZ
09.	MFM-S9	28.727±0.044	24.895±0.056	20.415±0.063	16.793±0.051	13.623±0.021	09.905±0.021	NZ
10.	MFM-S10	20.726±0.054	17.875±0.065	15.925±0.119	11.715±0.041	07.805±0.023	NZ	NZ
11.	UFM-S11	18.928±0.091	15.757±0.031	10.795±0.031	06.375±0.044	NZ	NZ	NZ
12.	UFM-S12	17.829±0.091	13.775±0.063	09.565±0.013	05.982±0.053	NZ	NZ	NZ
13.	UFM-S13	13.625±0.052	10.915±0.153	05.982±0.028	NZ	NZ	NZ	NZ
14.	MFF-S14	28.862±0.033	30.785±0.021	26.675±0.093	22.828±0.031	17.815±0.021	13.623±0.012	08.311±0.970
15.	UFM-S15	20.253±0.013	17.931±0.131	13.725±0.047	07.895±0.054	NZ	NZ	NZ

16.	MFF-S16	19.725± 0.065	13.955±0 .011	06.715± 0.021	NZ	NZ	NZ	NZ
17.	UFM-S17	14.819± 0.121	11.972±0 .033	07.706± 0.041	NZ	NZ	NZ	NZ
18.	MFM-S18	10.815± 0.092	07.415±0 .101	04.765± 0.051	NZ	NZ	NZ	NZ
19.	MFP-S19	11.625± 0.021	08.718±0 .032	05.672± 0.047	NZ	NZ	NZ	NZ
20.	UFM-S20	09.728± 0.036	05.881±0 .071	NZ	NZ	NZ	NZ	NZ
21.	MFP-S21	10.726± 0.071	06.817±0 .031	NZ	NZ	NZ	NZ	NZ
22.	MFM-S22	15.885± 0.056	12.985±0 .013	07.625± 0.015	NZ	NZ	NZ	NZ
23.	UFP-S23	09.726± 0.042	04.817±0 .071	NZ	NZ	NZ	NZ	NZ
24.	UFM-S24	24.825± 0.041	21.751±0 .054	17.726± 0.071	13.554± 0.061	08.910± 0.023	NZ	NZ
25.	MFF-S25	26.828± 0.046	21.495±0 .111	18.819± 0.131	13.975± 0.064	07.525± 0.011	NZ	NZ

UFP=Unifloral honey from plains UFM=Unifloral honey from hills

MFP=Multifloral honey from plains MFM=Multifloral honey from hills

MFF=Multifloral honey from forests NZ= No zone of inhibition

Discussion

Nitric oxides have a number of biological functions like neurotransmission, blood flow, antimicrobial and antiturmour activities but they also cause considerable damage when they combine with superoxide to form peroxynitrite anions (Patel and Patel, 2011).Free radicals are responsible for causing a large number of diseases including cancer (Kinnula and Crapo, 2004), cardiovascular disease (Singh and Jialal, 2006), degenerative brain disorders like, Alzheimer's disease (Smith *et al*., 2000) and Parkinson's disease (Bolton *et al*., 2000) and

cardiovascular diseases (Upston *et al*., 2003). There is substantial evidence that food containing antioxidants can greatly help to protect the body against the harmful effects of free radicals.

The percentage of inhibition of the nitric oxide free radicals by the honey samples is an indication of their antioxidant activity. The antioxidant activities of the honey samples were directly proportional to the rate of inhibition of the free radicals by the honey samples. The results clearly indicates that the darker multifloral forest honey samples (Table 1) from the forest (MFF) with higher content of polyphenolic compounds (Fig. 1 and Fig. 2) exhibited consistently a higher percentage of nitric oxides inhibition at all concentrations as compared to the standard value and hence possessed better antioxidant activity than honey samples collected from other eco-habitats. All honey samples showed a linear increase in the free radical scavenging activities of nitric oxide radicals in a dose dependent manner; the higher the dosage of honey used the greater was the percentage of inhibition as shown in Fig. 3 to Fig. 7 for nitric oxide scavenging activity. The study proved that there is a significant correlation between the antioxidant capacity of honey and the polyphenolic and flavonoid content (Table 2) which depends on their floral and geographical origin as shown in the earlier works of Aljadi and Kamaruddin (2004) and Beretta *et al* (2007).

Honey is one of the oldest traditional medicines important in the treatment of several human ailments. The bactericidal and bacteriostatic property of honey has been known for more than a century and this quality has been attributed to the high osmolarity, high acidity (3.4 to 6.1), hydrogen peroxide, phenolic acid, flavonoids, lysozyme and certain phytochemicals (Molan, 1992; Cooper, 2008; Gulfraz *et al.*, 2010 and Irish *et al*., 2011) and

also on botanical and entomological origin. Due to the above mentioned antioxidant and antibacterial properties of honey and the development of antibiotic resistant varieties of pathogens there has been a renewed interest in the therapeutic value of honey. However, it is important that when honey is to be used as an antimicrobial agent it is selected from honeys that have been assayed in the laboratory for antimicrobial activity.

The honey samples varied widely in their antimicrobial activity. The dark multifloral honey samples MFF-S6, MFF-S7 collected from the forests of Western Ghats showed the greatest antimicrobial activity as compared to the other unifloral and multifloral honey samples collected from the plains and hills of Karnataka. It was also observed that the zone of inhibition of all the honey samples decreased with decrease in concentration. The MIC of honey also exhibited great variations between the honey samples for the different microbes tested as depicted in the Table 3 for the *S. aureus* bacterium and Table 4 for the *E. coli* bacterium. Most of the honey samples did not show any antibacterial activity after 70 percent dilution.

Hence, in conclusion it can inferred from the present *in vitro* studies that honey possesses antioxidant and antibacterial activity but their potency varies considerably depending on the botanical origin, physico-geographic parameters of the honey samples as also revealed by the research works of Molan (1992), Gulfraz *et al* (2010), Irish *et al* (2011) and Alexander and Daniel (2014). Though it is a well established fact that honey inhibits a broad spectrum of bacterial species the present *in vitro* study indicates that honey can be recommended for therapeutic purposes after investigating the efficacy of their antibacterial activity in the laboratory. Honey with its free radical scavenging activity may be of great benefit in preventing or postponing the onset of

degenerative diseases. Thus, including a spoonful of honey to our daily diet would substantially improve the quality of life and greatly cut the cost of healthcare in our country.

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CONSUMPTION OF BAKERY PRODUCTS: SCHOOL CHILDREN'S PREFERENCES AND ITS OUTCOME

Resmi Devi S.R.
Assistant Professor, Naipunnya School of Management, Cherthala, Alappuzha
Email id:resmi.devi@yahoo.com

Abstract

The present project aims to analyze the threats present in the consumption of bakery productsin school children and to find out the awareness level of their parents about its health impacts.The area selected for this study is school children belonging to standard I to V and their familymembers in Alappuzha district. Personal interview was conducted with parents, teachers andstudents, observation check list was filled up to collect information regarding the consumption.Body mass index of the students were noted down. The study showed that majority of thestudents consumed bakery products regularly, at their interval time in school. In most cases thestudents were introduced bakery products by their parents. The reason for consuming bakeryproducts were easy availability, variety and taste. Majority of the parents were unaware of thehealth hazards of the bakery products. Once made aware of the hazards they were willing tochange. The emphasis should be more on strategies to raise the awareness level of parents.Theschool management should be made vigilant to develop good food habits in students.

Key Words: Bakery Products, Body Mass Index, Food Habit

Introduction

A healthy life style is corner stone of good health, physical fitness, energy and reduced risk for disease. Good nutrition, daily exercise and adequate sleep are foundations for healthy life style. School-age children (ages 6-12) need healthy food and nutritious snacks. They have a consistent but slow rate of growth and usually eat four to five times a day (including snacks). Many food habits, likes, and dislikes are established during this time. Family, friends, and the media (especially TV) influence their choices and eating habits. School – age children are often willing 3 to eat a wider variety of foods than their younger siblings. Eating healthy after – school snacks is important, too, as these snacks may contribute up to one –third of the total calorie intake for the day.

Consumption of bakery product is steeply increasing both in urban and rural areas. The ease of availability, taste and advertisement make them popular with children. Food items such as burgers, pizzas, are not considered right choice to meet nutrient need. The diet should provide around 50-60% of total calories from carbohydrate, about 10-15% from proteins, & 20-30% from visible and invisible fat. Since food consumption at school is significant part of the over all daily diet, school should not allow the canteens/students to carry food to promote food habits that negatively impact the health of children.

Parents play very important role to create healthy food habits in children. Always serve break fast even if it has to be on the run. Educate the children the importance of balanced diet. Food menu need to include rich in nutrients, - fruits, vegetables, grains and cereals.

Objectives

1) To study the food habits of school children of class I to V and their interests to use bakery products.
2) The time intervals of consumption of bakery product by students.
3) The consequences of usage of bakery products by students
4) To create general awareness on the adverse effect of the consumption of bakery products among the parents and school management. To promote a life style that include healthy diet and minimum level of physical activity.

Methodology

The district of Alappuzha was selected for the study. Government schools and CBSE Schoolslocated in town and village were selected. The core sample consists of 100 students of bothgender (criteria-physical appearance). The student's food habits were obtained by observing thefood consumption at school mainly during intervals, morning break and lunch break for oneweek period. To arrive at a sound feedback, personal interview was conducted with care takers inschool and teachers .Body Mass Index (BMI) of selected children were noted down.Information regarding the food habits of students were also collected by interaction with theparents of students and family members.

Results and Discussions

The major findings were as suspected –High consumption of unhealthy bakery products. Sincemost of the parents are employees, and are busy in the mornings, many children don't get a goodbreakfast. Hence, they are hungry and tend to eat as much as they can during the first availablechance, i.e. during the

first interval in the morning. Since this is a short interval, the students tend to grab ready to eat bakery products like, puffs, pastries, buns, and packaged snacks likelays, kurkure, etc. Some of them have a cola or other similar soft drink as well.

The result is that the students are not hungry during the lunch time and tend to eat less for lunch, which makes them hungry again during teatime, making them to eat more bakery products.

It is also observed that those students who have a good breakfast do not have the habit ofsnacking compared to others. They also tend to eat their lunch well, making them less hungryduring teatime.

Another highlight of this study is that the students like the noon meal scheme (in the government schools) and look forward to a hot cooked meal for lunch, rather than having the cold contents of the lunch box prepared and packed many hours ago.

Since Indian cuisine is best served hot and that it becomes rather un appealing when it is cold, children tend to eat bakery products which taste good, even when they are served cold. Moreover accompaniments like sauce, chutney etc makes the products taste even better. (Table I).

A general awareness meeting was conducted at school auditorium with the help of school management, canteen staff and parents During the meeting the adverse effect of bakery products were explained. Though the initial symptoms were not visible, it will have a consistent adverse effect on them. The energy out put, of these products are high which lead to child hood obesity, (Body Mass Index of around 50% of the selected students were unsatisfactory.)

cardiovascular diseases, diabetes, cancers and chronic respiratory diseases. (Table II).

Table 1: Observation Checklist Used for Data Collection

Parameters	**School 1(Town)**	**School 2(Town)**	**School 3(Village)**	**School 4(Village)**
Total number of students observed	25	25	25	25
BMI of students	US=50%	US=50%	US=50%	US=50%
Bakery products consumption during interval time	100%	98%	98%	100%
Fast food consumption during lunch	20%	30%	25%	22%
Schools providing canteen facility	Yes	Yes	Yes	Yes
Awareness of school management regarding the present problem	No	Aware	No	Aware
Canteens are providing bakery/snacks	Yes	Yes	Yes	Yes
Bakery/Snacks consumption evening tea time	50%	55%	60%	52%
Employment status of parents	Parents employed	Parents employed	Around 95% employed	Employed
Awareness of parents regarding the present problem	Aware	Aware	Around 95%aware	Aware
Watching T.V while consuming food	Yes	Yes	Around 95%	Yes

Table 2: Problems associated with bakery products

Sl No	Food items	Problems	Adverse health Effect
1	Maida based products	Decrease appetite	Child hood obesity, cardiovascular diseases, diabetes, cancers and chronic respiratory diseases.
		Cause constipation	
2	Colour used in bakery product	Addition of non food gradecolour	
		Colour addition without anymeasurement	
3	Fat used for preparation	Saturated fat	
4	Oil used for preparation	Repeated thermal treatmentchange colour of oil	
5	Sugar Used	Synthetic sugar ,not good forhealth.	
6	Salt	Salt content found to be high.	
7	Preservatives	Added in excess, not as specifiedby FSSA .	

Literature Review

Higher consumption of bakery products,sweetened soft drinks and yogurt is associated with higher intake of energy, saturated facts ,sugars and worse overall diet quality among Spanishchildren. This is a cross –sectional study covering 1112 children aged 6.0-7.0 years in fourSpanish cities. Nutrient and food intake were obtained through a food- frequency questionnaire,and overall diet quality calculated using the healthy- eating index (HEI) developed by Kennedy etal (1995).Standardized methods were used to measure anthropometric variables. Associationsof interest were summarized as the difference in nutrient and food

consumption between thevalue of fifth and the first quintile of consumption (dq) of bakery products, sweetened soft6drinks or yogurt,adjusted for energy intake and BMI. Bakery products ,sweetened soft drinks or yogurt, adjusted for energy intake and BMI. Bakery products ,sweetened soft drinks and yogurtsupplied 15,5,1.0 and 5.6 % energy intake respectively .Higher consumption of these foods wasassociated with greater energy intake ($p<0.001$) , but not with higher BMI. Consumption ofbakery products was associated with the proportion of energy derived from intake of totalcarbohydrates (dq 4.5%,$p<0.001$) and sugars (dq 2%,$p<0.001$) ,but not with higher BMI .

Consumption of bakery products was associated with a lower consumption of milk (dq-88 ml,$p<0.001$) and ca (dq -175 mg/d,$p<0.001$) and worse HEI (dq -2 ,$p<0.01$).Consumption ofyogurt ,while associated with higher energy intake from saturated fats (dq 1.77% , $p<0.001$) andsugars (dq 2.02 % , $p<0.001$) , showed no association with the HEI. Differences in the intake ofnutrients and foods across quintiles of consumption of bakery products, sweetened soft drinksand yogurt were usually very small. We conclude that the impact of the consumption of bakery products, sweetened soft drinks and yogurt were usually very small. We conclude that the impact of the consumption of bakery products, sweetened soft drinks and yogurt on the qualityof the diet of Spanish children is most only modest, although it may contribute to aggravatingcertain unhealthy characteristics of their diet, particularly excess energy, saturated fats and sugars. Therefore, consumption of bakery products and sweetened soft drinks should bemoderated, and priority given to consumption of low -fat, low – sugar yogurt. (Consumption ofbakery products, sweetened soft drinks and yogurt among children aged 6-7 years :association with nutrient intake and overall diet quality-Rodriguez-Artalejo F',Garcia EL, Gorgojo L, Graces C, Royo MA ,

Martin Moreno JM, Benavente M, Marcias A,De Oya M;Investigators of the Four Provinces Study.)

Assessment of application of the enriched bakery products in nutrition of school students have been presented. Composition and technology of the enriched bakery products have been developed . The influence of enrichers on the basis of proteins of whey, plasma of blood, hemoglobin calcium and iodinated components and food fibres on the nutritive and energy value of bakery products a significant amount of protein –12.5 – 23% of the recommended daily intake (RDI), to satisfy daily need of school students in calcium up to 13.4% , in iron—up to 20% , iodine –12.5 % and food fibres – 17.3%.When comparing blood hemoglobin content in school students after inclusion in a diet of the enriched bakery products , the lacks of significant efficiency of ant changes of this indicator in children with normal hemoglobin content has been determined that is the confirmation of safety of use of the products enriched wit hem iron. At the same time, normalization of hemoglobin level in children (9.7%) with the initial threshold andlowered hemoglobin indicators is noted. The reliable increase in the content of hemoglobin inthis group of children from 112+-3 to 131+-6 g/l was established ($p<=0.05$).Positive dynamicsof the content of iodine in urine at school students with initial deficiency of iodine underadministration of the bread enriched with iodine has been defined . Ioduria indicatorsauthentically increased from 88+-10 to 116 + - 9 mkg/l ($p<0.05$). Before diet correction in 53from 59 children surveyed in the Stavropol region , a mild lack of iodine has been revealed (iodine levels less than 100 mkg/l urine), while after bread intake –-only in 7 (11.9%) students.

(Efficiency of application of the enriched bakery products in children nutrition,Koryachkina SY, Ladnova OL , Lublinsky SL, Kholodova EN.)

Recommendations

Children are not the best judge of their food choice. They are not aware of the balanced diet.

Plan the food as per family's choice .Make a wide variety of healthful foods available in thehouse .This practice will help the children learn how to make healthy food choices.

Teach Good Food Habits: Serve water with meals . Encourage children to eat slowly. Eat mealstogether as a family as often as possible Encourage children to drink more water. Pay attentionto portion size and ingredients.

Involve children in food shopping and preparing meals. These activities will give hintsabout children's food preferences, an opportunity to teach children about nutrition, andprovide kids with a feeling of accomplishment. In addition, children may be more willing to eator try foods that they help prepare.

Plan for snacks. Continuous snacking may lead to overeating, but snacks that are planned atspecific times during the day can be part of a nutritious diet, without spoiling a child's appetiteat meals times.

Always serve breakfast, even if it has to be "on the run" Some ideas for a quick , healthybreakfast include : Fruit, Milk, sandwich etc. Include grains, cereals, vegetables, fruits, dairy, different protein food like more fish, nuts, seeds, peas, and beans in selecting food. Nuts canbe included. Animal fats are solid fats and should be avoided.

Children need at least 60 minutes of moderate to vigorous physical activity on most days tohave good health and fitness and for healthy weight during growth. Encourage physical activityto schoolchildren.

School should promote nutrition education and awareness among children through varioustools such as posters.

A school food committee could be set up comprising of teachers, parents, students and schoolcanteen operatior, who will prepare guidelines for safe food.

Government authority should regulate the promotion of such food items that are injurious to human health.

Conclusion

The Government of India, by order implemented, that the consumption /availability of most common bakery products in school and area within 50 meters. The shift from traditional to modern foods, changing cooking practices have affected peoples perception of foods as well as their dietary behavior. This necessitate systematic nutrition educational intervention on a massive scale. Schools are a place to learn right values and constructive behavior for a life time. Theschool management must ensure regulation of such food through canteen policies that promote healthy, wholesome and nutritious food. The school management, in association with parents carry a social responsibility towards implementing healthy eating behavior in children. It is well known that eating too much bakery products will lead to health complications. Parents should keep this in mind while choosing food for the family. Make sure that all members of the family have a healthy breakfast. Including home cooked food and fruits will make the lunch better. Locally available vegetables, cereals and pulses should be included in the menu. A generous helping of dried fruits and nuts is also suggested.

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ISOLATION AND CHARACTERIZATION OF POTENT MARINE MANGROVE YEAST Y17 FOR ANTIMICROBIAL ACTIVITY

Arya R. J and **K. Manjusha** *
Department of Microbiology, St. Xavier's college for Women, Aluva.

Abstract

Mangroves are a unique ecosystem.This marine habitat is unparalleled by virtue of harbouring multifarious, flora and fauna. Even though there has been considerable research on marine bacteria, studies on marine yeasts are limited.Yeasts as a eukaryotic microbe, is of importance for its nutritional quality and bioactivity. The antagonistic property of yeast has been put to use in various food and beverage processing industries as well as in agriculture as natural bio-control agents. The present study was focused on the isolation, characterization of yeasts with antimicrobial activity. For the study sediment samples were collected from mangrove areas of Vyppin. Isolation of yeasts was done on Malt-yeast-glucose-peptone agar (Wickerham's Media) supplemented with 200 mg/l chloramphenicol. Identification of the isolates up to generic level was done based on morphological, physiological and biochemical characteristics. The growth of the isolates at various pH, temperature and salinity was also investigated. The screening for bioactive compounds revealed that, the yeast strain Y17 was capable of producing hydrolytic enzyme lipase and also exhibited antimicrobial activity against bacteria. The detection of antibacterial activity using disc diffusion showed that the yeast isolate was capable of inhibiting the test strains of *E.coli, Staphylococcus* and *Bacillus*sp. The culture was further studied using well diffusion method. Molecular identification of the potent isolate based on ITS region revealed that isolate Y 17 was *Candida tropicalis*. From this study it can be concluded that marine yeasts of mangroves are a promising source of bioactive compounds. Of course, further studies are required before they can be used industrially.

Keywords: Marine yeast, bioactive compounds, antimicrobial activity, mangroves, *Candida* sp.,

***Corresponding author :**manjushak@stxaviersaluva.com

Introduction

Mangrove biome or 'mangal', a part of the vast marine habitat that has a distinct saline wood land or shrub land territory characterized by depositional coastal environments where fine sediments often with high organic content gather in areas protected from high energy wave action. Mangroves harbor diverse groups of organisms. Marine yeasts are an integral part of the biodiversity of mangrove microbial community and play vital role in their stability and functioning.

Yeasts are described as a polyphyletic group of basidiomycetous and ascomycetous fungi with an exclusive feature of unicellular growth.They participate in a range of ecologically significant processes in the sea, such as decomposition of plant substrates, nutrient-recycling phenomena and biodegradation of oil and recalcitrant compounds, particularly in estuarine and near shore localities. It has been reported that marine yeasts are able to produce various bioactive substances, such as amino acids, glucans, glutathione, toxins, enzymes, phytase and vitamins with potential application in the food, pharmaceutical, cosmetic and chemical industries as well as for mariculture and environmental protection (Chi et al., 2009). Yeastsas a eukaryotic microbe is of more importance for its nutritional quality and bioactivity. The antagonistic potential of yeast has been used in various food and beverage processing industries (Romano et al., 2006; Viljoen 2006 Pimenta et al., 2009) and in agriculture as natural bio-control agents.

Several workers have observed that bioactive compounds from marine microbes are more potent than their terrestrial counter parts.However, our knowledge on the biodiversity and economical relevance of these marine yeasts is inadequate when compared to their terrestrial counter parts. To this extent,

mangrove environment presents a largely untapped source of novel microorganisms with the potential to produce bioactive compounds. As studies on bioactive potential of marine mangrove yeasts is limited, especially in our state, for better understanding the present study was taken.

Materials and Methods

Study area

The study was confined to the Vyppin mangroves of Ernakulam District.

Sample collection

Sediment samples were collected during the period April to September 2015.Three samples each were collected from the Vyppin station.The substations were located few meters apart. Sediment samples for microbial analysis was collected and transferred aseptically into sterile containers.

Isolation of marine yeast

For the isolation of yeasts, plating of the sediment samples were done employing spread plate method. About 1 g of the sediment sample collected was suspended in 9 ml sterile water collected from mangroves was used as inoculum. Five hundred microliters of the inoculum was spread plated on Malt-yeast-glucose-peptone agar (Malt extract 3g/L,Yeast extract 3 g/L , Peptone 5g/L , Glucose 10 g/L, Agar 20g/L) (Wickerham, 1951) supplemented with 200 mg/l chloramphenicol.

The plates were incubated at 28°C for 1 day. The colonies developed were purified by quadrant streaking and transferred to malt extract agar slants for further studies.

Identification of the isolates

The isolates were identified up to genera as per Barnett et al. (1990). The characters studied were microscopic appearance of the cell, mode of reproduction and biochemical characteristics.

Physiological Characters

Assessment of the growth isolates at different temperature (4^{o}C, 28^{o}C and 37^{o}C), salinity (0, 5, 10, 15, 20, 25, 30 %) and pH(3, 5, 6, 7, 8 and 9) was done. Growth was estimated by measuring the optical density at 540 nm.

Screening for Bioactive Compounds

A. Detection of antibacterial activity

a) Disc Diffusion Method; Yeast cultures Y14, Y15, Y16, Y17 were inoculated into Wickerham's broth and incubated for 48 hrs. Tubes with Peptone water were inoculated with test cultures (*Staphylococcus, Pseudomonas, Bacillus and E.coli)* and incubated for about 2 hrs.The 48 hr grown yeast cultures were centrifuged and the cell free supernatent collected in sterile tubes. Nutrient aga (NA) plates were inoculated with well grown bacterial cultures by swabbing and sterileWhatmanfilter paper discs impregnated with cell free supernatant agar plates were placed on the plates. Each disc was pressed down to ensure complete contact with the agar surface and distributed evenly. The plates was incubated for 24 hrs at 37^{0}C.After incubation plates were observed for the development of clearance zones and the diameter of the zones were noted. The diameters of the zones of complete inhibition were measured.

b) **Well Diffusion Method:** NA plates were inoculated with bacterial cultures by swabbing. Wells were made on NA plates under sterile conditions, and 35 microliters of the yeast culture supernatant collected (as mentioned above) were transferred inside the well aseptically. Plates were incubated at 37^0 C for 24 hrs.After incubation plates were observed for clearance zones.

B. Hydrolytic enzyme production

The isolates were tested for the production of enzymes viz. amylase, lipase, protease, cellulase, pectinase and urease.

Molecular Identification of the potent yeast isolate

The total genomic DNA was isolated as per Harju et al. (2004). The identity of the potential isolate was ascertained by sequencing the 580 bp PCR amplicon containing the ITS 1, 5.8 S and ITS 2 regions. Internal Transcribed Spacer (ITS) sequences was amplified using ITS primers (Forward ITS 1-5' TCC GTA GGT GAA CCT GCG G 3' and Reverse ITS 4- 5' TCC TCC GCT TAT TGA TAT GC 3') as per White et al. (1990). DNA amplification was carried out in a thermal cycler which involved1 X 95°C for 5 minutes, followed by 30 cycles x (94°C for 1 minute, 56°C for 45 seconds, 72°C for 1 minute) and a final extension 1 x72°C for 10 minutes.

The PCR products were analysed by electrophoresis on 1% agarose gel prepared in 1X TAE buffer and stained with ethidium bromide. The PCR product was sequenced at SciGenom Labs Pvt Ltd, Kerala. The sequence obtained were subjected to BLAST analysis at the NCBI and based on similarity index the isolate was identified as *Candida tropicalis.*

Results and Discussion

Sample Collection and Isolation

Spread plating of samples on Wickerham'smediayielded small colonies.A total of 4 isolates were obtained one each from substations VY1 and VY3 and 2 from substation VY2. The strains were labeled as Y14, Y15, Y16 and Y17.

Identification of the isolates

Macroscopic appearance

All the isolates were cream in colour and had the typical yeasty odour. The colony characteristics of the isolates are summarized in Table 1.

Table 1: Morphology of yeast isolates

Isolate	Microscopic appearance	Macroscopic appearance					
	Cell shape	Form and size	Elevation	Margin	Colour	Density	Texture
Y14	Ovoidal,multilateral buds	Small, circular	Convex	Entire	cream	Opaque	Buttery
Y15	Ovoidal,multilateral buds	Small , circular	Convex	Entire	cream	Opaque	Creamy
Y16	Spherical with multilateral buds	Small ,circular	Convex	Entire	cream	Opaque	Creamy
Y17	Ovoidal,multilateral buds	Small , circular	Convex	Entire	cream	Opaque	Creamy

Microscopic Appearance

Microscopic examination of vegetative cells revealed the cells had different morphology; they were either, ovoidal, ellipsoidal or spherical in shape. It was also noted that all the isolates reproduce by budding and none exhibited fission (Table 1, Figure 1)

Table 2: Biochemical characteristics of yeast isolates

cltr.no	Glucose/ MOF	Sugar Utilisation					Nitrate	Probable isolate
		Galactose	Maltose	Xylose	Raffinose	Lactose		
Y14	F	A	A	K	K	K	-	UI
Y15	F	A	A	K	K	K	-	UI
Y16	F	A	A	K	K	K	-	*Candida*
Y17	F	A	A	K	K	K	-	*Candida*

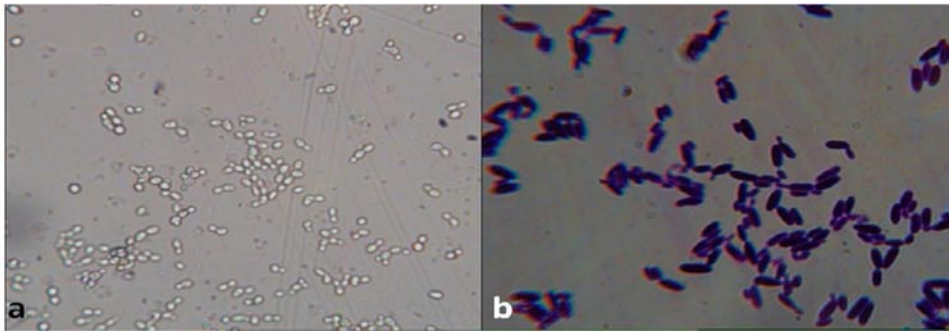

Figure 1: Microscopic appearance a) Wet mount b) Gram staining-budding yeast cells

Physiological characters

All the isolates were able to grow at all the temperatures tested (Table 3). Room temperature (28^{o}C) was found to be optimum for the growth of yeast isolates. Significant amount of growth was also seen at 37^{o}C.All the isolate exhibited low growth at 4^{o}C. The isolates Y15 and Y16 showed decrease in growth at 37^{o}C whereas; they grew well at room temperature.Kuriakose (2012) observed that irrespective of the temperature of the marine habitat where from the yeasts were isolated, the isolates exhibited considerable growth within a temperature range of 18 - 30 ^{o}C, pointing to the fact that they are mesophiles, probably of terrestrial origin and got adapted to marine environments.

Table 3: Physiological charaecteristics of yeast isolates

	Temperature			NaCl							pH					
cltr.no	**4^0C**	**28^0C**	**37^0C**	**0%**	**5%**	**10%**	**15%**	**20%**	**25%**	**30%**	**3**	**5**	**6**	**7**	**8**	**9**
Y14	+	+++	+++	+++	++	++	+	+	+	+	+++	+++	++	+++	++	++
Y15	+	+++	++	++	++	++	+	+	+	+	+++	+++	++	+++	++	++
Y16	+	+++	++	++	++	++	+	+	+	0	+++	+++	+++	+++	++	++
Y17				+++	++	++	+	+	+	0	+++	+++	+++	+++	+++	+++

OD range: 0-0.5= +, 0.6-1.00= ++, 1.01-1.5=+++

Considerable growth was observed in the salinity range 0-25% (Table.3).However, growthdecreased with increase in salinity. Roth et al. (1962) stated that almost all the yeasts were able to grow at wide range of NaCl concentrations. Salinity tolerance does notdistinguish marine species from terrestrial species because almost all yeasts can grow in sodium chloride concentrations exceeding those normally present in the sea.

The isolates were all able to grow at a pH range of 3-9(Table 3). Yeasts generally prefer a slightly acidic pH, which was evidenced in the case of these estuarine isolate also.

Based on the morphological, physiological and biochemical characters the isolates Y 16, Y17 were identified as *Candida* sp., whereas the cultures Y14 and 15 could not be ascertained to any specific genus therefore labeled as unidentified (UI). *Candida* is reported one of the most common genera in marine environments by workers (Fell and Van Uden, 1963; Kriss et al., 1967). Further identification of the unidentified isolates could probably be made on the molecular basis.

Testing for antibacterial activity

The primary screening for antibacterial activity using disc diffusion revealed that the yeast isolatesY15, Y16, Y17 (Table4) were capable of inhibiting the test strains of *E.coli, Staphylococcus* and *Bacillus*sp. . But none of the yeast strains were capable of inhibiting*Pseudomonas.* Y17 which was the most potent isolate; which was further screened using well diffusion method at 12 hrs interval (Figure 2). It was found that it performed a little better when the culture supernatant was taken after 24 hrs of incubation. Diameter of zones of inhibition of secondary screening were 17.6 mm, 11mm and 18 mm against *E.coli, Staphylococcus* sp. and *Bacillus* sp. respectively.

Table 4: Screening for antimicrobial activity

Cultr.no.	Probable Isolate	*E.coli* (mm)	*Staph* (mm)	*Baciillus* (mm)	*Pseudomonas* (mm)
Y14	UI	8	11	11	0
Y15	UI	14	7	12	0
Y16	*Candida*	11	8	10	0
Y17	*Candida*	15	13	13	0

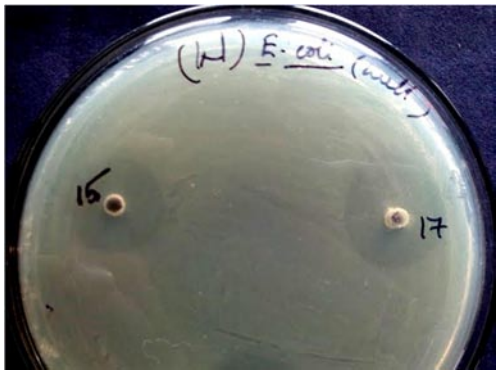

Figure 2: Antibacterial activity testing by well diffusion method

Hydrolytic Enzymes

All the isolates from Vyppin were lipolytic but did not produce any other enzymes. The ability to produce lipase indicates that are capable of being put to applications in industrial and biotechnological fields.Lipases produced from yeasts *Candida rugosa*was used for the production of fatty acids from castor bean. Pandey et al. (1999) investigated the production of flavour in concentrated milk and cream, using microbial lipase.

Molecular identification

The isolateY 17, that exhibited both antimicrobial activity and lipaseproduction capability was further identified to the species level based on the sequence of ITS region.Use of ITS or DNA sequences are considered to be the best tools for rapid and accurate identification of yeast isolates (Kutty and Philip,2008). The ITS region was amplified using ITS primers and a amplicon of ~580 bp was obtained (Figure 3). The PCR products were purified and sequenced. The sequence obtained were subjected to BLAST at the NCBI and based on similarity index the isolate was identified as*Candidatropicalis*.

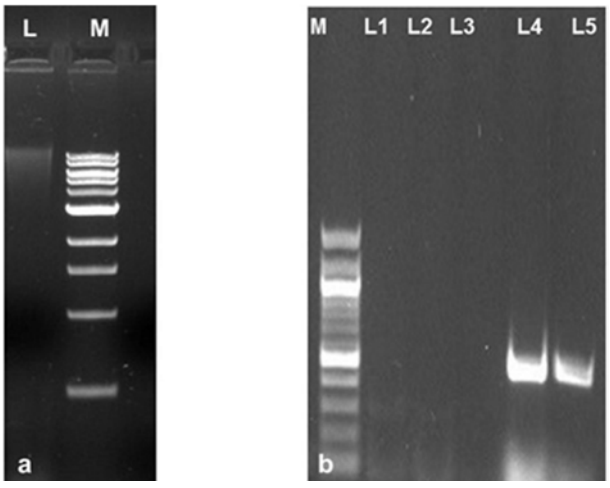

Figure 3: Molecular Identification. a) Isolated genomic DNA: L –sample, M-marker b) PCR: M-Marker, L4&5 PCR product of ITS region 580 bp size

Conclusion

Mangroves are unique ecosystem that harbours a diverse group of marine microbes with a potential producing a variety of bioactive compounds. Even though there has been considerable research on marine bacteria, studies on marine yeasts are limited. Therefore the present study was focused on the isolation and characterization of marine yeasts from the mangroves of Vyppin. An attempt was made to identify the isolates up to generic level based on morphological, physiological and biochemical characteristics. However all the isolates could not be identified, this indicated the need for molecular identification. The growth of the isolates was examined at various pH, temperature and salinity to find out their optimal range. Bioactive potential of these marine yeast isolates was investigated this revealed that, these yeasts are good producer hydrolytic enzymes specifically lipase and are also capable of producing compounds with antimicrobial properties. The strain Y17 (*Candida* sp.) which was both highly lipolytic and synthesised antimicrobial compounds was identified further on molecular basis by sequencing ITS region, revealed that the yeast was *Candida tropicalis*. From this study it can be concluded that marine yeasts of mangroves are a promising source of bioactive compounds. Of course further studies are required before they can be used industrially.

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A STUDY ON THE ANTIBACTERIAL ACTIVITY OF COBALT FERRITE NANOPARTICLES

Thahreem Fathima[1], Vandhana T.M.[1], Aiswarya Lakshmi.S[1] and K. Manjusha[2*]

[1] Department of Zoology, St. Xavier's College for Women, Aluva
[2] Department of Microbiology, St. Xavier's College for Women, Aluva

Abstract

Nanotechnology is a key area of research in modern material science. This technology is capable of providing applications that range from innovative fabric compounds to sophisticated medicinal techniques. Studies on the antimicrobial property of nanoparticles have gained importance in the recent studies by medical researchers, due to the interest towards particles of nanometer scale and an increase in antibiotic resistance among microbes. It has become important to find substitutes which can act against the pathogens without disturbing the immunological status of human body. The nanoparticles used in this study are those of cobalt ferrite oxide as such and in their silver doped form. These particles were synthesized and provided by the Department of Physics, St.Xavier's College for Women. These nanoparticles are considered important because of past experiments done in antibacterial studies and taking into account their magnetic properties. In this work, the antibacterial activity of ferrite nanoparticles against Gram positive and Gram negative pathogens were investigated using well-diffusion method. The preliminary investigations suggest that the silver doped ferrite nanoparticles were more efficient than the un-doped particles in inhibiting bacteria. Silver doped ferrite nanoparticles were effective against both Gram positive and Gram negative bacteria. Water was found to be ideal medium of dispersion. It clear from this study that, silver doped cobalt ferrite NP holds great promise as antibacterial agent and scope of being employed in therapy as they are also suited for targeted drug delivery.

Keywords: Cobalt ferrite nanoparticles, silver nanoparticles, antibacterial activity, well diffusion method

Introduction

Nanotechnology is a state of the art field that has begun touch every aspect human life including health and medicine. The re-emergence of diseases and the continuous development of drug resistance among microbial pathogens are posing a serious threat to public health worldwide (Desselberger, 2000). In this scenario, it has become inevitable to identify and develop new drugs or agents to control infections. With the recent advances in the field of nanotechnology particularly in the ability to prepare highly ordered nanoparticles (NP) of any size and shape, have led to the development of new biocidal agents. Studies have shown that NP formulations can be used as effective biocidal materials (Jones et al., 2008). The aim to improve the diagnosis and treatment of human diseases has led to development of a new field called nanomedicine.

NP can be classified as organic and inorganic nanoparticles. Organic NP consist of carbon , inorganic NP consist of magnetic NP, noble metal NP (like gold and silver) and semi-conductor (titanium oxide and zinc oxide) NP (Moghaddam et al., 2015). The most significant biomedical agents are metallic nanoparticles whereas magnetic nanoparticles are suited for targeted drug delivery and hyperthermia applications (Chaloupka et. al., 2010; Moghaddam et al., 2015). As the biocidal property of silver NP is well established (Xavier et al.,2014) and as cobalt ferrite NP are magnetic, Silver doped ferrite NP can serve as ideal therapeutic agents in nanomedicine. This study is a preliminary investigation on the antibacterial property of these NP and selection ideal medium for the dispersion of NP.

Materials and Methods

Test strains used

Four microorganisms were used in this investigation they include, Gram positive bacteria –*Staphylococcus* sp. and *Bacillus* sp., Gram negative bacteria *Escherichia coli and Pseudomonas aeruginosa*. The test organisms used were those that were maintained in the laboratory of the Microbiology department.

Nanoparticles

Nanoparticles of silver substituted cobalt ferrite ($Co_{X-1}Ag_X Fe_2O_4$, X=0.00, 0.025) for the study was kindly provided by the Physics department of the Institution. The Nano powders were labelled as C for the un-doped particle and CA_1 for the silver doped nanoparticles. The particles were synthesized in the as per the Sol-Gel process (Xavier et al., 2014) in the Physics laboratory. The stocks (100mg/ml) of the nanoparticles for the study were prepared in two dispersing mediums (solvents), water as well as Dimethyl sulfoxide (DMSO).

Antibacterial Susceptibility Testing

The doped cobalt ferrite nanoparticles were tested in vitro for their antimicrobial activities against the bacterial strains using the well diffusion method by using water and DMSO as dispersing medium.

Well diffusion Method

Peptone broth was inoculated with test cultures (*Staphylococcus* sp. and *Bacillus* sp., Gram negative bacteria *Escherichia coli and Pseudomonas aeruginosa*) and incubated for about 2-3 hrs at 37^{O}C. Mueller Hinton Agar (MHA) plates were inoculated with test bacterial cultures by swabbing. Wells were punched on MHA plates using a sterile well-cutter. 40 microliters of the nanoparticles (C and CA1) were transferred into the well aseptically from the

stock solutions prepared both in water/DMSO. Plates were incubated at 37^{O}Cfor 24 hrs.After incubation plates were observed for clearance zones. The diameter of the zones of inhibition (in mm) was noted.

Results and Discussion

In this investigation we analysed the antibacterial potential of cobalt ferrite NP and silver doped cobalt ferrite NP dispersed in two different mediums i.e water and DMSO. The study clearly revealed that cobalt ferrite NP (C) alone did not have inhibitory action against the test strains of bacteria. However when they were doped with silver, the antibacterial action was noted (CA1). They extent of inhibition varied with strains used. Greatest inhibitory action was noted against *Pseudomonas* sp. whereas *Bacillus* sp. was inhibited the least (Figure 1). Silver NP is widely used as therapeutic agents where they find their application as antibacterial, antifungal, antiviral, anti-inflammatory and anti-angiogenic factors (Moghaddam et al., 2015). Several mechanisms of anti-bacterial action of these NP have been proposed. According Gao et al. (2013) silver NP attach to the cell membrane and release silver ions that alter the membrane permeability resulting in cell death. It has also been suggested by

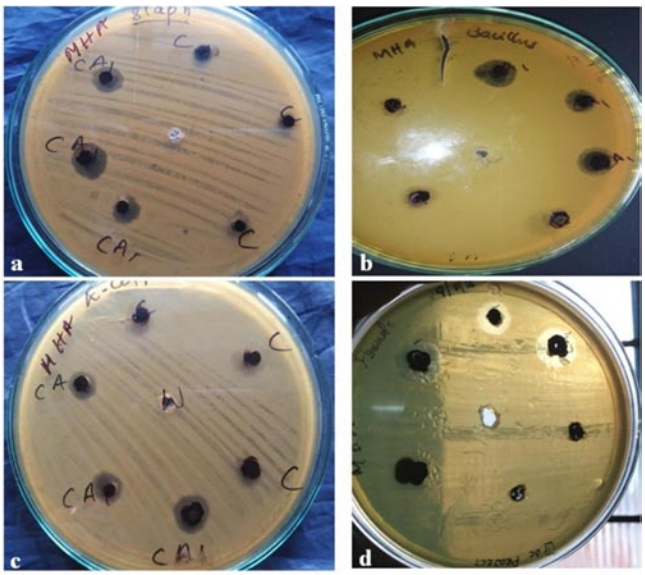

Figure 1: Antibacterial action of Silver doped NP dispersed in water against test strainsa) *Staphylococcus* sp. b) *Bacillus* sp., c) *E.coli* d) *Pseudomonas* sp.

workers (Sawai, 2003; Hashim et al., 2013) that the antibacterial action of silver NP is due to the release of reactive oxygen species (ROS) such as hydrogen peroxide (H_20_2) and superoxide (O^{2-}) from the surface of these particles.

The medium in which NP were dispersed (water/ DMSO) did not seem to significantly affect the antibacterial action (enhance nor reduce) against most of the strains (Figure 2). An exception to this was observed with regard to the action of NP against *E.coli* where antibacterial action of silver doped ferrite NP dispersed in water was significantly higher (*p value*=0.016) than those in DMSO. Xavier et al. (2014) have reported that the degree of dispersion in water plays a vital role the antibacterial properties of NP and it increases with particle size.

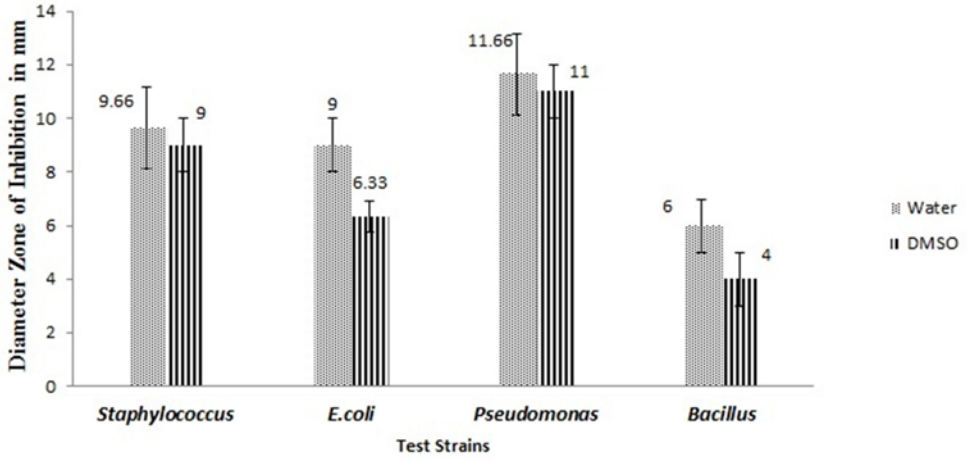

Figure 2: Effect of dispersing medium on the action of NP

Conclusion

Silver doped cobalt ferrite NP exhibited antibacterial action against both Gram positive and Gram negative bacterial pathogens; though the un-doped NP did not exhibit such bactericidal properties. The medium in which NP is dispersed did not seem to affect the antibacterial potential of the silver doped

cobalt ferrite NP. This indicated that water could serve as medium of dispersing NP as it is non-toxic and safe. It can be concluded from this preliminary study that, silver doped cobalt ferrite NP holds great promise as antibacterial agent and scope of being employed in therapy as they are also suited for targeted drug delivery.

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ANTAGONSTIC EFFECT OF*Trichoderma viride*AGAINST PLANT PATHOGENIC FUNGUS*Phytophthora capsici*

Sreelakshmi Rajesh and **Nisha P**
Department of Botany, St Xavier's College for Women, Aluva, Kerala, India

Abstract

Antagonistic effect is the ability to inhibit the life of an organism . Certain micro organisms act as a good antagonistic agent. This property can be very well used in formulating a biocontrol agent against a natural plant pathogenic agent and would be a very good step towards green agriculture. The present study covers antagonistic effect of *Trichoderma viride* against plant pathogenic fungus *Phytophthora capsici*.

Keywords: Antagonistic effect, *Trichoderma viride, Phytophthora capsici*

Introduction

Biological control is the use of natural enemies (biological control agents) to control a targeted weed, insect or a pathogen. The objective is to establish self-sustaining populations of the biological control agents that will proliferate and attack the target throughout its range. Biological agents are living organisms used for the purpose. The genus *Trichoderma* contains 9 species (Rifai, 1969) which are important saprobes found in soil,comprises a great number of fungal strains that act as biological controlling agents to other fungi including some pathogens. *Phytophthora capsici* is a fungus that affects solanaceous crops and cucurbits.

The present study is done to study the antagonistic effect of *Trichoderma viride* against *Phytophthora capsici* and to evaluate its antagonistic potential against fungal pathogen by dual culture techniques.

Materials and methods

Materials Required. Inoculums of antagonist and plant pathogenic fungus, petriplates, Potato dextrose Agar medium, inoculation loop.

Strains Used. The strain of ***Phytophthora capsici***was obtained from **Kerala Agricultural University, Mannuthy**.The antagonist ***Trichoderma viride*** was obtained from **Kaveri Fertilizers, Organic agri inputs Aluva**. The antagonists were selected based on their ability to suppress fungal pathogen and promote respective plant growth. Potato dextrose agar (PDA) was the choice of medium for sub culturing experiments.

Medium used. Potato Dextrose Agar (PDA):

Potato (peeled)	-	50.0 g
Dextrose (glucose)	-	5.0 g
Agar	-	5.0g
Water	-	250.0 ml

Method

Dual culture technique (Morton and Strouble 1995)**.** For this purpose two sterilized petriplates were prepared with PDA agar. This was prepared by pouring a small amount of molten water PDA agar and evenly spread over the plate to make a thin agar film. Culture of *Phytophthora capsici* was inoculated on PDA medium on petriplates 2mm away from the centre. These plates were incubated at 28^{o}c for four days. One plate was kept as a control .On the other plate after four days same plate was inoculated with *Trichoderma viride* 2mm away from the previous inoculums of the plant pathogenic fungus. It is again kept for incubation at 28^{o}C for four days.

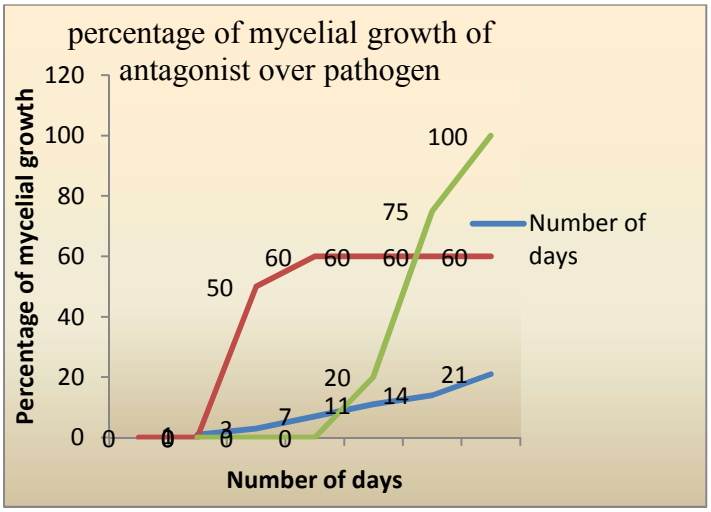
Graphical Analysis. Graphical analysis was made by observing the growth pattern of antagonistic agent *Trichoderma viride*against the plant pathogenic fungus *Phytophthora capsici* and is plotted on X and Y axis.

Result and Discussion.

Trichoderma viride inhibited mycelia growth of *Phytophthora capsici* which was well stabilized in the experimental plate. In dual culture plate growth of pathogen was normal initially but completely restricted growth of the mycelium in plate in the presence of *Trichoderma viride*.

The observation on experimental plate was tabulated as follows:

Number of days	**Mycelial growth of *Phytophthora capsici* (%)**	**Mycelial growth of Trichoderma viride over Phytophthora capsici (%)**
1	0	0
3	50	0
7	60	0
11	60	20
14	60	75
21	60	100



In dual culture plates *Trichoderma viride* completely colonized and showed over growth on *Phytophthora capsici* and inhibited its growth by 100% mycelial growth over it.

Trichoderma viride were able to reduce disease incidence of pathogen. It is due to the mechanism involved in the antagonistic activity by differential secretion of antifungal substances,various toxic and antibiotic metabolites (Dennis and Webster,1971; Claydon,1987;Lorito,1993) which are involved in the inhibition and lysis of pathogenic fungi. Similar reactions were reported (Barnett and Binder, 1973; Elad, 1983) which noticed inhibition of growth, lysis and parasitism by *Trichoderma* species against some species of *Phytophthora.* The isolation of a variety of mycoparasitic *Trichoderma* isolates have a potential to suppress root rot of Sugar beet caused by *Phytophthora* (Ezziyyani, 2007). These results also demonstrated a productive antagonistic effect against *Phytophthora capsici*'s root rot of pepper, especially on PDA medium enriched with laminarine – glucose (3:1,v/v), which is reported to increase the antifungal activity through secretion of the hydrolytic enzyme , β-1,3-glucanase.

Trichoderma viride inhibited mycelial growth, disorganized the host cell contents, lysed hyphae of *Phytophthora capsici* in the culture plates. Similar reactions were reported (Barnett, Binder) in which inhibition of growth, lysis and parasitism of some species of *Phytophthora* by *Trichoderma* species were observed. Hyphal lysis is due to an enzyme activity of *Trichoderma* isolates at the contact points (Elad). Five *Trichoderma* isolates *Trichoderma asperellum, Trichoderma harzianum* FG4, *Trichoderma harzianum* M53, *Trichoderma virens* DB2, *Trichoderma virens* DB6r showed the best result in reducing root rot. These isolates were selected and used in green house experiments. Progress colonization and sporulation of *Trichoderma* isolates on *Phytophthora* colonies

were observed. Similar results with other fungi have also been reported (Etebarian, 2006).

Non volatile metabolites produced by some of our *Trichoderma* isolates inhibited the growth of plant pathogen. Volatile metabolites also had inhibitory effects but the non volatile metabolites seem to be more effective in growth inhibition. Mycoparasitism, antibiosis, competition, promotion of plant growth and induction of systematic resistance in plants are mechanisms suggested to be involved in the antifungal activity of *Trichoderma* species (Vinale F, 2006).

Members of Trichoderma species are known to be active hyperparasites of several fungi, hence have been used as biocontrol agents (Wells H D,1972). Five possible modes of interacting colony growth are follows: (a) mutually intermingling growth was both fungi grow into one another without any microscopic signs of interaction, (b) intermingling growth were the fungus being observed is growing into the opposed fungus either above or below its colony, (c)intermingling growth were the fungus under observation has ceased growth and is being overgrown by another colony, (d)slight inhibition where the fungi approached each other, (e) mutual inhibition at a distance of 2 mm. Here, in dual culture plate growth of pathogen was normal initially but completely restricted growth of the mycelium in plate in the presence of *Trichoderma viride*. This is due to the mode of interaction where the fungus under observation, *Phytophthora capsici* has ceased growth and is being overgrown by another colony of *Trichoderma viride*.

Conclusion

The current study assures the efficiency of *Trichoderma* as a biocontrol agent against fungal soil pathogens such as *Phytophthora capsici*. It indicates the need of production and development *Trichoderma* based biocontrol agents to

serve as a model for environment friendly biocontrol agent. It also act as an avirulent opportunistic symbiotic. Antagonistic Trichoderma enhances resistance against the secondary infection of pathogen in the same plant.

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IN-SITU HYBRIDIZATION OF TAU mRNA IN TRANSGENIC DROSOPHILA MELANOGASTER THIRD INSTAR LARVAE

Princy Mary Paulose
Physician Assistant, 4802 10th ave, Brooklyn NY 11219.

Abstract

The purpose of this research was to study the transcription of human tau DNA in Drosophila melanogaster third instar larvae, and to detect the presence of tau mRNA wherever it was expressed. Drosophila flies transgenic for human tau were mated with flies having specific tissue-specific promoters which directed tau expression to specific tissues in the progeny. The promoter lines have specific GAL-4 transcription factors, which binds to UAS enhancer sequence upstream of tau promoter in the transgenic flies, inducing the transcription of tau in specific tissues in the latter. The two GAL-4 patterns that were used in this research were elav and eyeless. The expression of tau mRNA, if any, has to be detected using in-situ hybridization using an anti-sense RNA probe. The target-probe binding will be detected by antibodies, which specifically bind to regions where the probe mRNA (anti-sense) has bound to target mRNA (sense). In the end, differential interference contrast microscopy technique will be used for imaging of the desired gene expression. In this experiment, a negative control was also performed which involved a sense probe, which is not complement to the target mRNA (sense) of our interest. There will not be any target binding in this case.

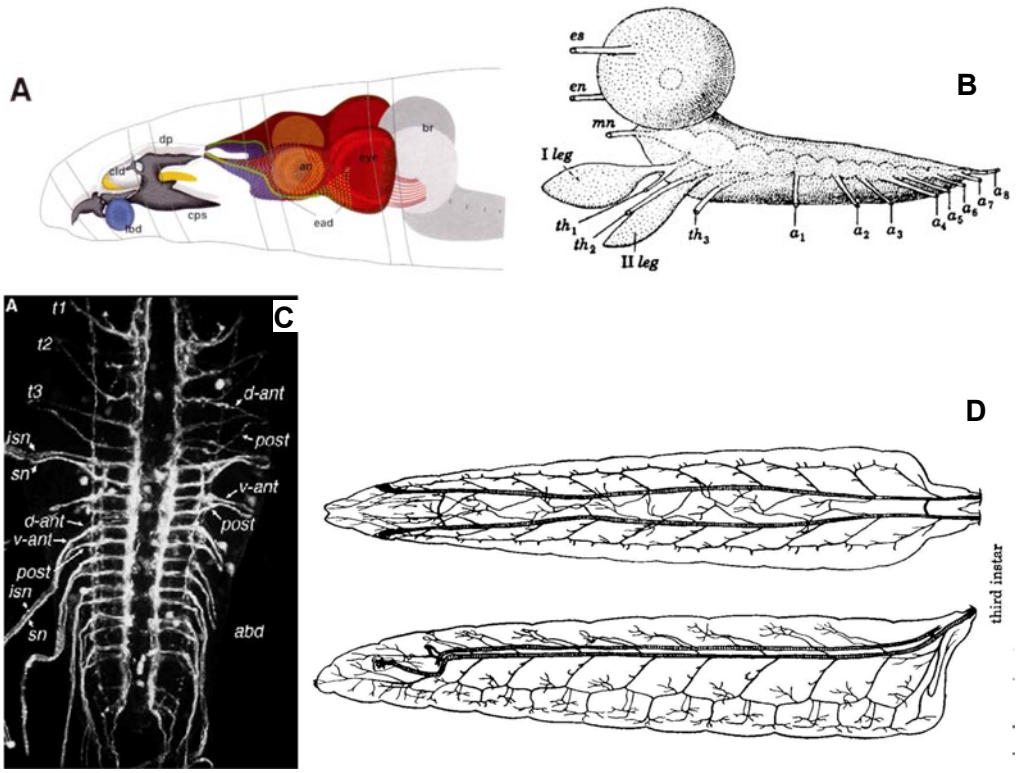
We expect that the F1 progeny formed by crossing elav GAl4 lines and transgenic tau flies will express tau in the elav pattern. Similarly, the eyeless driver lines will result in expression of tau in the same pattern in F1 generation.

Background and Significance

1. Drosophila as the model organism:

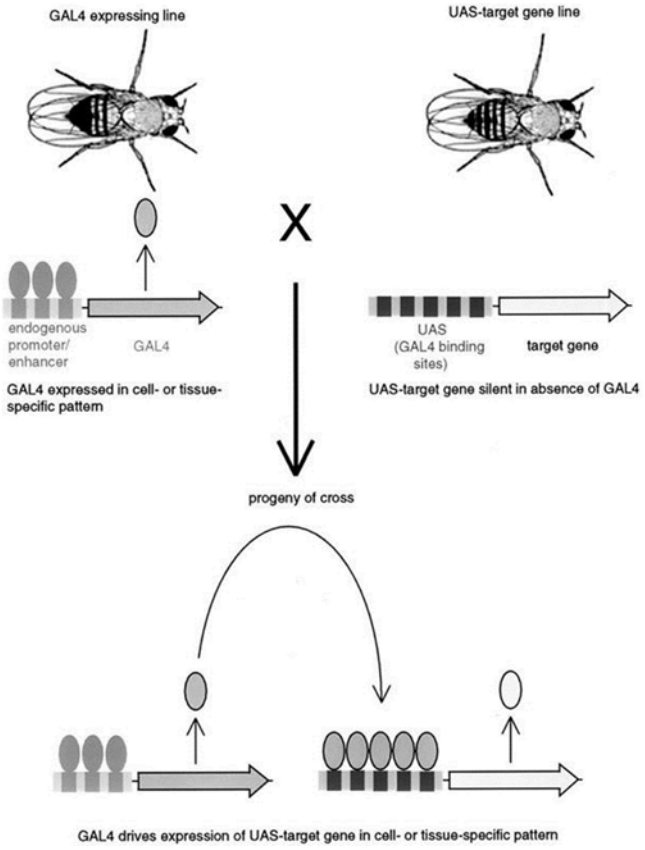
The use of drosophila melanogaster, commonly known as fruit fly, as a model organism for genetic studies has notable success. It is said that studies in fruit flies have changed our estimate of evolutionary relationship between vertebrate and invertebrate organisms (Reiter et al., 2001). About 75% of all known human disease genes have their counterparts in the Drosophila genome.

It is very easy and inexpensive to maintain large amount of fruit flies in the laboratory, and hence they have the longest history as model organisms in genetic studies. Another advantage that make them good candidates for sophisticated genetic screens include their rapid life cycle, and as a result large number of individuals are generated (Beir, 2005). The life cycle of Drosophila takes only about 10 days at an optimum temperature of 25 degree Celsius. Fruit flies begin their life cycle as embryo inside eggs (Reeve, 2001). After about one day, the embryo develops in to a larvae. The first instar larvae crawls out of the egg, and eats for one day until it molts in to second instar stage. After two days, the larva molts again to form the third instar larval stage. The larvae crawls out of the food source after three days and molts again in to a pupae, an immobile stage. Drosophila stays in the pupal stage for about five days, and metamorphosis in to an adult. Females emerging from pupae become receptive to adult males after 10 hours, lay eggs, and a new life cycle begins. We selected the third instar larval stage over adult fly for our particular study because it was easier and more convenient to fix the former group.



A. Diagram of the anterior end of third instar larvae showing mouth parts, and parts of imaginal disks such as antennal, eye, and brain disks. B. Central nervous system of larvae showing brain and ganglions. C. Ganglionic chains D. Larval tracheal system.

2. **GAL-4 induced ectopic gene expression in Drosophila:** The GAL4 system allows the targeted expression of any cloned gene in a wide variety of cell and tissue specific patterns in Drosophila (Duffy, 2002). The system involves two parts, the yeast GAL4 transcriptional activator protein and a responder gene. The GAL4 protein is formed by the transcription of GAL4 gene which is present in the GAL4 activator fly lines. The other part involves an upstream activator sequence, UAS, which is situated upstream of the promoter of the gene which has to be expressed. There is no target gene



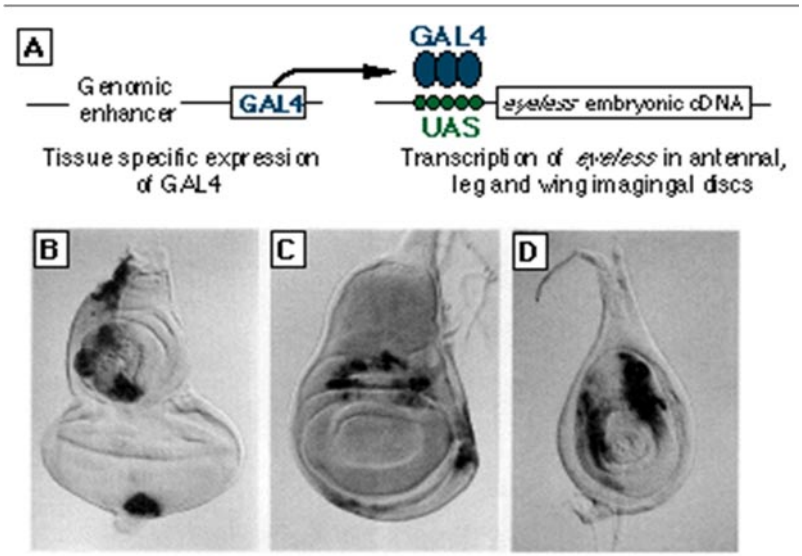
presentin the activator line. Similarly, transcription of the responder gene requires the presence of GAL4 protein. So in order to activate the responder gene transcription, responder lines are mated to flies expressing GAL4 in a particular pattern, called the driver. The target gene is turned on in the F1 progeny obtained by crossing of the activator line with L4 protein binds to UAS upstream of the geneand activates its transcription. (Phelps and Brand, 1998) the UAS-target gene line. The progeny will express the gene in a transcriptional pattern that reflects the GAL4 pattern of the respective driver (Phelps and Brand, 1998). GAL4 activator protein recognizes a 17 base-pair long sequence in the UAS sequence of the responder gene, binds to it, and promotes its transcription. The two driver lines that we used in this research were elav and eyeless.

Eyeless (*ey*) mutation was first described in 1915, and is a loss of function mutation, that have shown to lead to a reduction or absence of eye structures (Halder et al., 1995). This suggests that *ey* may be the master control gene for eye morphogenesis in Drosophila. In Drosophila, *ey* is first expressed in embryonic ventral nerve cord and in particular regions of brain. During larval

stages, it continues to be expressed in developing imaginal discs. However, in third instar larval stage *ey* expression becomes restricted to part of the eye disk that is anterior to the morphogenetic furrow. Hence the eyeless GAL4 inducer lines will direct tau expression as discussed above.

The embryonic lethal abnormal visual system (elav) gene in Drosophila is required for the correct differentiation and maintenance of the nervous system, and is expressed exclusively in neurons (Lisbin et al., 2001). The RNA binding protein encoded by elav promotes the generation of the neuron-specific isoform of Neuroglian by the regulation of pre-mRNA alternative splicing. Studies show that the ectopic expression of ELAV in imaginal disk cells is sufficient to mediate this neuron-specific splicing (Kaushika et al., 1996). Elav is expressed in the central nervous system as well as the peripheral nervous system of embryos, larvae, pupae, and adults. However, during the third instar larval stage, the elav expression is restricted to central nervous system and eye disks. Studies show that in third instar larvae, elav is not expressed in neuroblasts. It has to be noted that thus the elav GAL4 inducer lines will express tau according to elav pattern in the central nervous system and eye imaginal disks.

A. Representation of ectopic expression of ey by GAL4-UAS system. B through D. ß-galactosidase staining of third instar imaginal discs shows the activation of a UAS-LacZ reporter construct by the GAL4 enhancer-trap line E132. ey expression in several antenna disks (top) and posterior part of eye disk (bottom). C. ey expression in proximal regions of future wing blade. D. Leg imaginal disk with expression in regions corresponding to tibia and femur. (Halder et al., 1995).



3. Why do we need to study expression of tau? Tau is a microtubule associated protein which is formed by the alternative splicing of a single gene. In humans, it is found primarily in neurons of the central nervous system. It interacts with tubulin subunits, and promotes their assembly in to microtubules. It has six isoforms with microtubule binding domains, and is thus also responsible for the stabilization of microtubules. The hyperphosphorylation of tau plays a critical role in Alzheimer's disease (AD). Studies show that various kinases and phosphatases that regulate tau phosphorylation are responsible for this abnormality (Yu et al., 2009). Tau hyperphosphorylation results in formation of insoluble aggregates referred to as paired helical filaments and straight filaments (Iqbal et al., 2005). Self-assembly of these tangles result in the formation of neurofibrillary tangles which is associated with the pathogenesis of Alzheimer's disease and several other neurodegenerative diseases (Goedert, 2006).

4. Whole Mount In Situ Hybrisization to detect the ectopic expression of tau in Drosophila larvae by elav-GAL4 and eyeless-GAL4 inducer lines.

This technique aids in observing changes in gene expressions that occur throughout the development of an organism. It involves using a labelled RNA or DNA to detect specific DNA or RNA in an organism. The labelled RNA or DNA, known as probe, is complementary to the target and thus binds to the target sequence. This binding takes place at elevated temperatures, and excess of probe (unhybridized probe) is washed away. The probe-target interaction can be further localized by labeling with radioactive or fluorescent molecules or even with antibodies. Many factors such as probe concentration, temperature, and pH have to be taken in to account for an efficient probe-target binding. Higher probe concentrations result in increased probe-target binding, and the maximum rate of hybridization occurs at 25 degree Celsius below the melting temperatures of the target. It is also proven that the maximal rate of hybridization is achieved with long probes (Magliano et al., 2001).

Materials and Methods

Obtaining F1 generation with both GAL4 activator and UAS-target gene.

The first step in this research was to isolate virgin females from both eye inducer (ey) and brain inducer (elav) GAL4 lines. This was achieved by obtaining the females as soon as they came out of their pupal shells. Three females that were isolated in each case were then mated with 5 male flies that are transgenic for human tau. The latter ones are the UAS responder lines. The flies were reared at 24 degree Celsius in tube vials and their growth and maintenance was continuously monitored. After fertilization the pregnant females laid eggs (F1 generation) in the fly food at the bottom of the vials. The third instar larvae were crawling up the tube vials after about five days. The larvae were isolated and fixed at this stage.

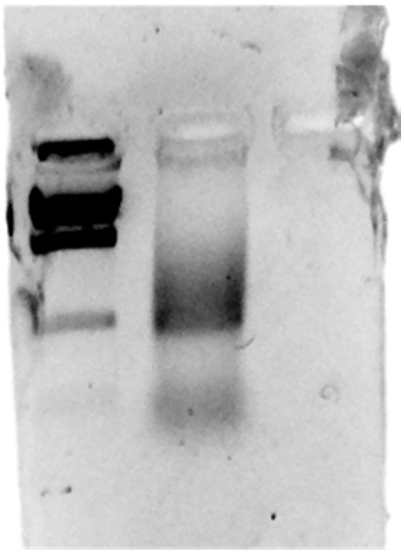
Fixation of larval tissue:

The anterior part of the Drosophila larvae was separated from the posterior part, with mouth parts and some imaginal disks in the former. The larval tissues were then fixed in 4% formaldehyde, 0.5 M NaCl and 0.1 M MOPS buffer (pH 7) for one hour at room temperature. After three washes with at least 1.5 ml of 0.1 MOPS at pH 7, 0.5 M NaCl, and 0.1% Tween-20 (MOPS buffer), the fixed tissues were stored in 70% ethanol at approximately 20 degree Celsius (Arenas-Mena et al., 2000).

Preparation of the riboprobe (RNA probe):

Primers (forward and reverse) were designed to amplify the tau DNA that was incorporated in a plasmid vector. The purpose of these primers were to select specific regions that will be amplified. Using primers TauT7F1 (5' - ATC CCA GAA GGA ACC ACA GCT GAA - 3') and TauT7R1 (5' - TGT TTG GTC AAC TGG ACT CGT TCC - 3'), PCR reactions (based on standard PCR protocols) were employed to amplify the tau DNA region in the plasmid. 25 µL of master mix with the plasmid template DNA was treated with 1 µL each of forward and reverse primers, and 25 µL of water was finally added to make it to a total volume of 50 µL. Spectrometric analysis and agarose gel electrophoresis were conducted to analyze purity of the PCR product. From the gel electrophoresis, we found that the PCR product was about 600 bps. Spectrometric analysis helped us to find the concentration of amplified DNA which was about 65 µL/mL. The next step was to produce digoxigenin-UTP labelled RNA probe by in vitro transcription of PCR amplified tau DNA with T7 RNA polymerase. About 4 µL of template DNA was added to a sterile, RNase-free reaction vial. The total sample volume was made up to 13µL by adding water. The reaction vial was placed on ice, and was added 2 µL each of 10x NTP labeling mixture, 10x transcription buffer, and T7 RNA polymerase.

1μL of protector RNase inhibitor was also added, and the contents in the vial were mixed gently and incubated for 2 hours at 37 degree Celsius. To remove the template DNA after transcription, 2 μL of DNase I was added and incubated for about 15 minutes at 37 degree Celsius. The reaction was stopped by adding 2 μL of 0.2 M EDTA at pH 8. Probe RNA was precipitated by centrifuging at 14,000 rpm at 4 degree Celsius for thirty minutes. The RNA transcripts were then analyzed by spectrometric and gel electrophoresis techniques. Spectrometric results showed an absorbance of 0.371 A at a wave length of 257 nm. We then calculated the concentration of RNA as 14.84 ng/μL, which was enough for our experiment. The riboprobe thus produced is an anti-sense RNA sequence which is complementary to the mRNA transcript (sense RNA) of our target tau gene in the transgenic fly larvae.

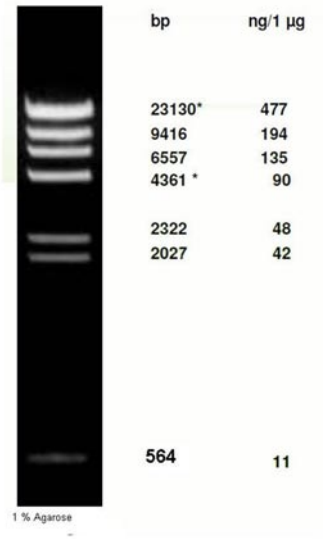


a) Gel electrophoresis results showing the size of riboprobe as about 600 bps. 2 μL of sample RNA plus same amount of loading buffer was loaded in the right side well, whereas in the left side well 2 μL of a marker was loaded. b) Right side picture shows a conventional lambda hind III plus marker. (The bottom light band in the marker corresponds to 600 bps as in the lambda hind III marker diagram). (Voytas, 1998).

Hybridization of the riboprobe to target tau mRNA and further staining of the hybridized probe:

The fly tissues were rehydrated with 3 washes of about 1.5 ml of MOPS buffer. Pre-hybridiation of the tissues were performed in a solution (hybridization

solution) containing 70% deionized formamyde at pH 7, 0.5 M NaCl, 0.1 M MOPS buffer at pH 7, 0.1 mg/ml Bovine serum albumin, BSA (solubilized in water first), and 0.1% Tween-20 for about 3 hours at 55 degree Celsius. In the hybridization step, the tissues were kept in 0.1 ng/µL of digoxigenin-UTP labelled riboprobe in hybridization solution at 55 degree Celsius for two days. After hybridization, tissues were washed 5 times in MOPS buffer at room temperature to remove any excess probe, incubated for another 3 hours in hybridization conditions, and washed three more times in mops buffer. The samples were then blocked with 10 mg/ml BSA in MOPS buffer for 20 minutes at room temperature, and then with a100 µL solution of 10% goat serum plus 1 mg/ml BSA in MOPS buffer at 37 degree Celsius for 30 minutes. Incubation of samples with 1/4000 dilution of alkaline phosphatase conjugated antibody solution was then performed overnight at room temperature. The excess antibody (non-specific binding) was removed by washing the tissues about 6 times in MOPS buffer for at least 12 hours. This was followed by further washing in alkaline phosphatase buffer which contains 100mM Tris base at pH 9.5, 100 mM NaCl, 50 mM MgCl, and 0.1% Tween-20. The reaction was then developed for 2-3 days by conventional method of adding 0.337 mg/ml NBT, 0.175 mg/ml BCIP, and 10% dimethylformamide in alkaline phosphatase buffer. The latter ingredient (dimethylformamide) was added to enhance the staining reaction. The reaction was stopped by diluting in MOPS buffer. (Arenas-Mena et al., 2000). Then we mounted the tissues in 50% glycerol, and a cover slip was placed on top of it followed by sealing with a red nail polish.

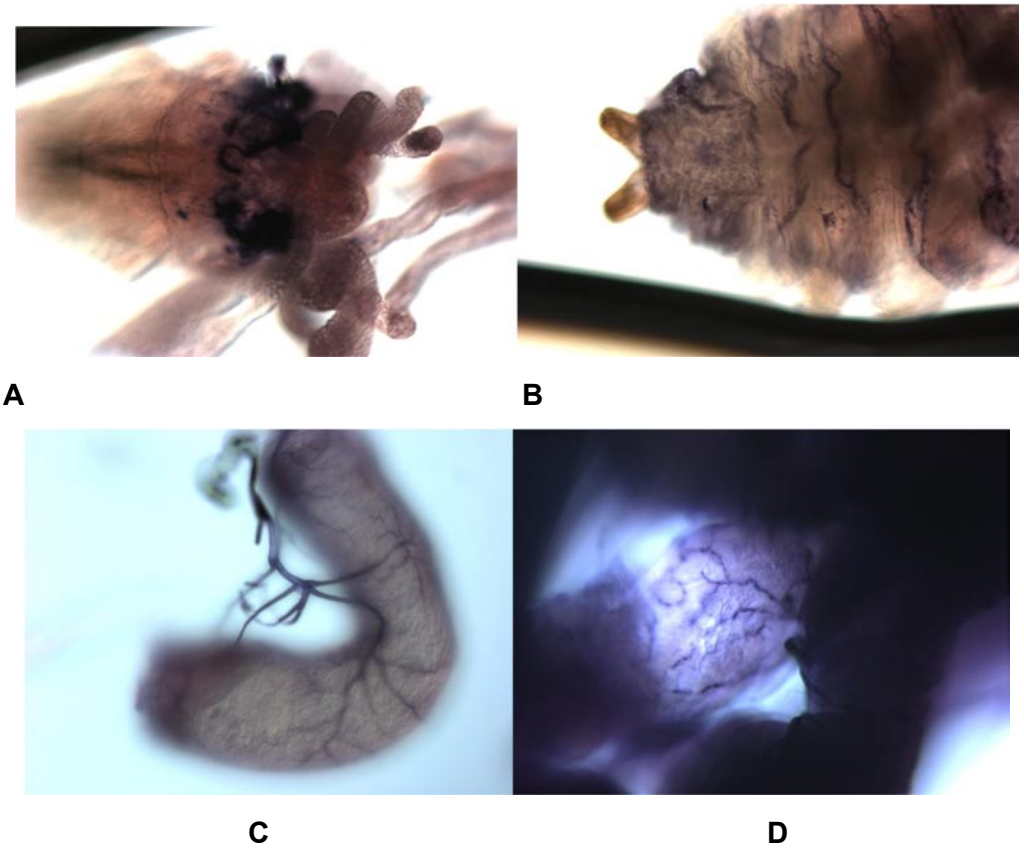


Differential interference contrast microscopy optics was employed to obtain images:

This technique allows live imaging, and involves higher intensity of light than standard conventional microscope. (Amos et al., 2003). This optics is employed to view transparent specimens such as living tissues, which are normally difficult to observe under traditional bright field illumination techniques. The basic setting resembles that of a traditional polarized light microscope with specialized beam-splitting prisms. The prisms can accommodate objective lenses with varying focal lengths and aperture sizes. A polarizer and analyzer are inserted into the optical pathway before the condenser and after the objective, respectively. This microcopy technique converts gradients in specimen optical path length and convert it in to amplitude differences that can be viewed as difference in contrast in the resulting image. Differential interference microscope produces an image with high resolution, which can be manipulated using digital and video imaging techniques to further improve contrast (Matsui, 2003).

Results and Discussion:

We expected that the F1 generation formed by crossing eyeless GAL4 inducer lines with the transgenic tau gene responder lines would express tau in the eyeless pattern. That is, tau will be directed to express in specific pre-determined areas of eye, antennal, leg and wing imaginal disks. Similarly, elav GAL4 driver lines will express tau in same places where elav is expressed in the third instar larvae. The expression will be found in the central nervous system, and the eye imaginal disks of third instar larvae.



A. Shows the anterior part of the tissue in negative control treatment, with parts of gut sticking out. B. Posterior of the larval tissue in negative control. C. Gut from larval tissue of eyeless-GAL4 x UAS-tau. D. Posterior part of elav GAL4 x UAS-tau cross.

The tissue-specific expression in tissues was not really seen because of the thick cuticle covering that cover the tissues at many places. The probe, sticking on to the cuticle, was creating too much noise. The cuticle covering was restricting the view to the tissue interior. However, we identified that some structures were sticking out from the cuticle covering. Those structures are the gut of the organism, and the thread-like structures on it looked like the tracheal system. It was stained because the probe was bound non-specifically to the tracheal system. It was really difficult for us to finalize anything because of the lack of

enough negative control to compare to. In short, we could say that there was too much noise because of the probe binding to non-specific areas such as cuticle. No detection of any non-specific binding might be two reasons. One is the thick cuticle covering hindering proper inside view, and other is the lack of enough negative control.

The purpose of negative control was to see if there is any non-specific binding happening in the tissue. We could see the negative probe sticking on to parts of cuticle and the breakage area(formed when we dissected), which inferred that there are chances of non-specific staining in our experimental tissues which is not desirable.

Alternative plans

This experiment has to be repeated to get accurate results. Alternate dissection method should be employed, by which an efficient removal of the thick cuticle covering can be achieved.

In this experiment, we dissected the larvae in the middle by tearing it apart in to anterior and posterior tissues. However, the cuticle was in tact even after the dissection. Therefore, we should think of some way to get the internal structures out without the cuticle. One method is to grab the mouth and pull the imaginal disks and central nervous system out, which will be attached to the mouth parts. If this method is employed, we do not have to worry about non-specific binding of the probe to cuticle or the breakage area. In order to study the targeted expression of tau protein in fruit fly, we could also use the Drosophila embryos as an ideal specimen since it is much easier to handle. Further, there is no cuticle present in this stage.

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PROGNOSTIC SIGNIFICANCE OF PHOSPHORYLATED STAT3 AND SURVIVIN EXPRESSION IN BREAST CANCER

Prabha Pillai[1] and **Lakshmi S**[2]

[1]Department of Zoology, NSS Hindu College, Changanacherry. Affiliated to Mahatma Gandhi University, E-mail ID: prabharcc@gmail.com

[2]Laboratory of Molecular Medicine, Division of Cancer Research, Regional Cancer Centre, Thiruvananthapuram

Abstract

Breast cancer is the most common cancer among women worldwide. Understanding the molecular basis of breast cancer and increasing each patient's chance of survival is important. Transcription factors are incongruously activated in breast tumors. Among these are Signal Transducers and Activators of Transcription (STATs) which serves as both cytoplasmic signal transducers and transcription activators controlling gene expression. Apoptosis is a conserved genetic program essential for the development and homeostasis of the immune system. Survivin is a member of the inhibitor of apoptosis protein (IAP) family that is frequently expressed in tumors. RT-PCR, immunohistochemistry and western blot analysis of primary breast tumor samples and normal breast tissues revealed an elevated expression of STAT3 and Survivin at the mRNA level and phosphorylated STAT3 (p-STAT3) and Survivin at the protein level expression in breast tumor samples.Expression of p-STAT3 at the protein level was strongly associated with clinicopathological features of breast cancer patients such as patient's age, parity, metastatic lymph nodes and histology grade. Survivin demonstrated strong positive associations with tumor stage and tumor grade. Further, p-STAT3 significantly associated with Survivin in breast tumor samples. Expression of p-STAT3 and Survivin at the protein level demonstrated an inverse association with overall survival of cancer patients. Therefore, p-STAT3 along with its downstream target, Survivin may be used as molecular marker to predict poor prognosis in breast cancer.

Keywords: Phosphorylated STAT3 (p-STAT3), Survivin, breast cancer

Introduction

Signal Transducers and Activators of Transcription 3 (STAT3) is a member of latent cytoplasmic transcription factors that convey signals from the cell surface to nucleus on activation by cytokines and growth factors [1]. In response to these factors, STAT3 is phosphorylated by receptor associated kinase. This activated STAT3 (p-STAT3) can dimerize and translocate to the nucleus and regulate gene expression. Constitutive activation of STAT3 has been detected in numerous cancers and is found to mediate malignant transformation by up-regulating its downstream targets such as Survivin. Survivin is a member of Inhibitor of Apoptosis protein (IAP) and is involved in anti-apoptosis and in regulation of cell division. Normal cellular homeostasis is maintained by a balance between the process of growth and cell death. Imbalance in either can lead to uncontrolled cell growth and the development of cancer. The development of anti-apoptotic phenotype is one of the hallmark characteristic that is required for cells to become cancerous [2]. Breast cancer is a heterogeneous disease and therefore understanding its molecular basis is very important for employing better treatment regimes. In the present study, we have investigated the expression and prognostic significance of p-STAT3 and Survivin in breast cancer patients. Overexpression of p-STAT3 and Survivin was found in breast tumor samples compared to normal breast tissue and strongly associated with the clinicopathological features of breast cancer patients. We have found that p-STAT3 and Survivin were significantly associated with unfavorable outcome of breast cancer using Kaplan-Meier survival analysis and may be used as a prognostic factor for poor outcome in breast cancer.

Materials and Methods

Study Subjects and Sample Collection

Breast tumor samples and adjacent normal breast tissues were collected from a cohort of 100 breast cancer patients who were previously untreated and undergoing primary surgery for breast cancer at Regional Cancer Centre, Thiruvananthapuram. Of the 100 breast tumor and adjacent normal breast samples collected, 92tumor samples and 20 normal samples were subjected to mRNA analysis while 55 tumor samples and 10 normal samples were used for protein analysis. An informed consent was obtained from each breast cancer patient and this particular study was recognized by the Regional Cancer Centre Review Board and Human Ethical Committee. The patient details and clinicopathological features were obtained from their medical records maintained by the hospital. For mRNA analysis, the breast samples were collected in RNA*later* (Ambion) while for western blot analysis, samples were collected in phosphate buffered saline (PBS). For immunohistochemistry, tissues were fixed in 10% buffered formalin.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using TRI Reagent (Sigma) following manufacturer's protocol and was immediately quantified by spectrophotometry (A_{260}/A_{280} ratio). The integrity of mRNA was checked by agarose gel electrophoresis. 2 µg of total RNA was reverse transcribed to cDNA at 42°C for 1 h in a 25 µl reaction mix. PCR amplification was performed in a 20 µl reaction mix containing 0.2 µl Taq DNA polymerase in a 5X reaction buffer containing 1.2 µl dNTP mix and specific primers for STAT3 and Survivin. The amplified products were resolved on 1.2% agarose gel impregnated with ethidium bromide. The bands were visualized under a UV illuminator (Vilber

Lourmat) and photographs were taken using gel documentation and analysis system (Amersham Pharmacia Biotech Asia Pacific Ltd). The band width was quantified using ImageJ 1.47, USA.

Immunohistochemistry

Four μm thick sections of formalin fixed, paraffin-embedded breast tissues and adjacent normal breast samples were first subjected to Haematoxylin and Eosin staining (H&E staining) to confirm whether they were tumor and normal samples and to ensure that the number of tumor and normal cells were sufficient for IHC analysis. IHC analysis was carried out using standard procedures. Serial sections of breast tumor tissues and adjacent normal breast tissues were deparaffinized in xylene and hydrated through graded alcohol. Endogenous peroxidase activity was blocked using 0.3% H2O2 in methanol for 30 min. These sections were then subjected to antigen retrieval by boiling in 10 mM citrate buffer (pH 6.0)for 15 minutes. The slides were then cooled and non-specific binding sites were blocked by incubating with 3% bovine serum albumin (BSA) for 20 minutes in a humidified chamber at room temperature. The sections were then incubated overnight at 4°C with primary antibodies specific for STAT3 (1:800; Cell Signaling Technology) and Survivin (1:50; Santa Cruz Biotechnology). Negative controls were performed by substituting the primary antibody with 1% BSA in PBS. The bounded primary antibody was detected by addition of secondary antibody conjugated with horseradish peroxidase polymer (HRP) and diaminobenzidine (DAB) substrate using Super Sensitive Polymer HRP Detection System (Biogenex). The sections were then counterstained with haematoxylin, dehydrated through graded alcohol, cleared in xylene and were mounted using DPX mountant. The slides were evaluated for nuclear expression and cytoplasmic staining of p-STAT3 and Survivin.

Western Blotting

Tissue extract was prepared from adjacent normal breast samples and tumor breast tissues by using RIPA (Radio Immuno Precipitation Assay) buffer. After centrifugation at 12,000 rpm for 20 min at 4°C, the protein concentrations were determined by Bradford assay using Bradford reagent. For Western blot analysis, tissue extracts were mixed with 5X Laemlli bufferand were boiled for 5 min at 95°C. 60 µg of protein were separated by electrophoresis on a 6-12% SDS-polyacrylamide gel using mini-PROTEAN3 cell (Biorad, CA). The separated proteins were electrophoretically transferred on to polyvinylidinene difluoride (PVDF) membrane (Millipore Corp., MA) for 2-6 h at 40-80 V and blocked with 5% nonfat milk in tris buffered saline (TBS). After blocking, membranes were incubated at 4°C overnight with p-STAT3 (1:100, Cell Signaling Technology) and Survivin (1:200; Santa Cruz Biotechnology) primary antibodies in TBST containing 5% BSA. The expression of β-actin (1:400; Santa Cruz Biotechnology) was used as normalization control for protein loading. The membranes were then washed with TBST and were incubated with corresponding alkaline-phosphatase conjugated secondary antibodies (Sigma). Subsequently, membranes were washed and immune complexes were detected using Bromo-4-Chloro-3-Indoyl Phosphate (BCIP)/Nitro Blue Tetrazolium (NBT) (Sigma) in alkaline phosphatase buffer (Appendix 2.13). The specific proteins were detected with reference to prestained molecular weight marker (Sigma).

Statistical Analysis

Statistical analysis was carried out using SPSS software 17.0 (SPSS Inc., USA). To estimate the correlation between STAT3, p-STAT3 and Survivin markers, Spearman's rho correlation test (two-tailed) was used. This test was

also used to analyze the association of the above mentioned markers with clinicopathological variables of breast cancer patients. To assess the clinical features contributing to overall survival of breast cancer patients, Univariate Cox regression analysis was carried out. Univariate survival analysis was performed using Kaplan-Meier method (Log rank test) to predict the prognostic significance of p-STAT3 and Survivin with the overall survival of breast cancer patients. The significance for all the tests was set at $P<0.05$.

Results

Expression of STAT 3 and Survivin in Breast Tumor Samples and Normal Breast Tissues

92 breast tumor samples and 20 normal breast tissues were subjected to RT-PCR to analyze the mRNA expression of STAT3. The band intensity of STAT3 mRNA in tumor samples was higher compared with normal breast tissues. Concomitantly, a high frequency of STAT3 mRNA expression (89%) was observed in breast tumor tissues while only 10.9% of normal samples expressed STAT3 mRNA.The expression of Survivin was observed in 78% of breast tumor samples compared with normal breast tissues (40%) at the mRNA level and the tumor samples had a higher band intensity compared to normal tissues (Figure 1).

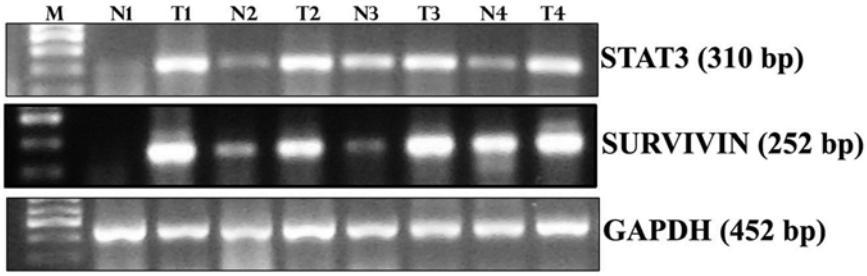

Figure 1: RT-PCR analysis of STAT3 and Survivin mRNA expression in normal breast tissues and breast tumor samples. Representative results of expression of STAT3 and Survivin mRNA in normal breast tissues (N) and breast tumor samples (T), M-100 bp ladder.

Immunohistochemical and Immunoblot Analysis of P-STAT3 and Survivin in Breast Tumor Samples and Normal Breast Tissues

The expression of p-STAT3 and Survivin proteins was analyzed in 55 breast tumor tissues and 10 normal breast samples (Figure 2). 58% of tumor samples expressed of p-STAT3 whereas only 30% of the normal samples expressed p-STAT3. IHC staining revealed both cytoplasmic and nuclear localization of p-STAT3 in breast tumor and normal samples. Most of the tumor samples positive for nuclear p-STAT3 showed moderate expression (16%) while in normal tissues, most of them demonstrated mild immunolocalization (20%). The expression of p-STAT3 was not detectable in the cytoplasm of majority of normal (80%) and tumor breast samples (71%). IHC analyses revealed a high frequency of Survivin expression in tumor samples (95%) compared to normal samples (50%).

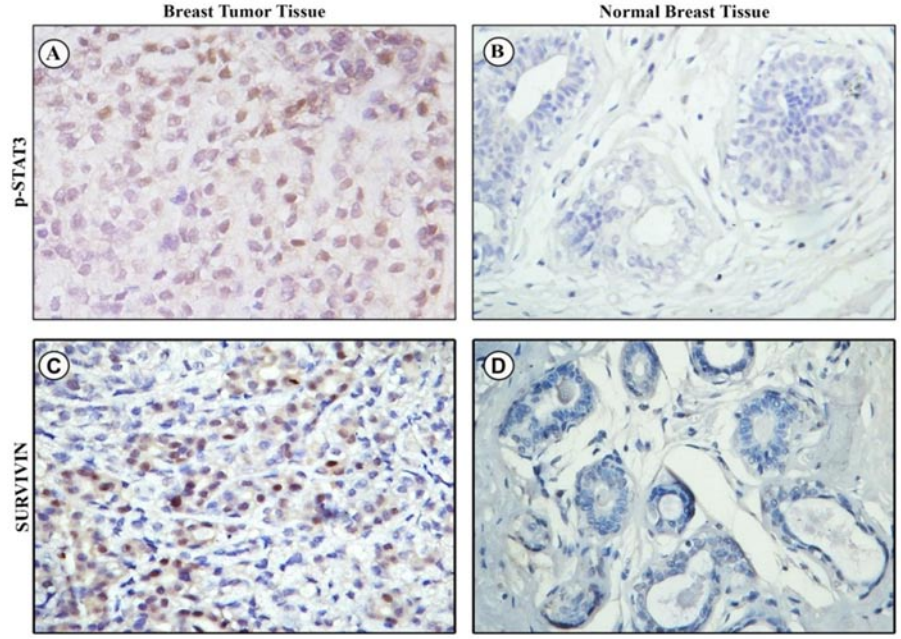

Figure 2: Protein expression levels of p-STAT3 and Survivin were elevated in breast tumor samples compared with normal breast samples.A and Brepresents immunolocalization ofp-STAT3 in breast tumor and normal breast samples respectively.C and Dindicate immunostaining of Survivin in tumor and normal tissues respectively.

The analysis of immunohistochemical staining pattern revealed both nuclear localization and cytoplasmic staining for Survivin. Half of the normal samples were negative for nuclear localization of Survivin and exhibited no intense expression for it. Most of the normal samples were also negative for cytoplasmic expression for Survivin. Mild nuclear expression for Survivin was predominant in most of the tumor samples. 10% of the normal samples showed strong cytoplasmic staining for Survivin. Similarly immunoblot analysis also revealed a high expression of p-STAT3 and Survivin in breast tumor samples compared to adjacent normal breast samples (Figure 3).

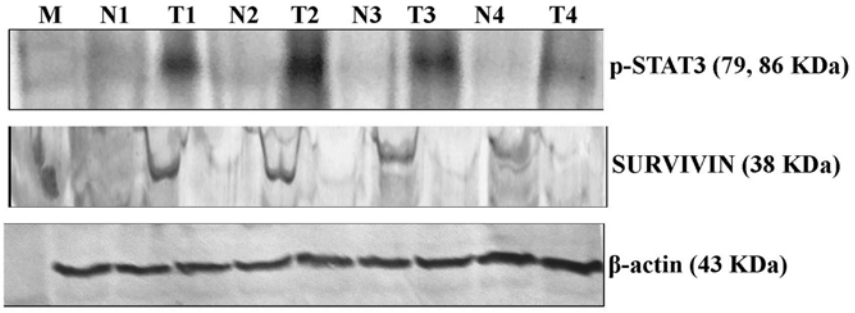

Figure 3: Western blot analysis of p-STAT3 and Survivin protein expression in normal breast tissues (N) and breast tumor samples (T) in representative samples. M- Protein molecular weight marker.

Association between p-STAT3 and Survivin Proteins in Breast Cancer

Activated STAT3 significantly associated with Survivin in tumor tissues (r=0.343, P=0.010).p-STAT3 did notdemonstrate any strong correlations with Survivin in normal samples.

Clinicopathological Significance of p-STAT3 and Survivin in Breast Cancer

Expression of p-STAT3 protein strongly associated with breast cancer patient's clinicopathological features such as patient's age *(*r = 0.107, P = 0.367), parity (r = 0.838, P= 0.024), metastatic lymph nodes (r = 0.325, P= 0.011) and

histology grade (r = 0.946, P= 0.008). Survivin demonstrated strong positive associations with tumor stage (r = 0.283, P= 0.036) and tumor grade (r = 0.366, P= 0.006) and a significant negative correlation with cancer therapy(r = -0.341, P= 0.011).

Survival Analysis Based on Expression Status of p-STAT3andSurvivin In Breast Tumor Samples

Univariate survival analysis was performed using Kaplan-Meier method to find out the overall survival of patients with breast cancer.p-STAT3 demonstrated a negative impact on the overall survival of cancer patients (Figure IV).Enhanced expression of Survivin drastically reduced the overall survival of breast cancer patients (Log rank, P = 0.009) (Figure V).To assess the clinical features contributing to the overall survival of breast cancer patients, univariate Cox regression analysis were carried out.Tumor size (P = 0.013) and tumor stage (P= 0.001) correlated significantly with overall survival of breast cancer patients.

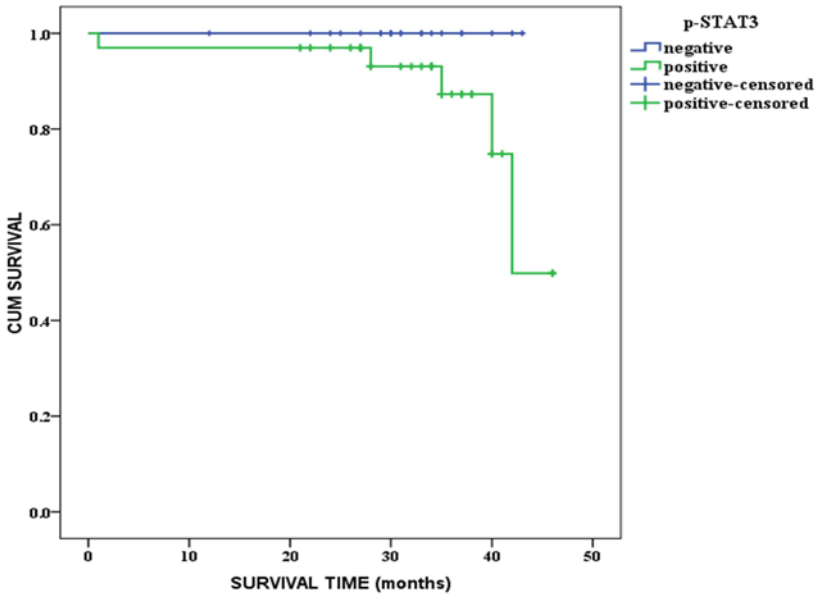

Figure 3: Kaplan-Meier curve of overall survival of patients with breast cancer, dichotomized based on the positive and negative expression of p-STAT3.

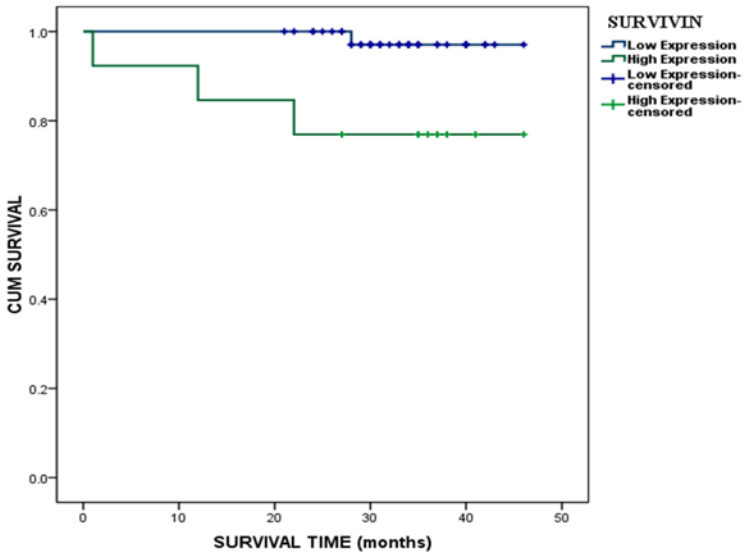

Figure 5: Kaplan-Meier curve for overall survival of patients with breast cancer, dichotomized based on high and low expression for Survivin protein.

Discussion

Breast cancer is a heterogeneous disease occurring from multiple genetic changes in oncogenes and tumor suppressor genes which play a potent role in maintaining the homeostatic control of mammary epithelial cell proliferation, differentiation and death. The constitutive expression of transcription factors such as STAT3 is capable of transforming normal cells into malignant forms. In the JAK-STAT paradigm, cytokine binding to its receptor results in activation of receptor associated Janus Kinases (JAKs) or activation of receptor-intrinsic tyrosine kinase activity. Homo or heterodimerization of STATs, its translocation to nucleus and binding to target genes induces transcription [3]. It may also contribute to tumor progression by up-regulating its downstream targets such as Survivin. In this particular study, the RT-PCR analysis revealed a high frequency of expression and greater band density for STAT3 and Survivin in breast tumor samples compared to normal breast tissues. This is in concordance

with the elevated expression of STAT3 and Survivin protein in breast tumor samples on immunohistochemical and immunoblot analysis. Persistent activation of STAT3 requires a cyclic process of phosphorylation in the cytoplasm and dephosphorylation in the nucleus and export through the nuclear pore complex [4]. Correlation analysis demonstrated a strong correlation between p-STAT3 and Survivin in breast tumor samples, suggesting Survivin as an immediate downstream target of STAT3 and their role in tumor progression. The most aggressive cell lines are found to have the highest DNA binding activities [5]. On examination of the association of STAT3 with clinicopathological features of breast cancer patients, we found that p-STAT3 protein strongly correlated with patient's age, parity, metastatic lymph nodes and histological grade. On the other hand, Survivin protein demonstrated strong associations with tumor stage and tumor grade of breast cancer patients. Univariate Survival analysis using Kaplan-Meier method showed that both p-STAT3 and Survivin had a negative impact on the overall survival of breast cancer patients. Further, tumor size and tumor grade significantly correlated with overall survival of breast cancer patients. Therefore, high expression of STAT3 and its downstream target, Survivin in cancer lesion may be a useful biomarker for poor prognosis in breast cancer. This poor outcome may be explained atleast in part by the multiple cellular function of STAT3 which is a critical component of diverse signal transduction pathway [6].

Conclusion

Breast cancer is the most common cancer worldwide and in India, it is the second most common cancer in women after cervical cancer. Since breast cancer is a heterogeneous disease, new prognostic markers for breast cancer are essential. STAT3 is constitutively expressed in most cancer and the expression of phosphorylated STAT3 in cancer tissues is important to understand the

tumor progression. In this particular study, the expression of STAT3, p-STAT3 and its downstream target, Survivin was found to be elevated in breast tumor samples compared to normal breast tissues. A strong association was also found between p-STAT3 and Survivin in tumor samples. Significant correlations of p-STAT3 and Survivin proteins with some of the important clinicopathological features of breast cancer patients accounts for their potent role in breast cancer. p-STAT3 and Survivin expression had a significant negative impact on overall survival of breast cancer patients, suggesting them as a useful biomarker for poor prognosis in breast cancer.

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SCREENING AND OPTIMIZATION OF BIOSURFACTANT PRODUCTION BY THE HEAVY METAL RESISTANT BACTERIA

Gisha Elizabeth Koshy [1] and **Elza John** [1]

[1]Department of Microbiology, Mar Athanasius College, Kothamangalam

Abstract

Biosurfactants are amphiphilic compounds produced by microorganisms as secondary metabolite. Spot inoculation method determined the bacterial heavy metal resistance to copper. Heavy metal resistant isolates also showed anitibiotic sensitivity except for penicillin. Biosurfactant production was confirmed by conventional screening methods, including haemolytic activity, oil spreading technique and emulsification capacity (E24) test. Biosurfactant production by two isolates using different temperature, pH and concentration of diesel was studied. Biosurfactants were then extracted from these two isolates. On the basis of 16S rRNA gene sequencing, two of the isolates were authentically identified as *Pseudomonasaeruginosa* and *Acinetobacter baumannii.*

Introduction

Biosurfactants are heterogenous group of surface active molecules produced by a wide variety of bacteria, yeast and filamentous fungi, which either adhere to cell surface or are excreted extracellularly in the growth medium.[1] Biosurfactants play a number of roles including increasing the surface area and bioavailability of hydrophobic water-insoluble substrates, binding of heavy metals, quorum sensing and biofilm formation.[3,4] Compared with synthetic surfactants, biosurfactants have higher surface activity, lower toxicity, higher biodegradability and better environmental compatibility.[18]

With their high surface activity and environmental compatibility, biosurfactants are widely used in environmental applications such as for enhancement of oil degradation,[2]

Materials and Methods

Sample collection and Identification of Bacterial Isolates

Industrial effluent was collected from Cochin Refinery. The selected isolates from the industrial sample were subjected to Gram's staining, motility and biochemical tests for identification.

Determination of Mtc's of Heavy Metal by Spot Inoculation[5]

Cu was added to sterilized nutrient agar medium at concentrations 1 ppm, 10 ppm, 50 ppm, 100 ppm. Plates were then spot inoculated and incubated at 37ºC for 2 days.

Screening For Biosurfactant Activity & Extraction of Biosurfactants

Biosurfactant production was identified using Haemolytic activity[6], Oil spreading technique[7], and Emulsification capacity (E24) test [8]. Biosurfactant was extracted[10]from the most promising isolates and dry weight was calculated

Optimization of Physical Parameters for Biosurfactant Production [9]

The process parameters were optimized using mineral salt broth in a series of experiments to obtain higher productivity of the biosurfactant. The observed variables were pH, temperature and concentration of diesel.

Molecular Characterization

The isolated bacterial DNA were analysed on 0.8% agarose gel and visualized under UV transilluminator.

Amplification of 16S rRNA Gene

16S rRNA gene was amplified using the standard PCR amplification technique using the isolated genomic DNA as a template. The DNA band was excised using a scalpel blade and transferred in to a pre weighed dry eppendorf and.

Sequencing of the Gene

ABI sequencing of the samples were done at SciGenom, Kakkanad.The obtained sequence were analysed using (BLAST), and highly similar sequences were downloaded in FASTA format.

Results & Discussion

From the industrial effluent water sample, a total of 20 isolates were obtained by subculturing. Among these, 8 isolates were identified by Gram staining, Motility test and other biochemical tests and the results were tabulated. The isolates were found to be *Pseudomonas* sp., *Acinetobacter* sp., *Staphylococcus* sp., *Escherichia coli*, *Vibrio* sp., *Proteus* sp., *Bacillus* sp.and *Klebsiella* sp. Seventeen isolates showed resistance to copper upto 100 ppm.The most efficient biosurfactant producing isolates were identified using Haemolytic activity, Oil spreading techniquue[1],and Emulsification capacity (E24) test.Optimum conditions of parameters for the growth of the two selected isolates were analysed. *Acinetobacter* sp. showed maximum growth at pH 8, temperature 37˚C and 5% diesel concentration. *Pseudomonas* sp. showed maximum growth at pH 6, temperature and 1% diesel concentration. The growth of *Acinetobacter* sp. increased with increasing concnetration of diesel. Hence it is of importance in areas with heavily diesel contamination.

Genomic DNA isolation (Figure 1) of the two of the biosurfactant producing isolates were performed. 16S rRNA gene was amplified with specific primers and resolved in 1.5% gel. An amplicon size of 750bp was observed and confirmed with the help of a DNA ladder.

The amplified DNA was eluted using GeneJET elution kit and sequenced. The 16S rRNA genes of *Pseudomonas* and *Acinetobacter* were analyzed and edited using the software, BIOEDIT. Homology search was performed using BLAST in order to authenticate the similar sequences in the database. Comparison of isolated organism with other organism reveals that the sequence of isolated organism has 100% similarity with that of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. (Table 2, 3, Figure 2, 3)

Biosurfactant was extracted from both *Acinetobacter* sp. and *Pseudomonas* sp. *Acinetobacter* sp. produced higher amount of biosurfactants (1.330 g) compared to *Pseudomonas* sp. (0.134 g). (Table 1).Hence the heavy metal tolerant *Acinetobacter baumannii* is a good source for biosurfactant production. The unique properties of biosurfactants make them possible to replace or to be added to synthetic surfactants which are mainly used in food, cosmetics, pharmaceutical industries and in environmental applications.

Table 1: Dry weight of biosurfactants

Isolates	Plate weight (g)	Plate weight after drying of biosurfactants (g)	Dry weight of biosurfactants (g)
Pseduomans sp.	43.160	43.294	0.134
Acinetobacter sp.	44.250	45.580	1.330

Table 2 : Homology comparison of isolate 1

Source culture	Closest relative	Gene bank accessionnumber	Similarity %
Isolate 2	*Acinetobacter baumannii* Naval -81 clone	JN668259.1	100
	Acinetobacter baumannii Naval -81 clone	JN668243.1	100
	Acinetobacter baumannii Naval -81 clone	JN668236.1	100
	Acinetobacter baumannii Naval -81 clone	JN668232.1	100

Table 3: Homology comparison of isolate 2

Source culture	Closest relative	Gene bank accession number	Similarity %
Isolate 2	*Acinetobacter baumannii* Naval -81 clone	JN668259.1	100
	Acinetobacter baumannii Naval -81 clone	JN668243.1	100
	Acinetobacter baumannii Naval -81 clone	JN668236.1	100
	Acinetobacter baumannii Naval -81 clone	JN668232.1	100

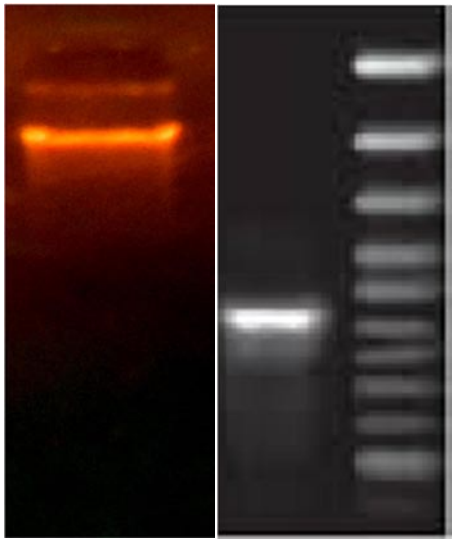

Figure 1: Genomic DNA

TCGGGGATTTCACATCCGACTTAATAAGCCGCCTGCGCACGCTTTACGCCCAGTAAATC
CGATTAACGCTCGCACCCTCTGTATTACCGCGGCTGCTGGCACAGAGTTAGCCGGGGC
TTATTCTGCGAGTAACGTCCACTATCTCTAGGTATTAACTAAAGTAGCCTCCTCCTCGCT
TAAAGTGCTTTACAACCATAAGGCCTTCTTCACACACGCGGCATGGCTGGATCAGGGTT
CCCCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCA
GTCCCAGTGTGGCGGATCATCCTCTCAGACCCGCTACAGATCGTCGCCTTGGTAGGCCT
TTACCCCACCAACTAGCTAATCCGACTTAGGCTCATCTATTAGCGCAAGGTCCGAAGAT
CCCCTGCTTTCTCCCGTAGGACGTATGCGGTATTATCATCCCTTTCGAGATGTTGCCCC
CCACTAATA

Figure 2: Sequence of *Pseudomonas sp*.

CTCTAGCTCAGTAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCAA
CTTGCTGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCT
TCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGTTGGTAACGTCA
AAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGA
AGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTC
CCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGATCAT
CC

Figure 3: Sequence of *Acinetobacter baumannii*

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