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## Current Trends in Biotechnology and Pharmacy

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## Information to Authors

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Mahavadi, S., Rao, R.S.S.K. and Murthy, K.S. (2007). Cross-regulation of VAPC2 receptor internalization by m2 receptors via c-Src-mediated phosphorylation of GRK2. *Regulatory Peptides*, 139: 109-114.

Lehninger, A.L., Nelson, D.L. and Cox, M.M. (2004). *Lehninger Principles of Biochemistry*, (4<sup>th</sup> edition), W.H. Freeman & Co., New York, USA, pp. 73-111.

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## Generation of FMDV recombinant 3ABC protein and development of monoclonal antibodies as reagents for 3ABC quantitative ELISA

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### Abstract

FMDV is one of the most contagious disease in cloven hoofed animals and it is known to cause lameness, vesicular lesions on tongue, feet, teats and snout with reduced milk production, loss of weight and high mortality in young animals. Many countries being endemic, continuous vaccination might help in attaining disease free status. The vaccination programme with a marker vaccine can establish differentiation among vaccinated and unvaccinated animals by eliciting immune response against the viral structural proteins and not against non-structural proteins (3ABC). Vaccine manufacturers should ensure the limited NSP content in their vaccine formulations, so that there will be no immune response against non-structural proteins. This can be evaluated by determining the concentration of NSP (3ABC) in the vaccine antigen batches. In the present study, we have developed a recombinant 3ABC antigen with minor modification at the 3Cpro catalytic site and cloned the amplification products into bacterial vector for expression. The bacterial expressed purified protein was used for the generation of monoclonal antibodies. The selected monoclonal antibodies were characterized against NSP antigen. The screening of 3ABC mAb towards antigen was performed by ELISA. The specificity of the mAb was established by its non-reactivity towards other sera samples by indirect ELISA (Enzyme linked Immunosorbent assay) and competitive ELISA. To identify the mAb binding epitope site on FMDV 3ABC, Phage display

dodecamer peptide library was used and selected the peptides based on the binding activity by Phage ELISA. Further analysis of the peptide sequence revealed the presence of four amino acids (QPKL) corresponding to 3ABC region of FMDV genome. We have further shown that the non-competing mAbs were used to develop immunocapture ELISA for the detection of NSP content in in-process samples during manufacture of the vaccine. This ELISA can be adapted to measure the NSP content in the final purified bulk antigen. The mAb based ELISA has the potential to use as a quality control in vaccine production.

**Key words:** Foot and mouth disease, monoclonal antibody, vaccine, ELISA, Non-structural proteins

### Introduction

Foot and Mouth disease (FMD) is a highly contagious viral disease that is affecting all cloven hoofed animals around the world (1) and it is known to be an economically important and devastating disease which includes the production loss and affecting the international trade to areas that are free from FMDV infection (2 and 3). It is endemic in several parts of Asia, Africa and Middle East. The virus is among those which belongs to the class *Picornaviridae* and genus *Aphthovirus*. The virus replicates with its positive sense single stranded RNA and co-translated into a polyprotein as an ORF. The polyprotein is then cleaved into structural proteins (SPs) that forms an icosahedral

capsid and Non-Structural proteins (NSPs) which play a vital role in virus replication and in host-cell interactions (4). The control of FMDV other than the usage of vaccine which is an inactivated whole virus, involves culling of infected animals and restriction of animal movement to avoid the spreading of virus. The currently produced inactivated FMD vaccine contain NSPs that interfere with DIVA in differentiating vaccinated from infected animals due to poor purification methods of inactivated viral bulk harvest (5). Differentiation of naturally infected from vaccinated animals is essential to evaluate the effects of disease control measures and to detect early signs of FMD incursion or transmission in epidemic regions. A disease control program in endemic countries like India needs a marker vaccine that could assist in distinguishing infected animals among vaccinated ones (DIVA)(5 and 6).

3ABC is considered as most immunogenic and expressed abundantly during infection and could be a marker for viral replication (7, 8, 9 and 10). It shows unique characteristics, with an encryption of 3 copies of viral genome-bound 3B protein that serves as a primer for RNA replication in cell culture and for virulence in natural host (11). A marker vaccine anticipating for a DIVA concept should be NSP (3ABC) free in association with the assays that detect anti-3ABC antibodies (8 and 12).

Several researchers and the diagnostic kit manufacturers consider that polyprotein 3ABC, 3AB and 3B antigen would be best options in the use of DIVA assays (7, 8, 13 and 14). Evaluation of NSP free vaccine is performed according to the guidelines of OIE that articulates tests for seroconversion against NSP in susceptible animals after booster vaccinations, which is a long-term process to pass the vaccine batch. Hence it requires an in vitro assay to assess the content of 3ABC in the in-process vaccine antigen batches that could reduce time of testing in vivo and replace the usage of animals (15 and 16). All current foot-and-mouth disease virus (FMDV) DIVA tests rely mostly on polyclonal serum antibodies which is difficult to prepare and maintain in a quality-

assured manner and in the quantities required for post-outbreak surveillance (17). Monoclonal antibodies can guarantee the supply of a consistent and well-characterized reagent that can be produced in large quantities. Therefore development of monoclonal antibodies against NSP provides an alternative reagent in vaccine quality control, to improve the efficacy and decrease the vaccine production cost.

In the present study, we have cloned the non-structural protein 3ABC and expressed in bacterial system. The expressed and purified r3ABC protein was used as a standard for the estimation of 3ABC antigen content in vaccine in-process samples. Further, we describe the identification of an anti-r3ABC antibodies isolated from hybridoma secreting cells generated from immunized mice. The selected monoclonal antibodies were characterized for their binding activity and specificity towards 3ABC by ELISA based methods. These monoclonal antibodies were qualified to be used in the development of ELISA as a quality control in vaccine manufacture for determination of residual 3ABC protein content.

#### **Materials and Methods:**

**Reagents and chemicals:** We procured reagents like high fidelity platinum taq DNA polymerase, *E. coli* BL21(DE3) cells (Invitrogen, Carlsbad, USA), The bacterial expression vector, pET-22b (Novagen, Madison, USA), Nickel-nitriloacetic acid (Ni-NTA) agarose (Qiagen, Hilden, Germany), T4 DNA ligase (Merck, India), restriction endonucleases (NEB, UK), 2-Bromoethylamine hydro bromide and all molecular biology reagents and inorganic fine chemicals from Sigma Aldrich, USA.

**Cloning and expression of r3ABC in bacterial system:** The plasmid pTrOFL with full length genome of FMDV was constructed using the viral RNA isolated from the Type O IND R2/75 vaccine strain. 3ABC region was amplified using Platinum Taq DNA Polymerase-High Fidelity from the bacterial plasmid, pTrOFL and cloned into pET-22b (+) vector between NdeI (CATATG) and NotI (GCGGCCGC) sites to generate the plasmid

pET22b+ O IVRI 3ABC. The plasmid was transformed into BL21 (DE3) competent *E. coli* cells to express the protein with hexa histidine tag at C terminus. The positive transformants were selected for further study.

**Purification of recombinant 3ABC protein by immobilized metal affinity chromatography (IMAC):**

The bacterial pellet was first lysed and sonicated with 100 mM Tris-NaCl buffer containing 1mM PMSF (Phenyl methyl sulfonyl fluoride). The lysed culture was then solubilized in 100 mM Tris-NaCl buffer with 0.2% n-lauryl sarkosyl (NLS) and 1% Triton X100. The supernatant was loaded onto an IMAC gravity column (5 mL volume) equilibrated with 10 column volumes of 50 mM Tris, 300 mM NaCl, pH 7.6 with 15% glycerol (equilibration buffer) at a flow rate of 1 mL/min and washed with 20 column volumes of washing buffer (equilibration buffer with 30 mM Imidazole, pH 7.6). Bound 3ABC was eluted with 5 column volumes of elution buffer containing equilibration buffer with 300 mM Imidazole, pH 7.6, as 1 mL fractions. Fractions containing the recombinant 3ABC were pooled and dialyzed against phosphate-buffered saline (PBS). Protein concentration was determined by the BCA method (Thermo fisher scientific Ltd. USA) before storing it at -20 °C until further use.

**Development of monoclonal antibodies against 3ABC antigen:**

Six week old female BALB/c mice were hyper-immunized intraperitoneally with FMDV NSP 3ABC antigen (50µg/dose/animal) emulsified with equal volume of Freund's complete adjuvant (Sigma, USA) followed by booster doses after two weeks interval, administered intraperitoneally with FMDV NSP 3ABC (25µg/dose/animal) mixed with equal volume of Freund's incomplete adjuvant (Sigma, USA) and the seroconversion of mice against FMDV NSP 3ABC was tested by indirect ELISA. Spleenocytes were collected from the mice with high antibody titer and fused with mouse myeloma partner Sp2/m IL-6 followed by selection of the hybridomas and selected in HAT media. The confluent hybridomas were primarily screened against FMDV NSP 3ABC antigen by ELISA.

**Binding of hybridomas by Indirect ELISA:**

Recombinant 3ABC and *E. coli* cell lysate was immobilized on a 96 well Maxisorp plate (Nunc, Denmark) by dissolving in 100mM carbonate bicarbonate buffer, pH 9.6 and dispensing 100 µL in each well with a final concentration of 5 µg/ml. The plate was incubated overnight at 4°C. Next day, the plate was washed thrice with 40mM phosphate buffered saline containing 0.05% (v/v) Tween-20 (PBST) to remove any unbound antigen. The remaining active surfaces in the wells were blocked with 2% (w/v) skimmed milk powder in PBST for 1 hour at 37°C. The plates were again washed thrice with PBST and 100 µL of hybridoma culture supernatants, negative controls (PBST, unimmunized mouse serum) and the positive control (immunized mouse serum) were added to respective wells and incubated at 37°C for 1 hour. After completion of the incubation step, the plates were washed five times with PBST and goat anti-mouse IgG Fc specific HRPO conjugate (Sigma, USA) at 1:25,000 dilution in PBSA was added to each well (100 µL/well) and incubated for 1 hour at 37°C. The unbound secondary antibody was removed by washing with PBST and 3, 3', 5, 5'-Tetramethylbenzidine (TMB) with H<sub>2</sub>O<sub>2</sub> (0.03% v/v) was added to the wells as a chromogenic substrate. The reaction was stopped after 10 minutes with 1.25M sulfuric acid. Absorbance was recorded at 450 nm using ELISA plate reader (Molecular Devices, USA).

**Purification of mAbs by Affinity chromatography:**

The mAbs were affinity purified employing Protein G Sepharose™ 4 Fast Flow (GE health care, USA) as per manufacturer's recommendations. All the buffers required for the purification were prepared and filtered using 0.45 µm bottle top filter units and stored at 2-8°C until use. Briefly, the resin was packed into a suitable column (XK 16/20) and equilibrated with 5-10 column volumes of 10mM Tris, pH 7.5 (Buffer A) at a flow rate of 2 ml/minute. The filtered TCF was pre-diluted with the equilibration buffer (Buffer A), maintained on ice bath and passed through the resin at a flow rate of 1ml/minute to ensure higher residence time and a maximum binding of the



IgG to the resin. The unbound proteins were washed with 5-10 column volumes of Buffer A at a flow rate of 2 ml/minute. The bound mAbs were eluted in small fraction volumes of 10 ml using 5-10 column volumes of 0.1M Glycine, pH 2.5-3.0 (Buffer B) at a flow rate of 1ml/minute. The eluted fractions were neutralized with 1M Tris, pH 9.0 by adding 1/10<sup>th</sup> volume of the fraction. The eluted fractions were analyzed by SDS-PAGE and immunoblotting. Elution fractions containing the mAb were pooled and dialyzed against PBS, and were concentrated using PEG-4000. The protein concentration was estimated by bicinchoninic acid (BCA) method. The sample was stored at - 20 °C till further analysis.

**Immunoblot analysis of purified mAbs with 3ABC protein:** SDS-PAGE was performed as previously described. 3ABC protein (5µg) were loaded onto a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis under denaturing conditions. The proteins were electrophoretically transferred onto polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore) using standard techniques. The unbound active surfaces of the PVDF membrane were blocked with 5% (w/v) skimmed milk powder in PBST at 37 °C for 2 hours. The membrane was washed and incubated with the purified monoclonal antibody, 8C4, anti-r3ABC sera raised in cattle and sera from vaccinated and infected animals and infected animals at 37 °C for 2 hours. After incubation, all the cut membranes were washed and incubated with respective anti-antibodies/anti-sera conjugate (1:10,000) (Sigma-Aldrich) at 37 °C for 1 hour. The reaction was visualized with diaminobenzidine tetra hydrochloride (DAB) (Sigma-Aldrich) chromogen.

**Competitive ELISA:** Competitive ELISA was performed to identify the specificity of the monoclonal antibodies for FMDV 3ABC. A Maxisorp microtiter plate was coated with r3ABC dissolved in 100mM carbonate bicarbonate buffer, pH 9.6 at a concentration of 5 µg/mL and 100 µL/well was dispensed in to the wells of a Maxisorp 96 well microtiter plates. After overnight incubation

at 4°C and subsequent blocking with 2% (w/v) skimmed milk powder in PBST for 1 hour at 37°C, equal volumes of purified antibodies were added to respective wells and incubated for 1 hour at 37°C. After completion of the incubation step, these plates were washed thoroughly with PBST and streptavidin HRPO conjugate (1:25,000) was added to each well and incubated for 1 hour at 37°C and finally the reaction was developed using TMB substrate. The reaction was stopped after 10 minutes with 1.25M sulfuric acid. Absorbance was recorded at 450 nm using an ELISA plate reader (Molecular Devices).

**Biotinylation of mAbs:** Antibodies were biotin labeled as previously described by Gretch et.al, 1987 (18). Briefly, mAbs were dialyzed against 0.1 M Sodium bi-carbonate buffer and incubated overnight at 4°C. Biotin solution (EZ-Link™ Sulfo-NHS-SS-Biotin) was added to each antibody as per manufacturer instructions. The mixtures were incubated at room temperature for 1 hour and dialyzed against PBS to remove free biotin. Biotinylated mAbs were aliquoted and stored at -20°C for further studies.

**Identification of epitopes of mAb by using phage peptide library :** The mAb epitope was mapped using a Phage display dodecamer peptide library fused to a minor coat protein of M13 phage to identify the antibody interaction sites on the 3ABC antigen. Maxisorp Immunotube (Nunc, Denmark) was coated overnight at 4°C with purified mAb (50 ng/well) diluted in 50mM carbonate and bicarbonate buffer (pH 9.6). After overnight incubation at 4°C, the tubes were washed with 0.01% Tween 20 in Phosphate buffered saline (PBST) and blocked with 2% skimmed milk powder (Difco, BD, USA) in PBS. Tubes were washed for four times before addition of phage display random dodecamer peptide library (~1x10<sup>13</sup> pfu/mL) fused to a minor coat protein (pIII) of M13 phage to the immune tube at 1:100 ratio in blocking buffer. After incubation for 2 hours at 37°C, tubes were again washed for 10 times with PBST. The bound phages were eluted by 0.1M glycine (pH 2.2) and pH neutralized with 150 µL of 1M Tris-HCl buffer (pH 9.1). The eluted

phages were inoculated into 20 mL of *E. coli* ER2738 that were at log or mid log phase, cultured simultaneously. Infected cultures were titrated in log dilutions and spread onto the 2xYT-AG agar plate containing 100 µg/mL ampicillin, IPTG and Xgal for screening the eluted phages. The rest of the eluted phages were amplified and used for next round of bio panning/ELISA. The clones obtained after the final round of panning/titrations were sequenced and analysis of amino acid sequence of the presented peptide was deduced to determine the consensus-binding motif.

**Screening of specific phages by ELISA:** The affinity of the phages towards r3ABC antigen was performed by ELISA (19). Individual colonies after every round of bio panning were inoculated into 100 µL of 2xYT containing 100 µg/mL ampicillin and 2% glucose (2xYT-AG) and incubated in an orbital shaker at 37°C for overnight. 10 µL from the overnight culture were sub-cultured into 100 µL of 2xYT-AG media and incubated in an orbital shaker at 37°C till they reached an O.D<sub>600</sub> of 0.6. M13K07 helper phages at a concentration of  $2.5 \times 10^{10}$  pfu/mL was added and the cultures were further incubated in an orbital shaker for 2 hours and centrifuged at 4000 rpm for 20 min. The bacterial pellet was resuspended in 2xYT containing 100 µg/mL ampicillin, 50 µg/mL kanamycin, and 2% glucose and grown at 30°C overnight in an orbital shaker. Cells were pelleted and the supernatant-containing phages were collected for analysis by phage ELISA. Briefly, maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated with 8C4 mAb diluted in 50mM carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Blocking was performed using 2% skimmed milk (Difco, BD, USA) for 1 hour at 37°C using PBS-T followed by incubation with phages at a concentration of  $10^{11}$  pfu/mL (100 µL) of bacterial supernatant/well for 2 hours at 37°C. Background noise was estimated using non-specific phages in an uncoated microtiter plate. Binding of the phage particles to antigen was detected with the anti-M13 mouse antibody conjugated with HRPO (Pharmacia Biotech, USA) at 1: 5000 dilution and followed by

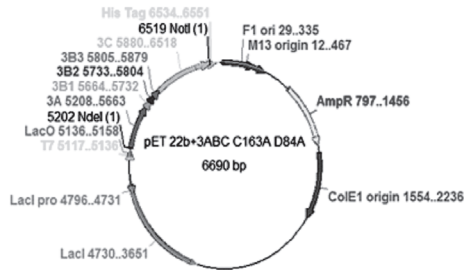
addition of 3, 3', 5, 5' tetramethylbenzidine (TMB). The plate was incubated at 37°C for 10 min, and the reaction was stopped by addition of 1.25 M H<sub>2</sub>SO<sub>4</sub>. The absorbance was measured at 450 nm using a microplate reader (BIO-TEK, USA).

**Sandwich ELISA:** Detection of NSP content in vaccine batches was performed by immunocapture ELISA. The 8C4 mAb, 1mg/mL were coated onto the maxisorp 96 well plate (Nunc) diluted in 50mM carbonate and bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Next day, the plate was washed five times with PBST and the plate was blocked with blocking buffer (10% Normal bovine serum (Gibco, USA) and 5% Normal rabbit serum (Gibco, USA) in PBST). Recombinant 3ABC protein was taken as a standard (500ng to 2ng) along with two fold diluted test samples (treated the test samples before adding to plate with 1% v/v triton X 100 in PBS and incubated at 4 - 8°C by gentle vortexing) were added in duplicate and incubated for 1 hour at 37°C on a plate shaker. The plate was washed as mentioned above. The 3ABC protein was captured by the biotinylated 8C4 mAb and detected by streptavidin conjugated with HRPO (Sigma, USA) followed by addition of TMB substrate. The plate was incubated at 37°C for 10 min, and the reaction was stopped by addition of 1.25 M H<sub>2</sub>SO<sub>4</sub>. The absorbance was measured at 450 nm using a microplate reader (Molecular devices, USA).

## Results

**Cloning and bacterial expression of recombinant 3ABC:** The PCR amplified 3ABC was cloned into pET22b vector and transformed into *E. coli* BL21 (DE3) cells (Fig. 1). The positive transformants were grown overnight and plasmids were isolated as per the manufacturer instruction. Sequence analysis of 3ABC by International ImmunoGeneTics information system (IMGIT) revealed the presence of 449 amino acids as shown in Fig. 2. The purified protein eluted fraction of 8C4 shows a ~ 50 kDa band on SDS-PAGE analysis as indicated in Fig. 3. The protein content was estimated by using Pierce BCA protein assay kit (Thermo Fisher Scientific, USA).

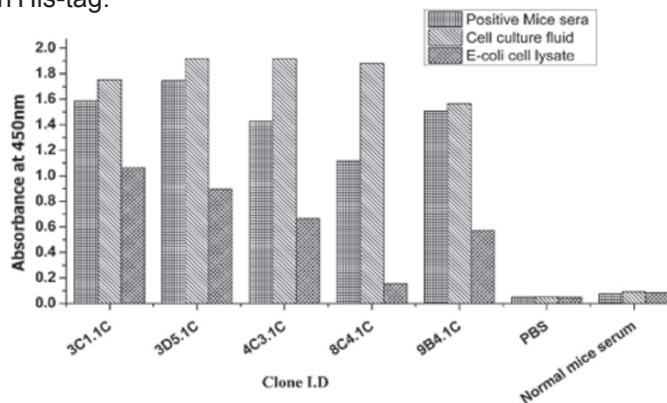




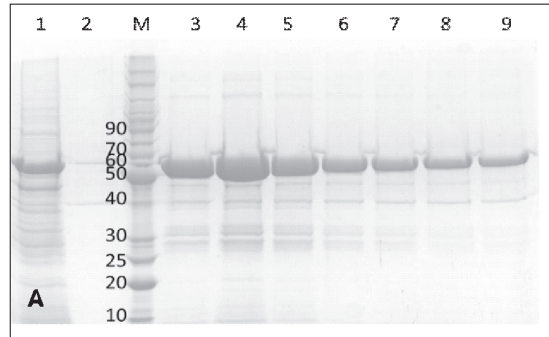
**Fig. 1:** Cloning of 3ABC into pET22b vector between NdeI (CATATG) and NotI (GCGGCCGC) sites.

MISIPSQKSVLYFLIEKGQHEAAIEF  
 F E G M V H D S V K E E L R  
 PLIQQTSFVKRAFKRLKENFEIVALCLT LL  
 ANIVIMIRETRKR QQMV DDAV NEYIEK  
 ANITDDDKTLDEAEKNPLETSGVST VGFR  
 ERTLPGHKVSDDDVNSEPTK PVEEQ PQA  
 EGPYAGPLERQKPLKVKAKLPHQEGP  
 Y A G P M E R Q K P L K V K A K A P V V K E  
 G P Y E G P V K K P V A L K V K A K N  
 L I V T E S G A P P T D L Q K M V M G N T K P  
 V E L I L D G K T V A I C C A T G V F G T A Y L V P R H L  
 F A E K Y D K I M L D G R A M T D S D Y R V F E F E I K  
 V K G Q D M L S A A A L M V L H R G N R V R D I T K H  
 F R D T A R M K K G T P V V G V I N N D V G R L I F S G E A  
 L T Y K D I V V C M D G D T M P G L F A Y R A A T K A  
 G Y A G G A V L A K D G A D T F I V G T H  
 S A G G N G V G Y C S C V S R S M L L K M K A H I D  
 P E P H H E A A A L E H H H H H H H

**Fig. 2.** Amino acid sequence of 3ABC clone with His-tag.



**Fig.4.** Binding activity of mAbs with r3ABC by indirect ELISA.



**Fig. 3:** SDS PAGE analysis of elution fractions of 3ABC-C163A D84A. Lane M is protein ladder, Lanes 1 and 2 is load and Flow through respectively and Lanes 3-9 are elution fractions.

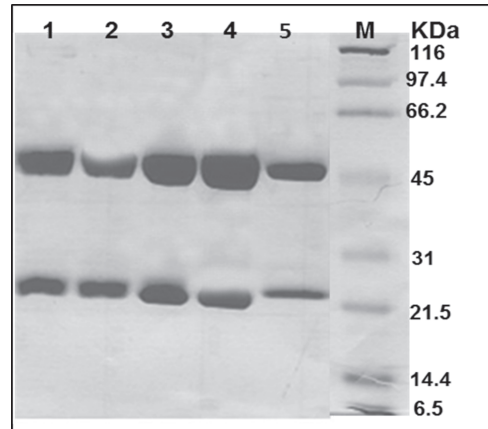
**Development of mAbs against r3ABC antigen:** The mAbs were generated and developed from mouse hybridomas according to standard techniques, as detailed in Materials and Methods. The hybridomas were developed by fusion of spleenocytes from mouse showing good seroconversion to r3ABC antigen by indirect ELISA. A total of 560 clones were selected on HAT medium and screened against r3ABC by indirect ELISA. Selected few clones that shown high binding activity with r3ABC by indirect ELISA. These were cloned for three rounds of limiting dilution for the establishment of monoclonality.

**Binding of mAbs to r3ABC by indirect ELISA:**

The selected mAbs were tested for cross reactivity towards r3ABC and *E.coli* cell lysate. Out of five selected mAbs, four mAbs (3C1.1C, 3D5.1C, 4C3.1C, and 9B4.1C) were found to cross react with *E.coli* cell proteins, while one mAb 8C4.1C showed specific binding towards r3ABC as shown in Fig. 4.

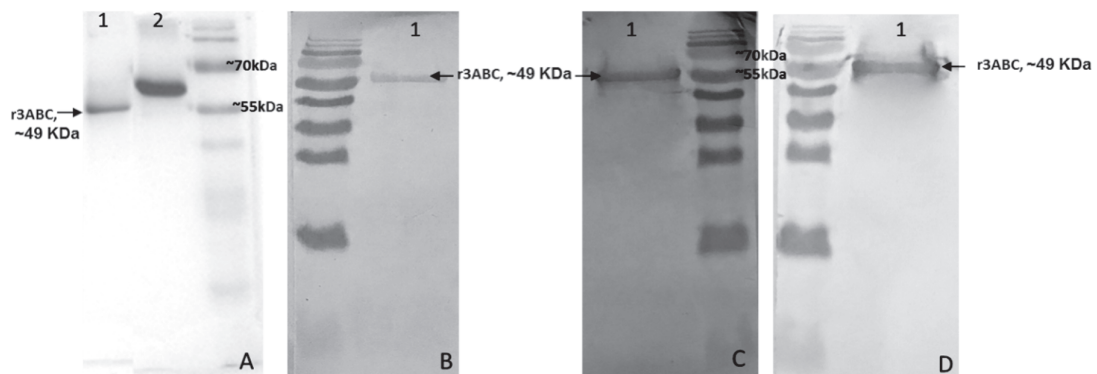
**Purification of monoclonal antibodies:** The matured culture supernatants of the selected clones were affinity purified by Protein-G Sepharose column as discussed in Materials and methods section. The purified mAbs were analysed under 12% reduced SDS-PAGE gel which showed bands at ~50 and ~25 kDa (Fig. 5). These purified mAbs were used for further studies.

**Specificity of the mAb by Immunoblot analysis:** The SDS-PAGE analysis revealed the presence of expected band at ~50KDa corresponding to 3ABC protein (Fig. 6A). The produced protein shows its specificity by binding with the convalescent sera (Fig.6B), vaccinated sera (Fig.6C) and the mAb 8C4 (Fig. 6D) for the r3ABC protein of the FMDV by Immunoblot analysis.



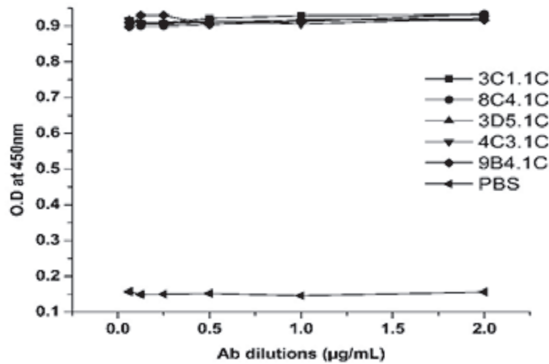
**Fig.5.** Detection of purified mAbs on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane 1 to 5 represents the mAbs 3C1.1C, 3D5.1C, 4C3.1C, 8C4.1C and 9B4.1C respectively and Lane M: protein molecular size standard (KDa).

**Competitive ELISA:** Competitive ELISA was performed to determine the competition between monoclonal antibodies 3C1.1C, 3D5.1C, 4C3.1C, 9B4.1C and biotinylated mAb 8C4.1C for the binding site on FMDV 3ABC. No competition could be seen when the constant amount of mAbs was allowed to compete with varying amount of



**Fig.6:** SDS-PAGE and Immunoblot analysis of r3ABC protein run on a 12% polyacrylamide gel with Page Ruler prestained protein ladder (Thermo scientific). **A.** Coomassie staining of the purified protein, lane 1: r3ABC protein and Lane 2: Bovine serum albumin and Lane M is marker (Pierce, Thermo scientific). **B, C and D.** Immunoblot analysis of r3ABC protein developed with convalescent sera, vaccinated sera and mAb 8C4 respectively.

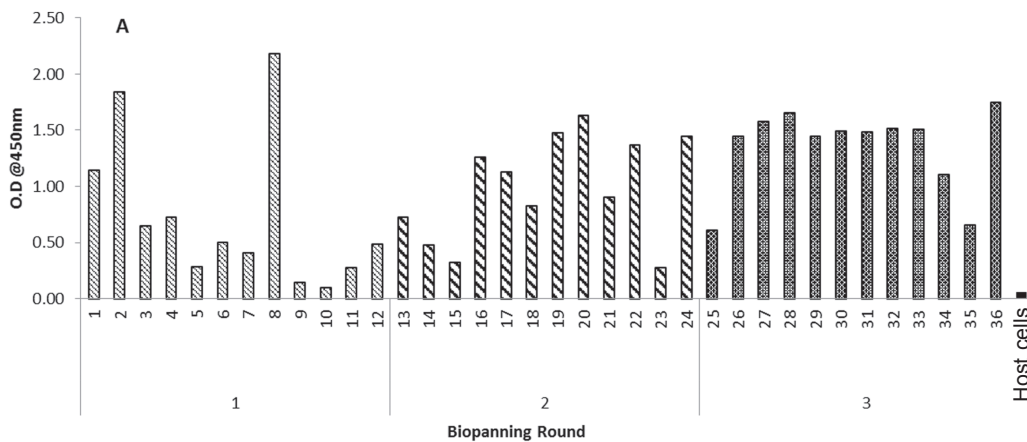
the biotinylated mAb 8C4.1C. Constant OD values (around ~0.9) in the dilution of both antibodies indicated that the mAbs were binding to different antigenic sites on FMDV 3ABC as shown in fig.7.



**Fig. 7:** Competition assay between Biotin labelled and in-house developed mAbs against r3ABC.

**Epitope mapping of monoclonal antibody by bio panning:** A Phage display dodecamer peptide library displayed on filamentous phage was screened on coated mAb 8C4. Following three rounds of panning /selection by phage ELISA (Fig.8A), four out of twelve phage clones at final round showing relatively higher response with the 8C4 mAb were selected and their sequence verified. The resultant translated insert sequence between the linker showed a recurrence of four amino acid sequence 'QKPL' (Fig.8B). A sequence similarity between the peptide recognized by 8C4 and the antigenic site of 3ABC at nucleic acid sequence 63 and 186 strongly suggested that 8C4 bound to the antigenic site of 3ABC.

**Quantification of 3ABC protein in vaccine antigen batches:** Immunocapture ELISA was developed to detect the NSP antigen in bulk vaccine samples. Monoclonal antibody 8C4 was used as capture and same mAb which was



**B**

Colony/Sequence I.D	Sequence 5'-3'
31	HVSPYQKPLLGD
32	TQKPLVANQGNT
34	TYNQKPLTGPK
36	TYQKPRRRWVLA

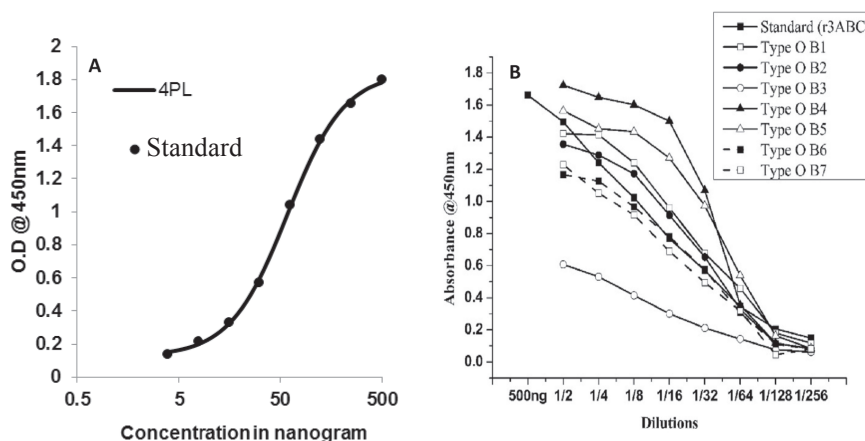
**Fig.8: A.** Eluted phages from each round of bio panning is sub cultured and used for ELISA, the average O.D values representing the specificity for each round. Labels on the X-axis denotes the colony number in each round of bio panning; **B.** Phage displayed peptide sequence of four colonies from third round of bio panning.

biotinylated as an antigen detection. The optimal dilutions of the monoclonal antibodies to be used for developing a sandwich ELISA were selected based on the checker board titration calibrated with the standard antigen. Internal reference standard, r3ABC with known concentrations was used to draw a standard curve starting from 500ng to 2ng using non-linear four parameter logistic curve fit software (Sigma Plot, version 12.5). Seven batches of virus cultures of serotype O were used for the qualification by ELISA method. The results shown that the mAb could able to detect the 3ABC antigen in the vaccine in process samples (Fig.9).

**Discussion:** Foot-and-mouth disease is the most contagious viral vesicular disease that affects cloven-hoofed livestock species. FMD has significant global socio- economic consequences, from national livestock industries suffering because of international trade restrictions to subsistence farmers suffering because of losses of stock productivity and livelihoods (20). Regions in which FMD remains endemic tend to be those of low economic capacity and the control and eradication of the disease mainly relies upon vaccination. Hence, FMD remains as an ongoing issue in

regions in which it is endemic, and it is a continuous threat to regions that are free from the disease. Sero monitoring of both structural and non-structural proteins would help in differentiating the vaccinated ones from the infected one (DIVA) and for identification of potentially virus carrier animals (21 and 22). The major problem comes from the unpurified vaccine antigen that consists of residual NSPs and interfere with DIVA test in detecting NSP antibodies(23, 24 and 25).

DIVA tests are important for serological surveys, provides evidence of FMD infection or freedom from FMD in vaccinated herds. Most serodiagnostic DIVA tests for FMD are enzyme-linked immunosorbent assays that use NSP antigens produced in either bacterial or baculovirus-mediated expression systems, in an indirect or competitive format. The NSP intermediate 3ABC is commonly used as an antigen for FMDV DIVA test because of its high immunogenicity and relatively low abundance in vaccine preparations generated from FMDV-infected cells (8 and 26). The removal of NSPs is required before emulsification of FMDV inactivated vaccine as the semi purified inactivated vaccine batches induce immune response against NSPs.



**Fig.9: A.** Non-linear 4 parameter logistic curve fit for r3ABC as standard and **B.** Testing of vaccine antigen batches by sandwich ELISA.

It is essential to have a quality test method that can bypass the usage of animals. Therefore, the development of mAb based ELISA against NSP would have the potential in manufacturing of quality vaccines.

The monoclonal antibodies have many potential applications and are more specific and sensitive when compared to the polyclonal antibodies. The monoclonal antibodies are being used in serological diagnosis of FMDV as described earlier (8, 27, 28, and 29).

In the present paper, we expressed the 3ABC in bacterial expression system as a recombinant protein with 6x His tag as described previously (30,31 and 32) and used this r3ABC antigen for monoclonal antibody generation, expression, purification and immunological characterization of an anti-r3ABC antibodies and their application for the quantification of antigen in in-process samples during vaccine production by ELISA. Monoclonal antibodies for r3ABC have been screened for their activity by competitive and indirect ELISAs. Based on the reactivity, one mAb was selected for further studies. The lack of cross reactivity confirmed the specificity of this mAb for the defined antigen. We used phage display peptide library to identify the epitope of 8C4 mAb on FMDV NSP 3ABC. Advances in phage display technology where displaying of peptides or proteins on the surface of bacteriophage particles has facilitated the isolation of specific peptides with affinity towards various targets (33) and potential to serve as prophylactic, therapeutic candidates or diagnostic reagents (34, 35, 36 and 37). Three rounds of biopanning were carried out to maintain the sequence diversity among the binding phage and enrich the specifically bound phages. Finally four clones were selected based on phage ELISA showing highest binding. On peptidesequence analysis, four clones revealed identical sequences as QKPL, as reported earlier and are known to form a linear epitope (38).

FMDV vaccination has become regular as a part of the national vaccination program in India (39). The production of FMDV vaccine is relatively

easier but to ensure consistency of batches and to maintain appropriate quality is found to be critical and routine testing requires both in vitro and in vivo assays. In vitro testing would be more beneficial as it helps in reduction and replacement of animal usage for batch release criteria. However these assays need to be validated and correlated well with in vivo assays. During the process of vaccine development, it is essential to establish vaccine characteristics as good quality and protective levels of antigen in the batches.

Quantification of NSP (3ABC) antigen has become essential tool for determining the quality of the vaccine batches. These quantifications can be performed through ELISA based methods using mAbs that offers a cheap, simple and convenient way of estimation without the loss of antigen. The mAbs developed were used in the development of an in-house ELISA for the quantification of NS protein (3ABC) in in-process samples during manufacture of vaccine. The ELISA showed a perfect linear fit with  $R^2$  value of  $> 0.99$ . This method can be further validated and used to quantify the NSP (3ABC) in the vaccine antigen batches of other serotypes.

**Conclusion:** Vaccination plays an important role in prevention and elimination of the FMD from the endemic countries. The batch clearance criterion for the FMDV inactivated vaccine requires quality parameters to be accomplished, especially presence of NSP in the vaccine antigen batches needs to be monitored for development of a quality vaccine. Though a commercial kit is available for the detection of NSP (3ABC), it limits the usage due to affordability. Non-structural protein (NSP) 3ABC antibody is known to be the most reliable indicator of FMDV infection in vaccinated animals. We have developed and characterized the r3ABC protein and the monoclonal antibody, 8C4 which could be used for in vitro immunoassays to detect the anti 3ABC serum antibodies and also to quantify the FMDV NSP (3ABC) content in the vaccine antigen batches. The developed ELISA method can be used for quantification of NSP in the vaccine in-process samples of other serotypes in future. Thus, these reagents would certainly aid



in developing and validating assays to regulate the 3ABC content in the vaccine antigen batches supporting DIVA.

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## Determination of enantiomeric purity of esomeprazole pharmaceutical products using validated HPLC method

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### Abstract:

This method was developed to perform a comparative study of enantiomeric purity of some products of esomeprazole (capsules, tablets) manufactured locally and in some neighboring countries for the determination the percentage of R enantiomer. A product of under license company was used for comparison of the results. The study was performed by using a validated HPLC method on chiral column Nucleocel Alpha S and a mixture ethanol: hexane (70:30, v/v) as a mobile phase, the flow rate was 0.65 ml/min, and the detection was carried out using UV detector at 302nm. The temperature of column was set at 25°C. The study showed that some samples were not polluted with R enantiomer, while the others contained this enantiomer in the range of 0.1-2.24%.

**Key Words:** Enantiomeric Purity, Omeprazole, Esomeprazole, HPLC.

### 1. Introduction:

Esomeprazole magnesium trihydrate (ES), bis (5-methoxy-2- [(S) - [(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulfinyl-1H-benzimidazole-1-yl) magnesium trihydrate is the S isomer of racemic omeprazole approved in February 2001 for use as a new pharmacological entity designed to improve the clinical outcome of available proton pump inhibitors in the management of acid-related disorders<sup>1,2</sup>.

Proton Pump Inhibitors are synthetic compounds that inhibit the enzyme H<sup>+</sup>/K<sup>+</sup> ATPase

that is responsible for pumping protons in the stomach and they used to treat gastric acid disorders such as ulcers that exist in the esophagus, stomach, and duodenum. PPIs include Omeprazole, Lansoprazole, Dexlansoprazole, Esomeprazole, Pantoprazole, Rabeprazole<sup>1-3</sup>.

The chemical structure of these compounds is derived from pyridyl methyl benzimidazole-sulfoxide, and their structures are contain an asymmetric sulfur atom, which allows them to present in the form of Enantiomers.

These drugs are used in the treatment in the form of racemic mixtures except of omeprazole and Lansoprazole (a racemic mixture of R and S Enantiomers), which are also given in the form of a single enantiomer called Esomeprazole and Dexlansoprazole, respectively<sup>1-3</sup>.

Since 1992 the FDA and the European Committee for Proprietary Medicinal Products have required that the properties of each enantiomer be studied separately before decisions are taken to market the drug as one of the enantiomers or as a racemate.

Several methods have been developed to allow the determination of enantiomers and enantiomeric purity of the chiral drugs, the most important of which are the chromatographic methods (eg, HPLC, GC) and electrical methods (eg. CE)<sup>4,5</sup>.

The methods of determination of R and S omeprazole in pharmaceutical forms and human

plasma focused on the use of HPLC, chiral column, and UV detector. Most of these methods were conform on plasma samples after oral administration of omeprazole, while a few of them applied on samples of pharmaceutical preparations<sup>6-13</sup>.

The United States Pharmacopoeia (USP38) issued in 2015 contains a monograph for the determination of enantiomeric purity of esomeprazole as a raw material using Chiral HPLC and L41 ( $\alpha$ 1-Acidglycoprotein) as a column, and a mobile phase of phosphate buffer pH = 6.0 and acetonitrile 15 : 85, v/v. In this method, a chiral column Nucleocel Alpha S was used which contains amylose triphosphate (5,3 diethyl phenylcarbamate).

The aim of this study was to development a validated HPLC analytical method that allows the separation and determination of S and R enantiomers of omeprazole. So that it can be used in a comparative study of the enantiomeric purity of pharmaceutical preparations for esomeprazole (tablets, capsules) that obtained from national industries and the industries of neighboring companies by determining the percentage of enantiomer R compared to a reference product obtained from an under- license company.

## 2. Materials & Methods:

**2.1. Equipment and instruments:** Use HPLC system with model of Agilent 1100, with G1311A Quaternary Pump, G1313AAutosampler, UV / Vis Detector, and G1379A Vacuum Degasser. Ultrasonic Device for Elma.Filters 0.45  $\mu$ m of Millipore Millex-LCR.Chiral column: Nucleocel Alpha S, (250mmx4.6mm, 5  $\mu$ m). This column contains amylose tri (5,3 Dimethyl phenylcarbamate) from Macherey-Nagel company, CN guard column (4mmx4mm, 5  $\mu$ m) fromMacherey-Nagel company, analytical Balance model Shimadzu AUW220D, with accuracy 0.1 mg.

**2.2. Reagents and solvents:** HPLC-Grade solvents were used:ethanol from Panreac, methanol from Panreac, Hexane from Sigma-

Prich, isopropanol from Riedel de Haën AG, Heptane from Scharlau, and diethylamine Extra Pure.

## 2.3. Samples:

**2.3.1. Standard Materials:** Omeprazole and esomeprazole Standards: Purchased from the European Council of the European Pharmacopoeia (EPH) Strasbourg, France.

**2.3.2. Pharmaceutical Products:** Esomeprazole capsules and tablets were bought from pharmacies in Damascus and belong to several national pharmaceutical companies and companies from neighboring countries, including the Nexium product produced by under-license company.

## 2.4. Preparation of solutions:

2.4.1. The mobile phase was amixture of ethanol and normal hexane 70:30, v/v.

2.4.2. The stock solution of omeprazole (1 mg / ml): Weigh 100.0 mg of omeprazole standard and take it into a 100 mL volumetric flask, dissolve it by adding 10 mL methanol and about 70 ml of mobile phase, add 0.1 ml diethyl amine, sonicate for 15 minutes, add mobile phase to volume. Stored this solution at -20 ° C away from direct light<sup>7</sup>.

### 2.4.3. The standard solution of omeprazole (100 mcg / ml):

Diluent 5.0 ml of stock solution to 50 ml Using mobile phase as a diluent.

**2.4.4. The stock solution of esomeprazole (0.5 mg / ml):** Weigh 55.5 mg of esomeprazole magnesium trihydrate standard and take it into a 100 ml volumetric flask, dissolve it by adding 10 mL methanol and about 70 ml of mobile phase, add 0.1 ml diethyl amine, sonicate for 15 minutes, add mobile phase to volume. Store this solution at -20 ° C away from direct light<sup>7</sup>.

**2.4.5. The standard solution of esomeprazole (50 mcg / ml):** Diluent 5.0 ml of stock solution to 50 ml using mobile phase as a diluent.

**2.4.6. The sample solutions of esomeprazole:** These solutions were prepared to contain a

concentration similar to the concentration of the standard solution of esomeprazole.

2.4.7. The chromatographic conditions to determination the percentage of R and S enantiomers of omeprazole:

Mobile phase: A mixture of Ethanol – normal Hexane 70:30, v/v.

Flow rate: 0.65 ml / min.

Injection volume: 20  $\mu$ l.

Column temperature was set at 25  $^{\circ}$ C.

Detector: UV at a wavelength of 302 nm.

Figure 2: shows the Chromatogram of omeprazole standard solution.

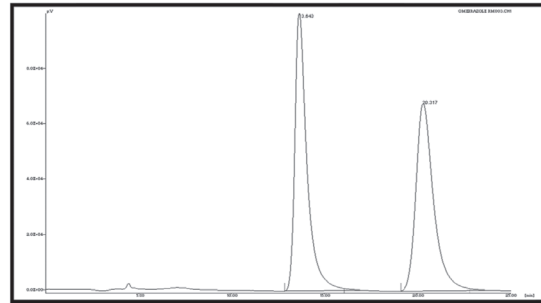


Figure 1: show the Chromatogram of omeprazole, standard solutions.

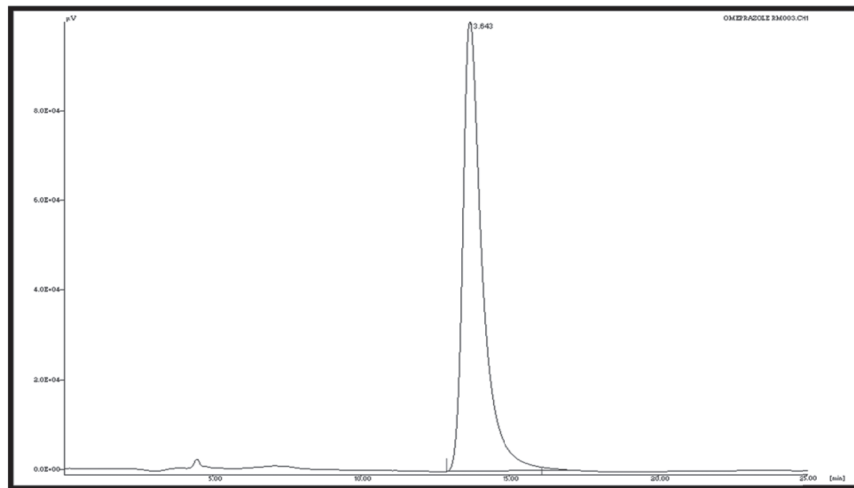


Figure 2: show the Chromatograms of esomeprazole standard solutions.

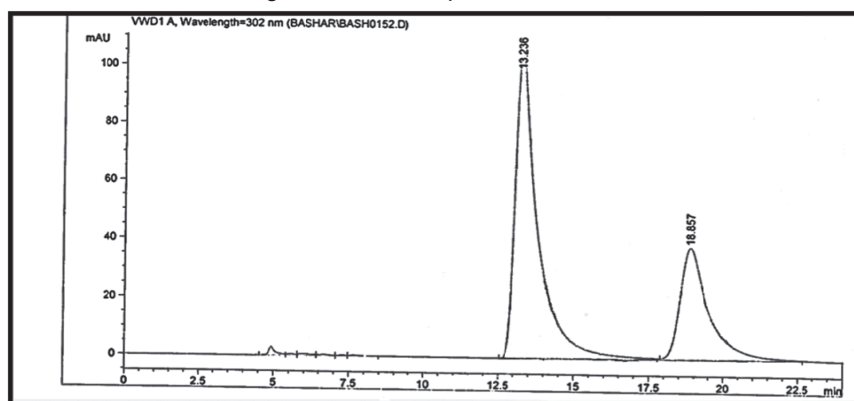


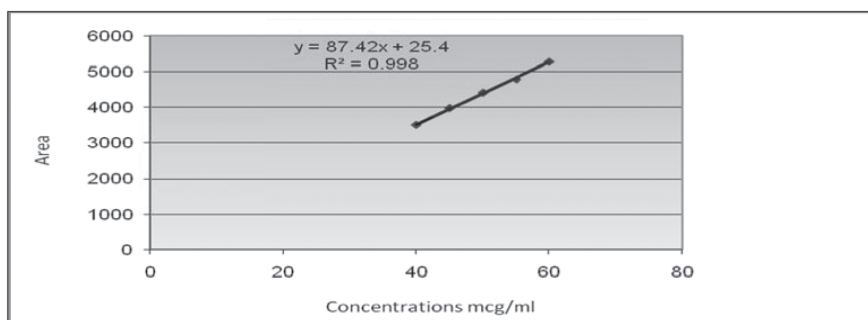
Figure (3): the Chromatogram of the mixture of esomeprazole standard solution and omeprazole standard solution.

Determination of enantiomeric purity of esomeprazole

**2.5. Determination of the peaks of omeprazole enantiomers in the chromatogram:**

The S and R peaks were confirmed by injecting a standard esomeprazole solution and observing the resulting chromatogram (Fig. 2), and by injecting a mixture of omeprazole and esomeprazole (the chromatogram in Fig. 3).

From the above, it was noted that the first peak with a retention time of 13.51 minutes was to the enantiomer S and the second peak with a retention time was 19.17 minutes was to the enantiomer R, table (1) shows the parameters of omeprazole enantiomers peak in figure (1).



The figure (4): the calibration curve of S-enantiomer.

**Table (1):** the parameters of peaks in Chromatogram of Figure (1).

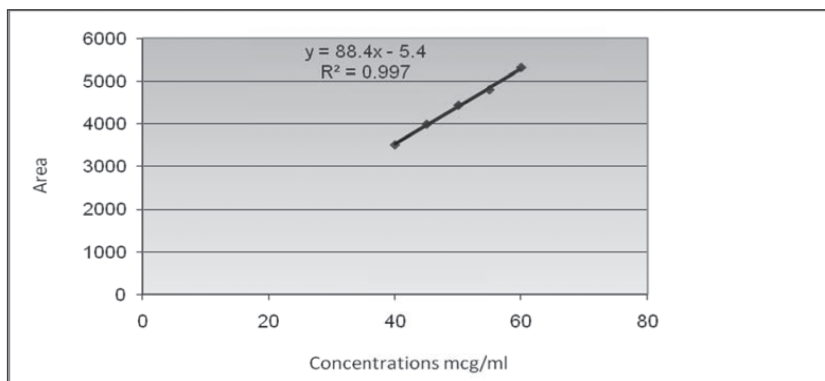
Compound	Retention time	Asymmetry	Theoretical plates	Capacity factor	resolution	Area %	Enantiomer
Enantiomer S	13.51	1.77	2551	2.52	-	5088	50.20
Enantiomer R	19.17	1.68	2390	3.40	5.55	5032	49.80

**3. Results:**

**3.1. Validation of method:**

The table (2) discusses the parameters of method validation.

Parameter	Enantiomer S	Enantiomer R
Retention Time (min)	13.64	20.32
Linearity (r <sup>2</sup> ), (figure .4 and 5).	0.9966	0.9958
Accuracy (Recovery %)	99.55±1.63	99.37±1.81
Repeatability (RSD, %)	1.52	1.80
Intermediate Precision (RSD, %)	1.16	0.99
Selectivity (Recovery %)	101.69±0.26	100.02±0.2
Robustness 0.55 ml/min	0.998	0.991
(Relative Retention 0.65 ml/min	0.999	0.998
Time) 0.75 ml/min	1.001	0.999
Detection Limit (ng/ml)	69.99	48.27
Quantitation Limit (ng/ml)	233.3	160.74



The figure (5): the calibration curve of R-enantiomer.

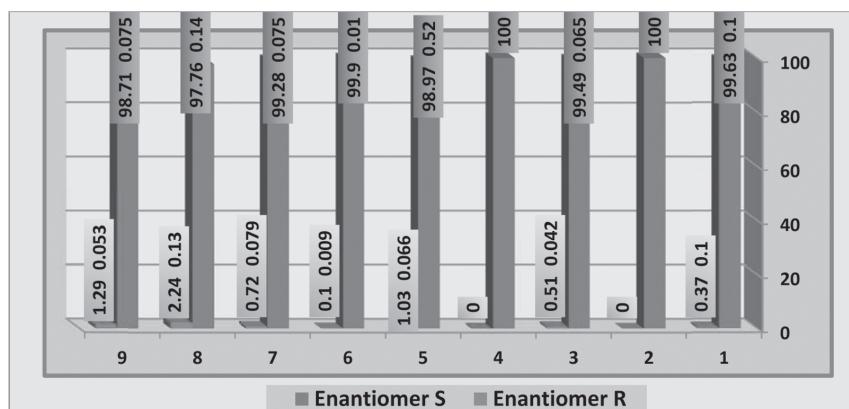


Figure (6): The percentages of S-enantiomer and R-enantiomer in pharmaceutical products of esomeprazole.

### 3.2. Study of enantiomeric purity of esomeprazole preparations:

The study was conformed on 9 pharmaceutical products of esomeprazole from 1 to 9, including the reference product (No 2). Figure 6 shows the percentages of both S and R enantiomers in the products.

The percentage of enantiomer-R was calculated in pharmaceutical products of esomeprazole by the following formula:  $100 * (r_u / r_s)$

$r_u$ : the peak response for the R-enantiomer

$r_s$ : the sum of the responses of the both the esomeprazole and R-enantiomer peaks.

The monograph of esomeprazole in USP 38, 2015

determined the enantiomeric purity of the material as a limit (NMT 0.2% of the R-enantiomer)<sup>14</sup>.

### 3. Results and discussion

A validated analytical method was developed using Chiral HPLC to determine the enantiomeric purity of esomeprazole products and the percentage of the R-enantiomer. The results of enantiomeric purity showed that two pharmaceutical product were free from enantiomer-R, while enantiomeric purity of other products was between 0.1-2.24 %.

The table 2 showed the parameters of validation of the analytical method, and the figures. 4,5 showed the calibration curves of S and R-enantiomers.



### 3.3. Discussion

1 - A validated analytical method was developed using Chiral HPLC to determine the enantiomeric purity of esomeprazole products and the percentage of the R-enantiomer.

2- The results of enantiomeric purity showed that two pharmaceutical product were free from enantiomer-R, while enantiomeric purity of other products was between 0.1-2.24 %.

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## Comparative Efficacy of Selected Disinfectants against Pathogenic Bacteria Isolated from Hospital Fomites and Disposal Wastes

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### Abstract

Prevention of health care associated infection has become an alarming concern and challenge for clinicians. This can be achieved by proper infection control practices, appropriate use of sterilization and disinfection procedures. In the current study eight bacteria were isolated from hospital wastes, collected from Government Hospital, Prem Nagar, Dehra Dun, by standard isolation methods. All the bacterial isolates were characterized by staining and biochemical tests up to the genus level. Among them two were gram-positive while six were gram-negative bacteria. The isolates were identified to be *Pseudomonas sp.*, *Klebsiella sp.*, *Micrococcus sp.*, *Enterococcus sp.*, *Alcaligenes sp.*, *Serratia sp.*, *Proteus sp.* and *Escherichia sp.* These isolates were subjected to bactericidal activity testing against three commercially available disinfectants namely Formalin (Formaldehyde), Pursue (Dimethyl ammonium chloride) and Phenyle (Phenol). Efficacies of these disinfectants were evaluated by Minimum Inhibitory Concentration using agar diffusion method. Among the three disinfectants, Formalin and Pursue showed better activity for both Gram-negative (Zone of Inhibition: 43 mm and 46 mm respectively) and Gram-positive bacteria (Zone of Inhibition: 38 mm and 32 mm respectively). Phenyle, mostly used in our hospitals, was found to be less effective for all the isolates (Zone of Inhibition: 25 mm for Gram-

negative and 17 mm for Gram-positive bacteria). Minimum Inhibitory Concentration of each disinfectant was also determined and was found to be best in Pursue (at dilution 1:512) followed by Formalin (1:64) and Phenyle (1:4).

**Key words:** Disinfection, Formalin, Health-care, Phenyle, Pursue

### Introduction

Nosocomial, or hospital-acquired infections, are defined by the US Center for Disease Control and Prevention as any localized or systemic condition that occurs in a patient as a result of the presence of an infectious agent or its toxin that was not present or incubating at the time of hospital admission.<sup>[1]</sup> It has been seen that immune compromised patients are at a threat of nosocomial infections (NI's) when they undergo surgery or have any underlying disease, and they are even worse affected when admitted in Intensive Care Unit, as the rate of NI's is almost three times higher than any other departments of the hospital.<sup>[2]</sup> To minimize the percentage of patients who acquire NI, several actions such as cleaning, washing and disinfecting hands and the use of isolation material are performed by health-care workers and these actions are called Infection Control Measures.<sup>[3]</sup> Different types of infections acquired in hospitals include bloodstream infections, surgical wound infections, ventilator-

associated pneumonia, urinary tract infection, lower respiratory infection, gastrointestinal, skin, soft tissue, ear, nose and throat infections. Although viruses, fungi and parasites are also recognized as sources of nosocomial infections, bacterial agents remain the most commonly recognized cause. In hospitals, inanimate surfaces and equipment like bedrails, door handles, drip stand, medical charts, stethoscopes, ultrasound machine may be contaminated by bacteria.<sup>[9]</sup> The agents that are usually involved in hospital-acquired infections include *Streptococcus sp.*, *Acinetobacter sp.*, *Enterococci*, *Pseudomonas aeruginosa*, coagulase negative *Staphylococci*, *Staphylococcus aureus*, *Bacillus cereus*, *Legionella* and *Enterobacteriaceae* family members, namely, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Escherichia coli* and *Serratia marcescens*. Out of these *Enterococci*, *P. aeruginosa*, *S. aureus* and *E. coli* have a major role.<sup>[1]</sup> Microorganisms causing nosocomial infection are transmitted in hospitals by several routes including direct or indirect contact, droplet spread, common vehicles like air and vector borne and alternative vehicles, such as blood plasma or food. The effective cleaning and disinfection of inanimate contact surfaces is therefore critical for the prevention and control of nosocomial infections within the health-care environment. Biocides such as antiseptics and disinfectants are widely used as an initial step to control or kill infectious agents from possibly contaminated equipment and specimens.

The effective use of disinfectants is an important factor in preventing nosocomial infections. However, the resistance of pathogens to disinfectants is an emerging problem and is due to the presence of integrons, super integrons, efflux mechanisms and genetic adaptations in the pathogens.<sup>[4,5,6]</sup> So, in spite of regular use of conventional disinfectants, surfaces and medical equipment are usually not effectively decontaminated and disinfected. There is a need to evaluate the efficacy of disinfectants which are commonly used in our hospitals and to look for newer and better options to disinfect the inanimate

surfaces and hospital environments. This study was therefore aimed at comparing the efficacy of three commercially available disinfectants on microorganisms isolated from clinical samples and to create an awareness for health workers to choose an effective disinfectant.

#### **Materials & Methods**

**Collection of samples** : Samples were collected from inanimate surfaces at Government Hospital Prem Nagar, Dehra Dun, Uttarakhand by swabbing 10 cm<sup>2</sup> surface areas with a sterile swab. The swabs were then immersed in 10 ml sterile normal saline (0.9% NaCl) and transported to the laboratory. Separate soil samples were also collected from hospital waste dumping site located at Sahastradhara, Dehra Dun in sterilized zip lock plastic bags and were transferred to sterile containers after reaching the laboratory, for further analysis.

**Isolation of bacteria** : All the collected samples were inoculated into Nutrient broth and were incubated at 37°C for 24 hours. 0.1 ml of the obtained bacterial culture from each sample was streaked onto Mannitol Salt Agar (MSA), Mac Conkey's Agar (MA) and Eosin Methylene Blue (EMB) Agar and the plates were incubated at 37°C for 24 hours. Different bacterial colonies so obtained were then marked and streaked on Nutrient Agar (NA) plates to obtain pure cultures of pathogenic bacteria. These pure cultures were then maintained on slants and preserved at 4°C for future use.

**Characterization of the isolates** : Characterization and identification of the isolates were done on the basis of their morphology, physiology and biochemical tests. Simple staining was performed to study bacterial morphology, and Gram staining for differentiation of bacteria. Indole production test, methyl red and voges-proskauer test, citric acid (citrate) utilization test, catalase activity test, nitrate reduction test, urea hydrolysis, hydrogen sulphide (H<sub>2</sub>S) production test, coagulase test and sugar mannitol fermentation test were performed for the characterization.

**Disinfectants used :** The disinfectants used in the current study were Formalin (40% Formaldehyde), Pursue, a product of Amway (Dialkyl/dimethyl ammonium chloride and Alkyldimethylbenzyl ammonium chloride-3.8%) and Phenyle (Phenol).

#### **Microbial analysis of the samples**

##### **Comparative efficacy of selected disinfectants on isolated pathogenic bacteria**

**Disinfectant dilution method :** A series of concentrations of disinfectants were obtained using serial dilution method into 10 test tubes. 5 ml of distilled water was first transferred into each test tube and 5 ml of concentrated disinfectants was transferred to the first tube containing 5 ml of distilled water. It was mixed thoroughly and then 5ml was transferred to the next tube to obtain dilutions in order 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512. Sterile distilled water was taken as control.<sup>[7]</sup>

**Agar diffusion method :** 0.1ml of 24 hours old culture of each bacterial isolate was spread on Muller Hinton Agar plates and the plates were allowed to dry for 5 minutes. A sterile core borer was used to make standard wells (of about 0.5 mm diameter) on the surface of the inoculated plate. Sections were made on the lid of the petri plate using ink marker. Micro pipettes were used to deliver the disinfectants to respective wells. The plates were incubated at 37°C for 24 hours and zones of inhibition were measured in millimetres using transparent meter rule.<sup>[8]</sup>

**Minimum inhibitory concentration (MIC) :** Minimum inhibitory concentration of disinfectants was evaluated by agar dilution method according to the CLSI guidelines 2010.<sup>[9]</sup> The dilution of MIC ranges from 1:1 to 1:512. Overnight culture suspensions of bacterial isolates were adjusted to 0.5 McFarland standard for turbidity and 10µl of suspension was inoculated onto Muller Hinton agar plates with above mentioned concentrations of disinfectants. Inoculated plates were incubated at 37°C for 24 hours and the lowest concentration of disinfectants that inhibited the growth of bacterial isolates was considered as MIC.<sup>[3]</sup>

#### **Result**

**Isolation of bacteria :** Eight different sites were selected for sample collection. Eight pathogenic bacteria were isolated, purified and preserved as shown in Table 1.

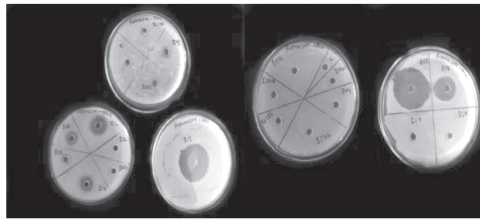
**Characterization of the isolates :** The isolates were identified on the basis of their morphology, staining and biochemical characterization as depicted in Table 2.

##### **Comparative efficacy of selected disinfectants on isolated pathogenic bacteria :**

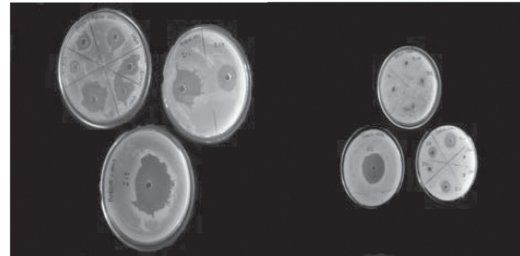
A wide divergence was observed in the responses of disinfectants among test bacteria. The effect of disinfectants against the isolates is shown in Tables 3 to 5. Figures 1 to 3 shows the effect of different dilutions of disinfectants on bacterial isolates and their respective zone of inhibitions. Table 6 shows the Minimum inhibitory concentration of disinfectants against bacterial isolates. Graph 1 shows the efficacies of the disinfectants against bacterial isolates at 1:1 dilution.

#### **Discussion**

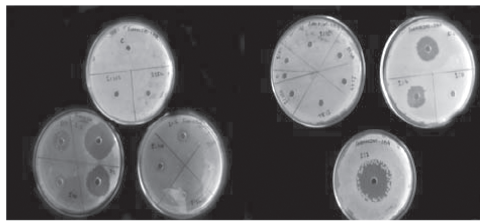
The current study was undertaken to compare the effectiveness of disinfectants namely formalin and pursue, with effects of phenyl, one of the commonly used disinfectant in hospitals. Eight pathogenic bacteria were isolated from inanimate surfaces at Government Hospital Prem Nagar, Dehra Dun and from hospital waste dumping site located at Sahastradhara, Dehra Dun, Uttarakhand. The microorganisms were identified to be *Pseudomonas sp.*, *Klebsiella sp.*, *Micrococcus sp.*, *Enterococcus sp.*, *Alcaligenes sp.*, *Serratia sp.*, *Proteus sp.* and *Escherichia sp.* It was observed that Pursue (Dialkyl/dimethyl ammonium chloride and Alkyldimethylbenzyl ammonium chloride-3.8%) showed its maximum zone of inhibition (46mm) against *Pseudomonas sp.* followed by Formalin (Formaldehyde) which showed its maximum zone of inhibition (43mm) against *Serratia sp.* and Phenyl (phenol) which showed its maximum zone of inhibition (25mm) against *Klebsiella sp.* at dilution of 1:1 respectively. Phenyl was observed to be least effective against all organisms studied at all



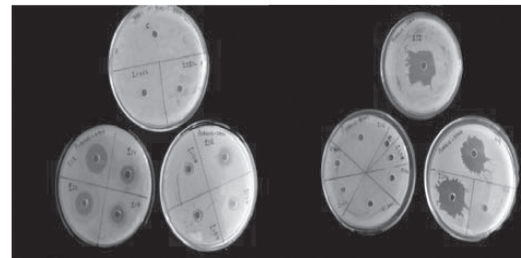
(a) (b)



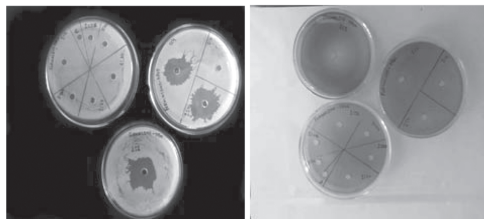
(a) (b)



(c) (d)



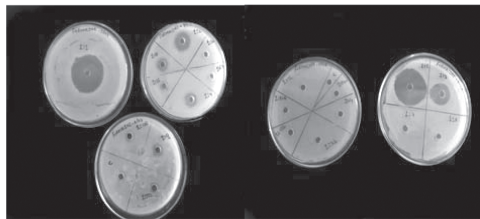
(c) (d)



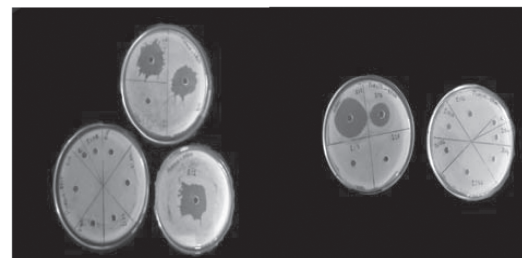
(e) (f)



(e) (f)



(g) (h)



(g) (h)

**Figure 1:** (a to h) Effect of formalin on bacterial isolates

concentrations as compared to effect of formalin and pursue. The lowest concentration that inhibited the growth of bacteria was seen best for Pursue at dilutions 1:512 against *Pseudomonas sp.* followed by 1:128 against *Micrococcus sp.* MIC of Formalin was recorded best at dilutions 1:64 against *Serratia sp.* followed by 1:16 against *Proteus sp.* and *Pseudomonas sp.* Phenyl showed least effect against all isolates with lowest

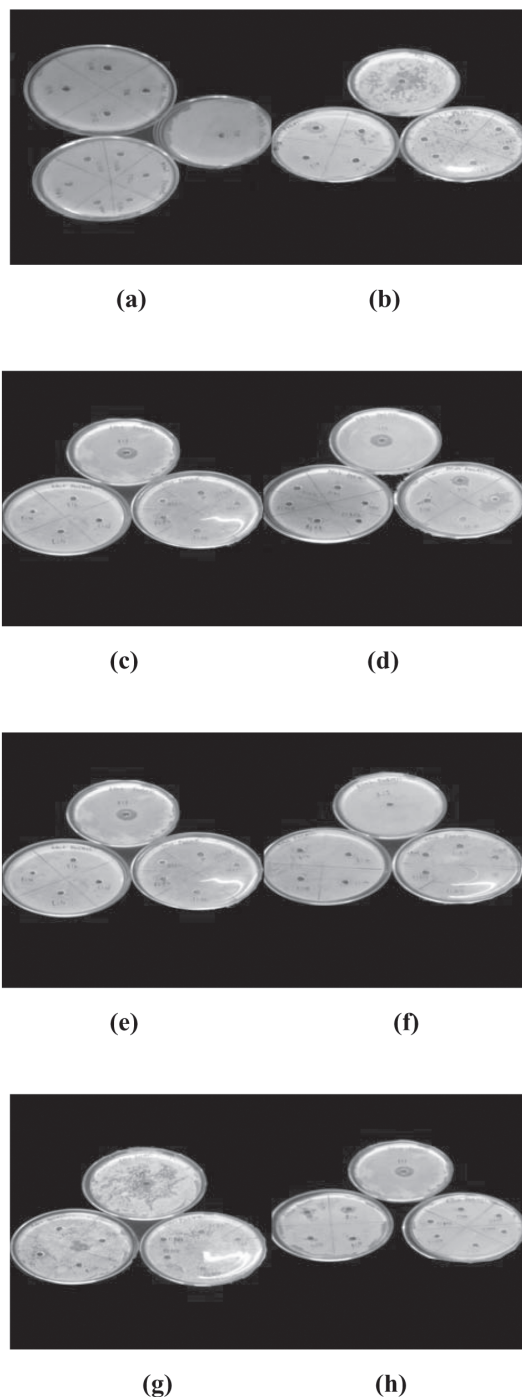
**Figure 2:** (a to h) Effect of pursue on bacterial isolates



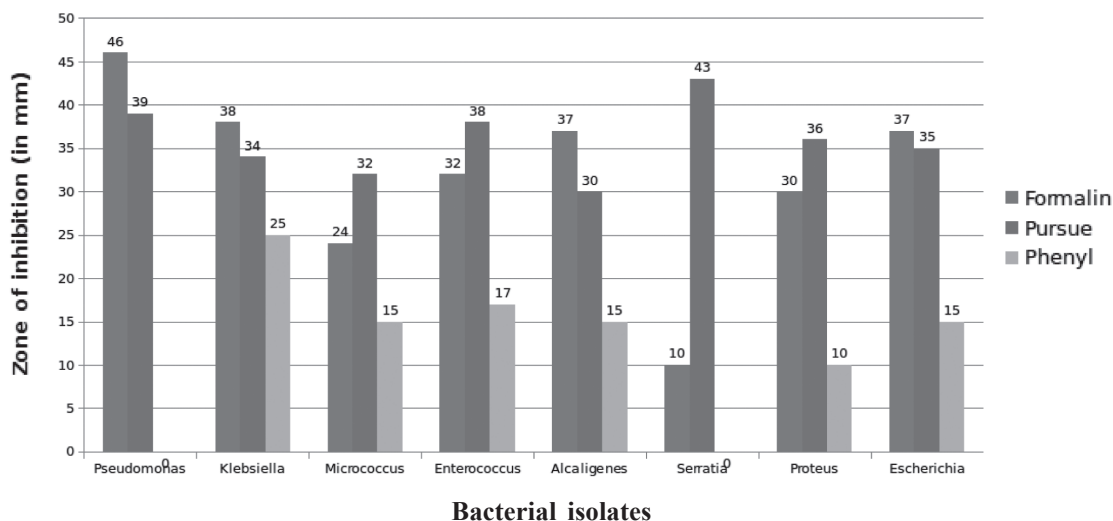
concentration inhibiting the growth of bacteria at dilutions 1:4 against *Enterococcus sp.* followed by 1:2 against *Klebsiella sp.*

In a similar study, efficacies of commonly used disinfectants namely Phenol, Chlorhexidine gluconate (CHX), Benzylkonium chloride (BZT), Chloroxylenol and Sodium hypochlorite were evaluated for bactericidal activity by minimum inhibitory concentration method and time kill assay. Among the five disinfectants, Benzylkonium chloride and Chloroxylenol inhibited growth of MRSA (Methicillin resistant *Staphylococcus aureus*), VRE (Vancomycin Resistant Enterococci), ESBL producing *E. coli*, *Proteus mirabilis*, *Proteus vulgaris*, MDR *Pseudomonas aeruginosa* and *Acinetobacter baumannii* and MRSA, VRE, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* respectively at dilution of 1:320. Sodium hypochlorite was found to inhibit MRSA and VRE at dilution of 1:320. Chlorhexidine Gluconate was found to be effective against all organisms studied at a dilution of 1:20. As in our study, here also phenol was found to be ineffective for all the isolates used showing growth till 1:20. Also, Chloroxylenol and Benzylkonium chloride showed 100% inhibition at contact time of 1 minute followed by Chlorhexidine gluconate and Sodium hypochlorite which showed 100% inhibition at 10 minutes of contact time. [2]

Similarly, antimicrobial effects of six antiseptics and disinfectants namely Dettol (Chloroxylenol), Savlon (Chlorhexidine Gluconate and Cetrimide), Iodine, Phenyl, Formalin and Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) at concentrations 100%, 50%, 25%, 10% and 5% were studied against five pathogenic bacteria (*Staphylococcus aureus*, *Salmonella typhi*, *Shigella dysenteriae*, *Klebsiella sp.* and *Escherichia coli*). Formalin was found to be most effective against all pathogens with zones of inhibition ranging from 20 mm to 51 mm. Hydrogen peroxide was also found to be highly effective to all the pathogens. Dettol was more effective against *S. aureus*, *S. typhi* and *E. coli*, Savlon worked better against *S. aureus*, *S. typhi* and *S. dysenteriae* whereas Iodine showed



**Figure 3:** (a to h) Effect of phenyle on bacterial isolates



**Bacterial isolates**  
**Graph 1:** Efficacies of the disinfectants against bacterial isolates at 1:1 dilution

**Table 1: Bacteria isolated on different media**

Acc. No.	Sample Site	Media Used	Possible Pathogens
DP01	Bed clothing (Hospital)	Mac Conkey's agar	<i>Klebsiella, Escherichia, Enterobacter, Pseudomonas, Salmonella and Shigella</i>
DP02	Drip stand (hospital)	EMB-agar	<i>Escherichia, Enterobacter, Pseudomonas, Salmonella, Klebsiella and Shigella</i>
DP03	Door handles (Hospital)	Mannitol Salt Agar	<i>Staphylococcus, Micrococcus and Enterococcus.</i>
DP04	Utensil tool (Hospital)	Mannitol Salt Agar	<i>Staphylococcus, Micrococcus and Enterococcus.</i>
DP05	Labour room (Hospital)	Mac Conkey's agar	<i>Escherichia, Enterobacter, Pseudomonas, Klebsiella, Alcaligenes and Shigella</i>
DP06	Soil (Hospital waste disposal site-1)	EMB-agar	<i>Pseudomonas, Salmonella, Klebsiella, Alcaligenes and Serratia</i>
DP07	Soil (Hospital waste Disposal site-2)	Mac Conkey's agar	<i>Escherichia, Proteus, Enterobacter, Pseudomonas and Salmonella,</i>
DP08	Soil (Hospital waste Disposal site-3)	Mac Conkey's agar	<i>Klebsiella, Escherichia, Enterobacter, Pseudomonas, Salmonella and Shigella</i>

**Table 2: Biochemical characterization of isolates**

Acc. No.	CATALASE	MR	VP	INDOLE	CITRATE	NITRATE REDUCTION	UREASE	H <sub>2</sub> S	MANNITOL FERMENTATION	COAGULASE	GENUS IDENTIFIED
DP01	NA	[-]	[-]	[-]	[+]	[-]	[-]	[-]	NA	NA	<i>Pseudomonas sp.</i>
DP02	NA	[-]	[-]	[-]	[+]	[-]	[-]	[-]	NA	NA	<i>Klebsiella sp.</i>
DP03	[-]	[-]	[-]	[-]	[-]	[+]	[-]	[-]	[-]	[-]	<i>Micrococcus sp.</i>
DP04	[+]	[-]	[-]	[-]	[-]	[-]	[-]	NA	[+]	[+]	<i>Enterococcus sp.</i>
DP05	NA	[-]	[-]	[-]	[+]	[+]	[-]	[-]	NA	NA	<i>Alcaligenes sp.</i>
DP06	NA	[-]	[+]	[-]	[+]	[+]	[-]	[+]	NA	NA	<i>Serratia sp.</i>
DP07	NA	[+]	[-]	[-]	[+]	[+]	[+]	[+]	NA	NA	<i>Proteus sp.</i>
DP08	NA	[+]	[-]	[-]	[-]	[+]	[-]	GAS PRODUCTION	NA	NA	<i>Escherichia sp.</i>

**Table 3: Zone of inhibition of formalin against bacterial isolates**

Bacterial isolates	Zone of inhibition of formalin in millimetres (mm)										
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	
<i>Pseudomonas sp.</i>	39	21	16	10	7	NZI	NZI	NZI	NZI	NZI	
<i>Klebsiella sp.</i>	34	21	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI	
<i>Micrococcus sp.</i>	32	25	20	14	NZI	NZI	NZI	NZI	NZI	NZI	
<i>Enterococcus sp.</i>	38	21	19	NZI	NZI	NZI	NZI	NZI	NZI	NZI	
<i>Alcaligenes sp.</i>	30	27	25	NZI	NZI	NZI	NZI	NZI	NZI	NZI	
<i>Serratia sp.</i>	43	25	20	15	14	10	8	NZI	NZI	NZI	
<i>Proteus sp.</i>	36	19	15	13	9	NZI	NZI	NZI	NZI	NZI	
<i>Escherichia sp.</i>	35	20	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI	

NZI= No Zone of Inhibition



**Table 4: Zone of inhibition of pursue against bacterial isolates**

Bacterial isolates	Zone of inhibition of pursue in millimetres (mm)									
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
<i>Pseudomonas sp.</i>	46	33	20	18	16	15	15	12	11	10
<i>Klebsiella sp.</i>	38	20	16	14	11	NZI	NZI	NZI	NZI	NZI
<i>Micrococcus sp.</i>	24	18	15	11	10	7	3	2	NZI	NZI
<i>Enterococcus sp.</i>	32	30	28	NZI	NZI	NZI	NZI	NZI	NZI	NZI
<i>Alcaligenes sp.</i>	37	23	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI
<i>Serratia sp.</i>	10	10	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI
<i>Proteus sp.</i>	30	27	25	NZI	NZI	NZI	NZI	NZI	NZI	NZI
<i>Escherichia sp.</i>	37	23	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI

NZI= No Zone of Inhibition

**Table 5: Zone of inhibition of phenyle against bacterial isolates**

Bacterial Isolates	Zone of Inhibition of Phenyle in millimetres (mm)									
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
<i>Pseudomonas sp.</i>	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI
<i>Klebsiella sp.</i>	25	14	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI
<i>Micrococcus sp.</i>	15	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI
<i>Enterococcus sp.</i>	17	14	12	NZI	NZI	NZI	NZI	NZI	NZI	NZI
<i>Alcaligenes sp.</i>	15	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI
<i>Serratia sp.</i>	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI
<i>Proteus sp.</i>	10	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI
<i>Escherichia sp.</i>	15	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI

NZI= No Zone of Inhibition

**Table 6: Minimum inhibitory concentration of disinfectants against bacterial isolates**

Bacterial isolates	MIC of disinfectants		
	FORMALIN	PURSUE	PHENYL
<i>Pseudomonas sp.</i>	1:16	1:512	Growth till 1:1
<i>Klebsiella sp.</i>	1:2	1:16	1:2
<i>Micrococcus sp.</i>	1:8	1:128	1:1
<i>Enterococcus sp.</i>	1:4	1:4	1:4
<i>Alcaligenes sp.</i>	1:4	1:2	1:1
<i>Serratia sp.</i>	1:64	1:2	Growth till 1:1
<i>Proteus sp.</i>	1:16	1:4	1:1
<i>Escherichia sp.</i>	1:2	1:2	1:1

better antibacterial efficacy against *S. aureus* and *E. coli*. Phenyl, again, was the least effective against all the pathogens under study with zone of inhibition ranging from 6 mm to 11 mm.<sup>[10]</sup>

### Conclusion

The aim of this work was to study the comparative efficacy of selected disinfectants against pathogenic bacteria. In most of the hospitals, cleaning is regularly conducted by less skilled workers. In addition, there are no rules or regulations for proper disinfection. Disinfectants are often misused and rationalization of their use in hospitals is desirable for control of infection and costs. Therefore, in health-care setting it is mandatory to choose proper disinfecting agent for controlling cross contamination in the hospital environment.

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## Comparative Modeling Studies of MPT51 Protein

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### ABSTRACT

Leprosy is caused by *Mycobacterium leprae* that secretes proteins that are immunogenic which are said to cause leprosy by causing host tissue binding. The proteins that are secreted are known as trimeric antigen 85 (Ag85) complexes and another protein that is related to leprosy is MPT51 which is also known as FbpC1. This protein binds to fibronectin that is present in the extracellular matrix. MPT51 also plays a role in virulence and host tissue attachment. It plays an important role in the activity of acyltransferase. Biosynthesis of lipids also takes place due to acyltransferase. They are not only required for production of energy but is also needed as building blocks for cell wall composition. MPT51 is a very important protein and there are no studies done on the structure of the protein and hence structure prediction was done by using comparative method. Considering the importance and lack of specific structure, of MPT51 we have predicted the structure by using I-TASSER and the model was validated by using PROCHECK.

**Keywords:** *Mycobacterium leprae*, MPT51 protein, acyltransferase, I-TASSER, PROCHECK

### INTRODUCTION

One of the important tools that help to prevent infectious disease is vaccines. One of the acute diseases that still remains as problematic public health issue is leprosy one of the mycobacterial diseases. 12 million people are affected all over the world by leprosy and tuberculosis and it results in death of 1 to 2 million

people every year (1). Hansen's bacillus is another term for *Mycobacterium leprae* and it is present within the cell, acid fast bacterium, pleomorphic that is said to cause leprosy (2). This bacterium is encompassed by a unique waxy coating and this bacterium is rod shaped aerobic and is gram positive. Based on the shape and size it looks similar to *Mycobacterium tuberculosis*. Due to the waxy coating thickness the bacteria instead of staining with normal Gram stain it stains with carbol fuchsin. *Mycobacterium leprae* was the first bacteria that was identified to cause disease in human beings (3, 4). As leprosy is not common the serological tests that are being used show less sensitivity there by reducing chances to find the disease and for detecting the infection (5). Dapsone, Clofazimine and Rifampicin are used combined as used for treating leprosy (6). Macrolides, Fluoroquinolones and Tetracyclines are the antimicrobials that have been introduced lately to treat leprosy (7). One of the most efficient ways to treat different kinds of leprosy is Multi-Drug Therapy (MDT) this should be strictly done under medical supervision (8). It can be said that occurrence of leprosy has become less common due to this therapy but still there is no decrease in the detection of leprosy cases in the world (9). In order to control the disease diagnosing patients with leprosy fast, giving them proper treatment and educating people about these diseases is necessary (10). Researchers attempting to find the variation within the strains between different isolates of *Mycobacterium leprae* have not met with much success. Even though leprosy is still a global health issue it is less prevalent due to

the drug therapy (11). MPT51 protein is said to be immune dominant that can be identified from leprosy and tuberculosis patients who have <“80% of human immunodeficiency virus-negative, smear-positive serum antibodies. *Mycobacterium leprae* produces MPT51 protein. It is a type of protein that is involved in the adhesion mechanism of *Mycobacterium leprae* and is defined to be a non catalytic á/â hydrolase. Expression of the protein takes place within the starting stage of the infection and can be identified in both patients suffering with leprosy and healthy infected people. In our study, we have modeled three dimensional structure of MPT51 protein using I-TASSER (12). The final model was validated by using PROCHECK.

#### METHODOLOGY

- A) The primary sequence of MPT51 Protein (Acc.ID. Q05861) was retrieved from Swiss Prot. BLAST software was used for homology search of MPT51 protein.
- B) MPT51 crystal structure was modeled by using the I-TASSER server with (PDB ID: 4QEK) as structural template.
- C) Stereo-chemical quality of all the chains in a protein within the given PDB structure can be done by PROCHECK analysis. The regions that have unusual geometry are highlighted and the overall structural estimation is also provided.

#### Comparative modeling

The steps to creating a comparative model are as follows (Fig.1.):

- \* Identify homologous proteins and determine the extent of their sequence similarity with one another and the unknown.
- \* Align the sequences.
- \* Identify structurally conserved and structurally variable regions
- \* Generate coordinates for core (structurally conserved) residues of the unknown structure from those of the known structure(s).
- \* Generate conformations for the loops (structurally variable) in the unknown structure.

- \* Build the side-chain conformations.
- \* Refine and evaluate the unknown structure.

#### RESULTS AND DISCUSSION

##### Query sequence of MPT-51 Protein

```
>sp|Q05861|_MYCLE antigen OS=
Mycobacterium leprae GN= PE=3 SV=3
M K F V D R F R G A V A G M L R R L V V E A M
G V A L L S A L I G V V G S A P A E A F S R P G L P V E Y
L Q V P S P S M G R D I K V Q F Q N G G A N S P A L Y L L D G L R A Q
D D F S G W D I N T T A F E W Y Y Q S G I S V V M
P V G G Q S S F Y S D W Y S P C
G K A G C Q T Y K W E T F L T S E L P Q Y L Q S N K Q I K
P T G S A A V G L S M A G L S A L T L A I Y H P D Q F I Y
V G S M S G L L D P S N A M G P S L I G L A M G D A G G Y
K A A D M W G P S T D P A W K R N D P T V N V
G T L I A N N T R I M M Y C G N K P T E L G G N N L P A K L L E G L V R T S N
I K F Q D G Y N A G G G H N A V F N F P D S G T H S W
E Y W G E Q L N D M K P D L Q Q Y L G A T P G A
```

##### Three-Dimensional Structure Prediction of Protein by I-TASSER :

By retrieving amino acid sequence from UniProtKB/Swiss-Prot database (Ref No.GenBank: Q05861) I-TASSER was used to build the protein structure of MPT51, based on the Normalized B-factor (Fig.2.) evaluated by using the stereo-chemical properties by using the catalytic domains of 4QEK (Fig.3.) was used as template and confidence value the model is built.

Three dimensional structure of MPT51 Protein (Q05861) as shown in (Fig.4.) was predicted by using the tool I-TASSER by taking 4QEK-A as template which was obtained through BLAST results by taking Q05861 as query sequence and performing Protein BLAST against the protein sequence database. Energy minimization of the modeled three dimensional structure of MPT51 protein is carried by using PROCHECK.

**Stereochemical Activity Analysis:** PROCHECK checks the stereochemical quality of a protein structure, producing a number of Post Script plots analysing its overall and residue-by-residue geometry. It includes PROCHECK-NMR for checking the quality of structures solved by NMR



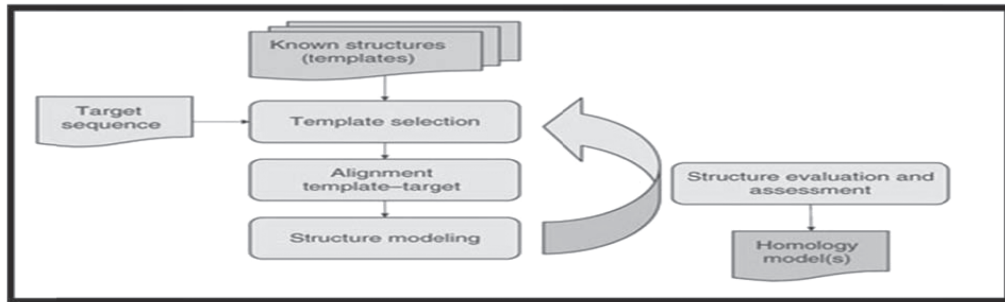


Fig.1. The four main steps of comparative protein structure modeling: template selection, target–template alignment, model building and model quality evaluation [13-18].

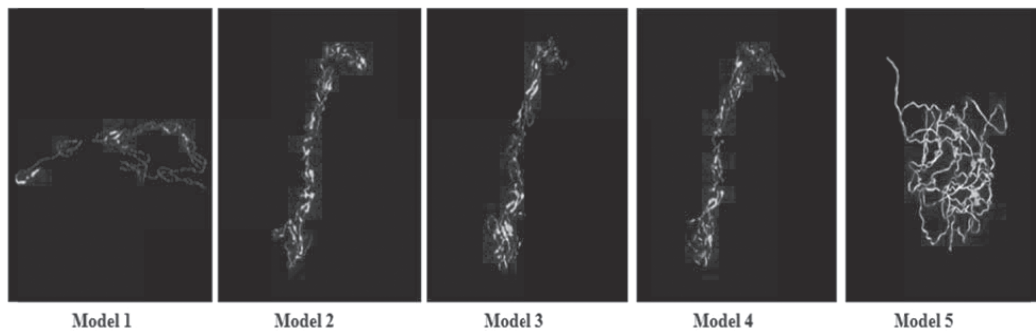


Fig.2. I-TASSER build models

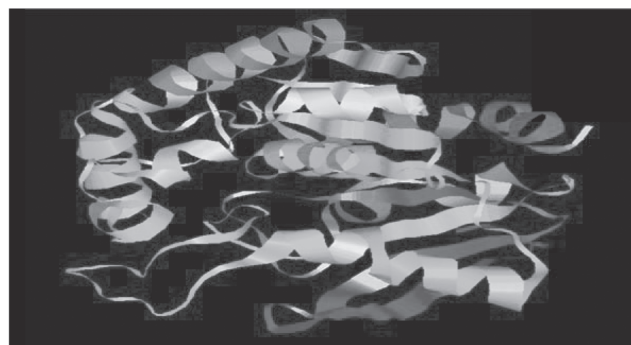
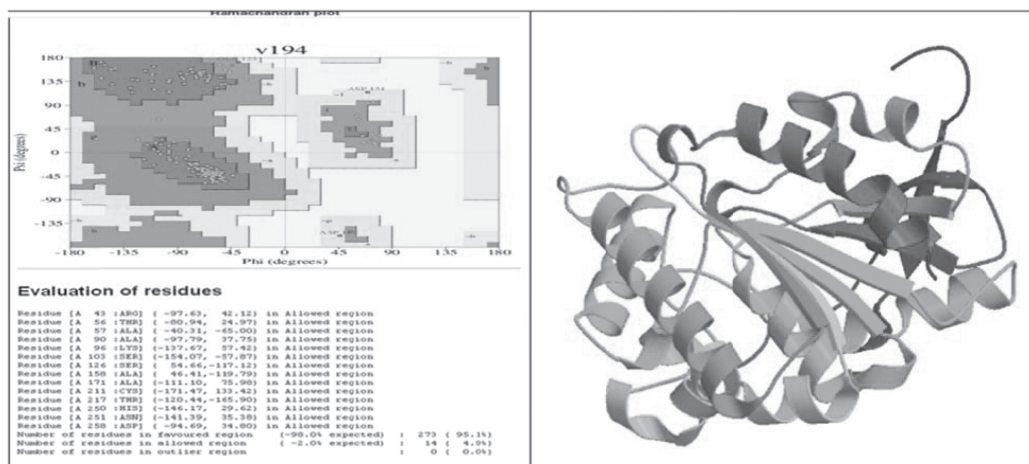


Fig.3. Crystal Structure of Antigen 85C-S124A mutant from *M. tuberculosis*

(PDB ID: 4QEK) as structural template.



**Fig.4.** Homology model of protein and validation of homology model using Ramachandran plot (PROCHECK)

and the results of procheck for protein structure after energy minimization is as follows (**Fig.3.**)

**Ramachandran Plot analysis by PROCHECK**  
 : PROCHECK is used for stereochemical assessment of the model. Ramachandran plot was developed by Gopalasamudram Narayana Ramachandran and Viswanathan Sasisekharan. It is used to visualize dihedral angles Psi and Phi of amino acid residues in protein structure and is commonly known as Ramachandran map or a Ramachandran diagram or a [Psi, Phi] plot. It depicts the possible conformations of Psi and Phi angles for a polypeptide. The model developed by PROCHECK was checked with the Ramachandran plot, MPT51 protein had 273 (95.1%) residues in the most favored region, 12 (4.9%) residues in allowed region and it can be seen in the (**Fig.4.**).

### CONCLUSION

In the current study we have modeled MPT51 protein, using I-TASSER server. By using BLASTP the template 4QEK was found. I-TASSER was used for building the homology model of MPT51 protein by using PROCHECK the protein structure validation was done.

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## An Evaluation of Antioxidant Potential of *Memecylon sisparens* Gamble Leaf in Doxorubicin- Induced Cardiotoxicity in Mice.

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### Abstract

*Memecylon* species has been reported for anti-cancer, anti-inflammatory, anti-hypertensive activities but *Memecylon sisparens* Gamble (MSG) activity is not yet reported. The present study is aimed for the first time to investigate on the antioxidant potential of MSG leaf ethyl acetate extract (EAE) on Doxorubicin (DOX) induced cardiotoxicity in mice model. The antioxidant activity was studied by total phenolic, flavonoid content, DPPH assay. Swiss albino male mice were treated with MSG leaf EAE for 9 consecutive days with DOX 15mg/kg, *i.p* on day7 to induce cardiotoxicity. ECG was recorded after 48h DOX administration, collected blood sample for CK-MB, LDH, AST, ALT and heart tissues for studying antioxidant parameters SOD, CAT, GSH, NO, MDA, histopathology. MSG leaf EAE showed good antioxidant activity with 93.67% DPPH radical inhibition at 250µg/mL. DOX significantly increased heart/bodyweight ratio along with CK-MB, LDH, AST, ALT levels in plasma, whereas treatment group significantly decreased the levels. In ECG recording, MSG leaf EAE decreased the ST segment which was elevated in DOX treated animals. In cardiac tissue, our extract decreased MDA, NO as DOX induced antioxidant markers

with an increase in SOD, CAT and GSH levels thereby showing protective mechanism through inhibition of oxidative stress. This is the first report that elucidated the antioxidant potential of MSG leaf EAE. Our results suggested that MSG leaf pre-treatment has an important therapeutic benefit during DOX therapy by inhibiting oxidative stress there by inhibiting lipid peroxidation, enhancing the cardio protective activity.

**KEY-WORDS:** Antioxidant, Cardiotoxicity, Doxorubicin, Electrocardiogram, Histopathology

### Introduction

DOX, widely used anticancer agent consists of a tetracycline moiety containing a quinone and a conjugated amino sugar residue in its structure which is prone to generation of free radicals leads to the oxidative stress that correlates with cellular injury by production of Reactive oxygen species (ROS) during its intracellular metabolism (1, 2). The increase in the release of ROS may lead to sub cellular changes in myocardium including the intracellular calcium dysregulation, loss of myofibrils and vacuolization of myocardial cells leading to release of cardiotoxic cytokines thereby causing cardiomyopathy and heart failure (1, 3, 4). The four cardiac chambers are dilated,

ventricular ejection fraction and contractile function is reduced (5). There is a concomitant diastolic dysfunction and increased wall stress leads to insignificant change in the LV wall thickness (5, 6). All these changes produce arrhythmias and leads to the elevation of ST segment of ECG. The DOX toxicity towards cardiac tissue is because of low levels of antioxidant defences compared to other tissues like liver (7).

*Memecylon sisparens* Gamble (MSG) is endemic to Western Ghats (8, 9) belonging to Melastomataceae family having pharmacologically active components- flavonoids, tannins, resins etc and needs experimentation. This family is proved for its anti-inflammatory, anti-HIV, anti-diarrhoeal, scavenging of free radicals (10, 11). Due to the occurrence of phytochemicals which exhibit best sources of antioxidant, anticancer activities towards the cure of many diseases, most of the developing countries are depending on the traditional medicine for their health care, The natural extract having potential bioactive phenolic compounds with more antioxidant capacity can be obtained by doing extraction in solvents such as ethyl acetate (12). Hence, we selected MSG leaf EAE for the first time in the present study by focusing on antioxidant activity and also to evaluate the cardio protective effect of MSG leaf EAE in DOX induced mice model of cardio toxicity as attention was focused on the naturally occurring antioxidants from medicinal plants because of their considerable efficacy without any adverse effect, comparative low cost and wide availability (13, 14).

#### Materials and methods

**Chemicals:** Ascorbic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), Rutin, TBA (Thiobarbituric acid), Gallic acid, TCA (trichloro acetic acid), 5,5 - dithiobis-2-nitrobenzoic acid (DTNB), Tetra methoxy propane (TMP), Folin-Ciocalteu (FC) reagent, Superoxide dismutase (SOD) kit, Sodium nitroprusside (SNP), DOX were purchased from Sigma-Aldrich Co., USA. AST, ALT, LDH, CK-MB kits were procured from Accurex Biomedical Pvt.Ltd, Mumbai.

**Preparation of plant extract:** The plant material was botanically authenticated by Dr. K.Madhavachetty, Department of Botany, Sri Venkateswara University, Tirupati against the voucher specimen No. 984 deposited in S.V.University herbarium. The MSG leaves was dried in shade, powdered and sieved coarse material of 150g were extracted with 600 ml of ethyl acetate in soxhlet apparatus. The extracts were filtered and evaporated by using rotary vacuum evaporator at temperature not exceeding 40<sup>o</sup> C and freeze dried the crude extract.

**Determination of total phenolic content:** The total phenolic content was spectrophotometrically determined by using gallic acid as a standard by *Scalbert method* with slight modification (15). To 0.1mL of MSG leaf EAE, add 0.2mL of FC reagent, 0.75mL of sodium carbonate solution (7.5%), incubated for 30 min at room temperature and read the absorbance at 765nm. The result was expressed as gallic acid equivalents (GAE) in milligram per gram of dry extract.

**Determination of total flavonoid content:** The total flavonoid content was spectrophotometrically determined by using rutin as a standard by *Brighente method* with slight modification (16). To 0.1mL of MSG leaf EAE, add 0.03mL of 5% sodium nitrite and incubate at 25°C for five minutes and then add 0.03mL of 10%aluminium nitrate. The reaction mixture incubated for five minutes at room temperature and then treated with 0.2mL of 1M NaOH. The reaction mixture was diluted with 0.5mL water and read the absorbance at 510nm. The result was expressed as rutin equivalents (RE) in milligram per gram of dry extract.

**DPPH radical scavenging assay:** The capacity of MSG leaf EAE to scavenge free radicals was spectrophotometrically determined by using Ascorbic acid, Curcumin, Butylated hydroxytoluene (BHT), Trolox as standard in DPPH free radical scavenging assay (17). To 0.1mL of MSG leaf EAE, add 1.0mL of methanolic solution of DPPH and incubate the reaction mixture for 20 minutes at room temperature and read the absorbance at 517nm and percentage



free radical scavenging activity (% FRSA) was calculated using the formula:

$$\%FRSA = (\text{Absorbance of control} - \text{Absorbance of sample}) / \text{Absorbance of control} \times 100$$

**Experimental animals:** Sixty male Swiss albino mice weighing between 23-30 g were procured from Teena biolabs, Hyderabad, India. The animals were housed in poly carbonate cages and fed with commercial pellet diet and water *ad libitum*. The animal experiments were performed in accordance with the CPCSEA guidelines. The Institutional Animal Ethics Committee (IAEC) approval with protocol number: NIP/02/2013/PC/43.

**Experimental design for DOX induced Cardiotoxicity:** The Swiss albino male mice were divided randomly into five groups (n= 12).

Group-I, Control group (C) receiving 2% sodium CMC orally for 9 days;

Group-II, MSG leaf EAE (H) receiving 500 mg/kg orally for 9 days;

Group-III, Disease control group (D) receiving DOX 15mg/kg, single *i.p* on day 7;

Group-IV, MSG leaf EAE receiving 250 mg/kg (LD) orally for 9 days with DOX 15mg/kg, single *i.p* on day 7;

Group-V, MSG leaf EAE receiving 500 mg/kg (HD) orally for 9 days with DOX 15mg/kg, single *i.p* on day 7.

After 48 h of DOX administration mice were anesthetized with ketamine and xylazine combination (60 mg/kg and 12mg/kg, *i.p*) and ECG was recorded by inserting the electrodes as per the Lead 1 position and ST-interval defined as the period from the S-peak to the end of the T-peak in seconds. The data were collected and analyzed by BIOPAC MP150 software, India. After recording ECG, mice body weights were taken and collected blood samples for plasma separation, heart tissues for studying antioxidant parameters, histopathology.

**Body weight and organ weight:** The animal body weights are recorded bi-weekly during the

experimentation and percent increase in body weight was estimated on the basis of initial weight. Animals were sacrificed and liver, heart, spleen was excised, washed with cold saline, dry blotted and weights were recorded. The relative heart weight for each animal in the groups was determined.

**Estimation of biochemical parameters:** Plasma was used to determine LDH, CKMB, AST, ALT by using respective biochemical kits from Accurex Biomedical Pvt. Ltd, Mumbai, India.

**Assessment of DOX induced oxidative stress markers:** Heart was dissected and washed with saline, blotted dry and weights were recorded. The heart tissues (n=6) was minced into small pieces and homogenized in ice-cold PBS, pH 7.0 to obtain 1:9 (w/v) whole homogenate. Homogenate of heart tissue was taken and mixed with same volume of 10% trichloroacetic acid (TCA) and centrifuged at 2900 × g for 10 minutes. Supernatant was collected and used for the estimation of GSH and MDA. GSH was determined by following *Ellman method* (18) where as MDA by following *Ohkawa and Yagi method* (19). Tissue homogenate was centrifuged at 17000 × g for 60 min at 4 °C, and supernatant was used for the CAT, SOD and NO. CAT was measured by following the method of *Aebi method* with slight modification (20). The SOD activity (cytosolic and mitochondrial) was determined using a SOD assay kit (Sigma-Aldrich Co., USA) by following the specifications of manufacturer. NO was determined by following the method of *Garrat method* with slight modification (21). Haematoxylin and Eosin staining was performed to see the histopathological changes in heart tissues after formaldehyde fixation, paraffin embedded.

**Histological analysis:** The heart tissues (n=6) was collected in 10% formal saline and embedded the heart tissue in paraffin blocks and sections were cut by microtome at 5µM thickness on a rotary microtome then stained by Haematoxylin and Eosin stain. These heart sections were evaluated for histological changes under NIKON light microscopy (Model: Ti.U).

**Statistical analysis:** The results were expressed as mean  $\pm$  SEM. The variation between various groups and inter-group was measured by one way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test compared control (C) with DOX (D) treated groups, DOX treated group (D) with MSG extract treated groups (LD, HD). Results were considered statistically significant when  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$  respectively.

**Results**

**Total phenolic and flavonoid content:** The total phenolic and flavonoid content for MSG leaf EAE was found to be  $238.99 \pm 28$  mg/g gallic acid equivalents,  $99.41 \pm 1$  mg/g rutin equivalents respectively.

**DPPH Free radical scavenging activity:** The antioxidant activity of MSG leaf EAE was explored by analyzing the free radical scavenging activity of DPPH as shown in Fig. 1. A steady rise in the

percentage inhibition of the DPPH radical by the EAE was observed up to  $250 \mu\text{g/mL}$  concentration, after that there was a levelling off with much slower increase in inhibition. In the presence of  $250 \mu\text{g/mL}$  of the test sample, the DPPH radical inhibition of MSG leaf EAE and the standard references like ascorbic acid, curcumin, BHT and trolox was in the following order: trolox (96.58 %) > MSG leaf EAE (93.67%) > ascorbic acid (90.23 %) > BHT (76.27%) > curcumin (70.24 %). The EC<sub>50</sub> values of ascorbic acid, curcumin, BHT, trolox, MSG leaf EAE were  $80.35 \pm 11.9$ ,  $172.91 \pm 7.28$ ,  $97.2 \pm 9.98$ ,  $1483.02 \pm 5.58$ ,  $64.40 \pm 3.45 \mu\text{g/mL}$  respectively.

**Effect on change in body weight and heart weight:** DOX enhanced the cardio toxicity markers like loss in body weight, proportionate heart weight. Upon 9 days continuous treatment with MSG leaf EAE (250 mg/Kg, 500 mg/Kg) enhanced the body weights as well as relative heart weights compared to DOX group as shown in Fig. 2.

**Evaluation of electrocardiogram:** The ECG recording of mice after 48h of DOX treatment shown the dilation of the ST segment in DOX group compared to the normal control group animals as shown in Fig. 3-A. Treatment with MSG leaf EAE incomparably declined the ST segment interval compared to the only DOX induced group animals as shown in Fig. 3-B.

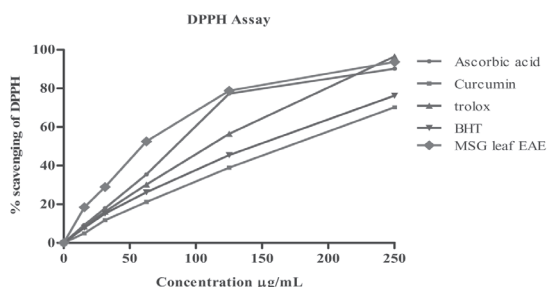


Fig. 1: DPPH free radical scavenging activity by MSG leaf EAE

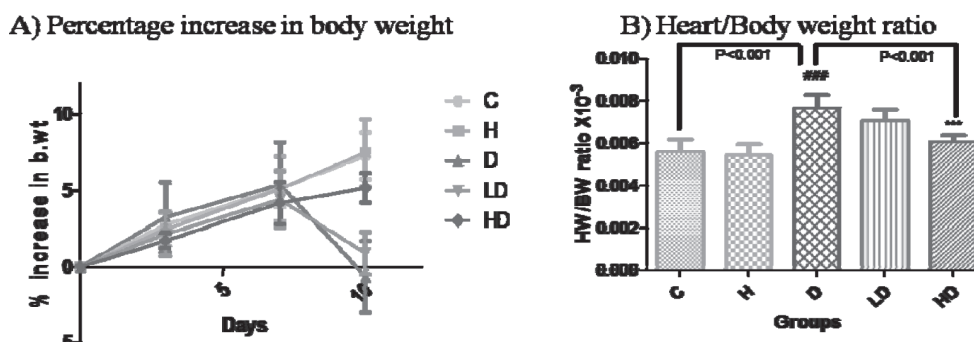
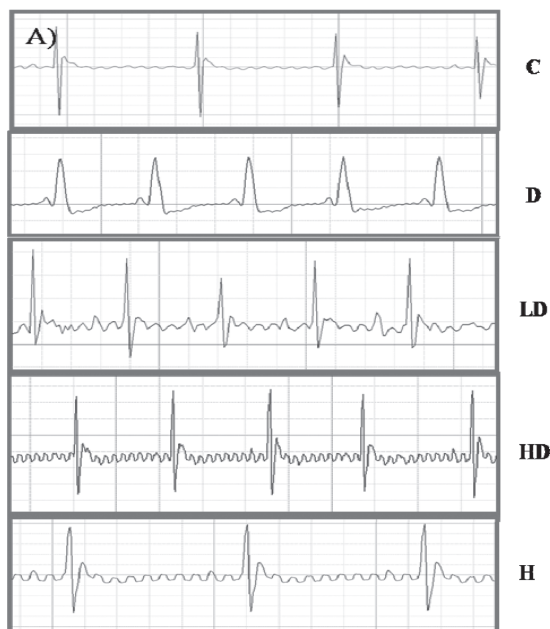
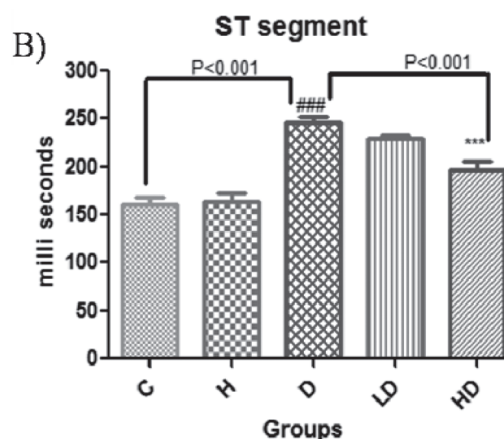


Fig. 2: Percentage increase in body weight and heart/ body weight of MSG leaf EAE against DOX induced groups: C=Control group; H= MSG extract (500mg/kg); D=Doxorubicin (15mg/kg); LD= MSG extract (250mg/kg) + DOX; HD= MSG extract (500mg/kg) + DOX. # DOX compared with Normal control and \* compared DOX with treated groups \*\*\*P<0.001 respectively.



**Fig. 3-A:** Effect of MSG EAE against DOX on ECG-recording in mice



**Fig. 3-B:** Graphical representation of effect of MSG leaf EAE against DOX on ECG-ST segment ECG determined by using BIOPAC system in Swiss mice. C=Control group; H= MSG extract (500mg/kg); D=Doxorubicin (15mg/kg); LD= MSG extract (250mg/kg) + DOX; HD= MSG extract (500mg/kg) + DOX.

**Effect on biochemical parameters:** The results of plasma biochemical parameters analysis in DOX treated mice are shown in Fig. 4. DOX treated mice showed significant levels ( $p < 0.001$ ) of LDH, CK-MB, AST and ALT levels in plasma compared to control group animals. Pre-treatment with MSG leaf EAE for 9 consecutive days along with DOX administration showed significant decrease ( $p < 0.001$ ,  $p < 0.01$ ,  $p < 0.05$ ) of LDH, CK-MB, AST and ALT levels in plasma compared to the DOX group there by showing protective mechanism.

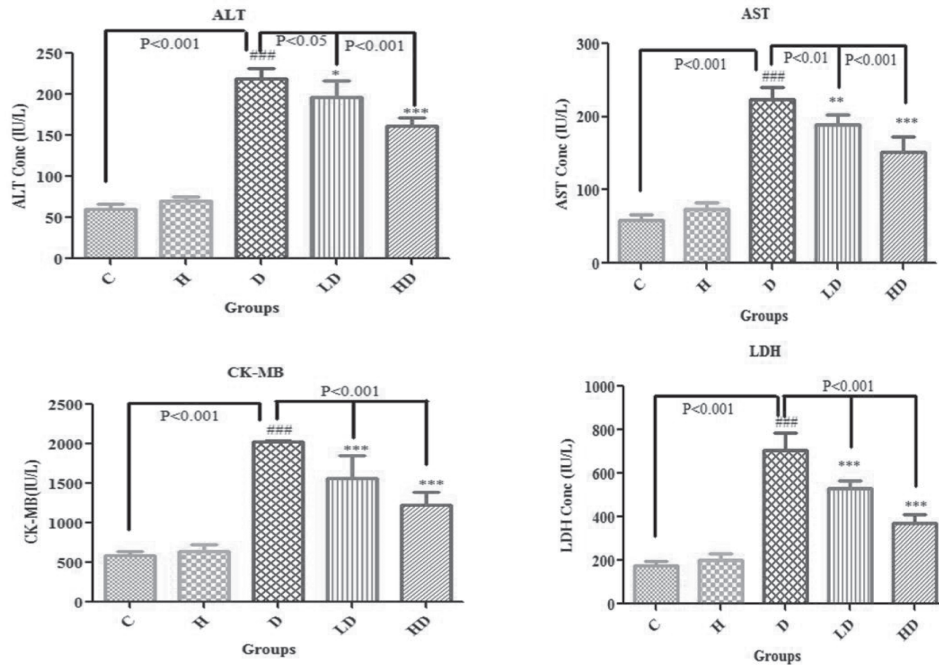
**Effect on antioxidant levels in the heart tissue:** The MSG leaf EAE (250mg/kg and 500 mg/kg) treatment for 9 days showed significant change in the levels of GSH, SOD and CAT in mice heart tissue as shown in Fig. 5. Compared to normal control group, DOX treated mice showed lower levels of GSH, SOD and CAT. The elevated levels of MDA indicate the lipid peroxidation in the DOX group compared with the normal control group and MSG leaf EAE treatment significantly ( $p < 0.001$ ) decreased the MDA levels in heart tissue. The

DOX induced mice showed higher levels of NO compared with control group mice where as pre-treatment with MSG leaf EAE significantly decreased the levels of NO in heart tissue.

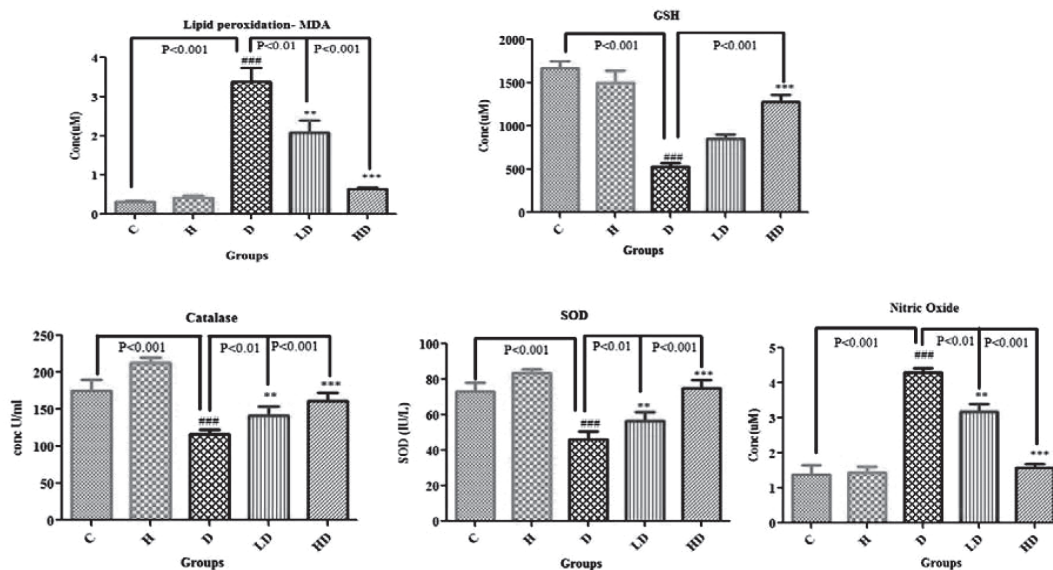
**Morphological changes in heart sections:** The heart sections after Haematoxylin and Eosin staining in normal control group mice showed the normal histology where as DOX group mice showed damage to muscle fibers and focal myocardial fibrosis, interfibrillar hemorrhages. Pre-treatment with MSG leaf EAE has decreased the DOX induced damage to muscle fibers as shown in Fig. 6.

### Discussion

In the present experiment, the potent free-radical scavenging activity of MSG leaf EAE *in vitro* showed comparable to that of ascorbic acid. DOX has more affinity towards the cardiolipin, phospholipids component of the mitochondria in cardiac myocyte, leads to generation of ROS thereby enhancing the lipid peroxidation and also

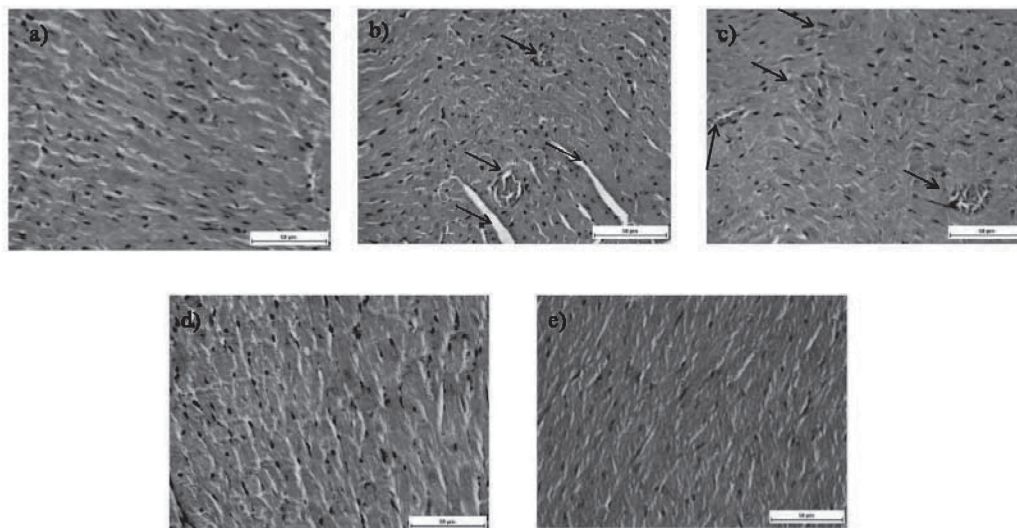


**Fig. 4:** Effect of MSG leaf EAE on DOX induced changes in biochemical markers: C=Control group; H= MSG extract (500mg/kg); D=Doxorubicin (15mg/kg); LD= MSG extract (250mg/kg) + DOX; HD= MSG extract (500mg/kg) + DOX. # DOX compared with Normal control and \* compared with DOX, treated groups. \*\*\*P<0.001, \*\*P<0.01, \*P<0.05 respectively.



**Fig. 5:** Effect of MSG leaf EAE on DOX induced changes of Antioxidant markers in heart tissue C=Control group; H= MSG extract (500mg/kg); D=Doxorubicin (15mg/kg); LD= MSG extract (250mg/kg) + DOX; HD= MSG extract (500mg/kg) + DOX. # DOX compared with Normal control and \* compared with DOX, treated groups. \*\*\*P<0.001, \*\*P<0.01 respectively.





**Fig. 6:** Photomicrographs of heart sections stained with haematoxylin and eosin (H&E). a) Control group mice showing normal histology b) Dox group mice showing damage to muscle fibres and focal myocardial fibrosis, inter-fibrillar haemorrhages c) Mice pretreated with MSG (500mg/kg, p.o) showing normal histology d) Mice pretreated with MSG low dose (250mg/kg,p.o) and Dox showing myocardial fibrosis and damage to muscle fibres along with inter-fibrillar haemorrhages e) Mice pretreated with MSG (500mg/kg, p.o) and Dox showing damage to muscle fibres.

causing energy depletion (22). In turn increase in ROS elevates the nitric oxide production through endothelial nitric oxide synthase. The cellular damage caused by lipid peroxidation is responsible for heart damage through the generation of ROS by elevating MDA levels in DOX group. The increased ROS flux has been shown to damage myocytes, impair contractile function leads to capillary leakage (23), there by leading to increase in the level of CK-MB in the plasma showing that the DOX causes cardiotoxicity through the generation of ROS (2). CK-MB is an enzyme found mainly during the myocyte injury in myocardium, used as a marker for evaluating the degree of the myocyte injury (24). Pre-treatment with MSG leaf EAE showed a decrease in the extent of lipid peroxidation in heart tissue there by reduction in circulating CKMB levels in plasma with an increase in the antioxidant activity in heart tissue such as SOD, GSH and catalase. Due to semiquinone moiety of DOX, increase in free radicals leads to increase in SOD enzyme

activity by dismutase, converts free radical to hydrogen peroxide. The decrease in the activity of catalase, GSH in DOX induced animals is ominous of enhanced oxidative stress. The DOX toxicity towards cardiac tissue is because of low levels of antioxidant defences compared to other tissues like liver because of the low levels of free-radical detoxifying enzymes like superoxide dismutase, GSH and catalase (25).

The mitochondrial injury induced by DOX in the cardiac myocytes is having a severe effect on the contractile functioning of the heart by alternating in the energy metabolism (26). Sarcomeres are the basic contractile units having thick myosin and thin actin which are present in the myofibrils of each cardiomyocyte, basically responsible for rhythmic contraction of heart along with calcium ion as key player. The calcium ions bind to troponin, thereby altering the tropomyosin position which covers the myosin binding site on actin filament leading to sarcomere shortening during relaxation (27). There is diastolic



dysfunction because of significant change in the wall thickness; an altered myocardial membrane leads to the wall stress is increased in DOX-treated animals by producing arrhythmias, dilating ST segment leading to ECG changes (28, 29).

In the present study, pre-treatment with MSG leaf EAE significantly increased the SOD activity compared to the DOX treated animal group. The level of GSH were also lowered significantly in DOX treated animals, while pre-treatment with MSG leaf EAE showed an increase in GSH levels. NO activity in DOX treated animals significantly decreased with the pre-treatment of MSG leaf EAE.

Our histopathological report suggests that MSG leaf EAE pre-treated groups attenuates the doxorubicin induced focal myocardial fibrosis, inter-fibrillar haemorrhages, loss of myofibrils. Though MSG leaf EAE attenuates DOX induced cardio toxicity, the mechanisms behind its protective action needs to be elucidated. The present investigation revealed that MSG leaf EAE possesses some bioactive constituents which are responsible for its antioxidant and cardioprotective activity against DOX induced cardiotoxicity mice model.

### Conclusion

This is the first report that elucidated the antioxidant and cardioprotective activity of MSG leaf EAE. Our results also suggested that simultaneous pre-treatment with MSG leaf EAE has an important therapeutic benefit during DOX therapy by inhibiting oxidative stress there by inhibiting lipid peroxidation, enhancing the cardio protective activity and there is a need for isolation of biologically active compounds.

### Acknowledgements

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## Genetic Algorithm Optimization of L-Glutaminase from novel mutated *Bacillus* sps.

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### Abstract:

L-Glutaminase is one of the most promising biocatalyst to treat Acute Lymphoblastic Leukaemia (ALL). The anticancer drugs which are available in the market are costly and have many side effects. Attempts have been made to overcome these problems. In addition, it has economic importance due to its flavour enhancing property in fermented food industry. A novel *Bacillus* sps was isolated from *Ocimum tenuiflorum* which is able to produce L-Glutaminase. Random mutation experiments were performed on this endophyte to maximize L-Glutaminase activity. Optimization studies were carried out by both traditional and statistical methods. Genetic Algorithm (GA) finds the global optimal solution by using Darwin's natural selection. Genetic Algorithm was employed on four components (Constraints)–Galactose (1-3 g/L), Time (24-48 h), Temperature (20-40°C) and pH (5.0-9.0) for optimization of L-Glutaminase activity. The polynomial equation obtained from RSM was used as objective function with above four constraints. Experiments were performed at GA optimized variable values for validation. At GA optimized variable values the L-Glutaminase activity was observed to be 27.8 IU/mL.

**Keywords:** Acute Lymphoblastic Leukaemia (ALL), Genetic Algorithm (GA), Endophyte, Biocatalyst, Global optimal solution.

### INTRODUCTION

Cancer is the second most leading cause for the number of deaths reported every year[1-3].

Up to some extent cancer can be treated by anti-proliferative medication and radiation therapy [4, 5]. The drawbacks for the above are anti-proliferative medication can be used only to treat reproducing cells, whereas radiation therapy is used to treat benign tumours[6-8]. There is a need to develop drugs which has high specificity and half-life with minimal side effects [9]. Enzymes are biocatalyst which has high specificity towards its substrate[10-12]. Different enzymes from different microorganisms have different therapeutically properties[11, 13, 14]. 80% of the industries are involved in the production of nearly 500 products which has been applied in food, medicinal and others [15].

Deamination of L-Glutamine by L-Glutaminase will play an important role to treat ALL[16, 17]. Enzymatic therapy by L-Glutaminase has gained importance due to minimal side effects [18-20]. In addition L-Glutaminase lowers the glutamine levels in the blood serum causing reduction in reverse transcriptase to treat HIV [21, 22]. The glutamine levels in the hybridoma mammalian cells can be monitored by L-Glutaminase [23]. Glucose and Glutamine are the precursors for cancer cells to meet their energy requirements [24]. L-Glutaminase degrades the excess glutamine in the serum making L-Glutaminase as therapeutic agent[25]. For urea synthesis Glutaminase will synthesize ammonia in hepatocytes [26, 27]. L-Glutaminase is also used for the synthesis of pyrimidine's and nitrogen metabolism [28, 29]. Acrylamide is the cancer causing agent which is

found in fried foods[30-32]. Treating processed foods with Glutaminase lowers the acrylamide formation[33, 34]. Glutaminase isolated from *Stenotrophomonas maltophilia* is used as flavour enhancer of soy sauce[35, 36]. The Glutamic acid formed by catalysing L-Glutamine will be used as flavour enhancer[16]. ADP is the strong inhibitor for L-Glutaminase[36, 37].

Kidney type (GLS1) and Liver type (GLS2) are the two isoforms of L-Glutaminase found in humans[38]. Both types of Glutaminases have different function and the percentage of occurrence in each organ is different[39, 40]. The most commonly found glutaminase in brain is GLS1[41]. In *E.coli* kidney type glutaminase is called Glutaminase A and liver type glutaminase is called Glutaminase B[42]. Both the Glutaminases differ in their function, structure and catalysis. In *Saccharomyces* two types of Glutaminases has been reported one is extra-cellular (Glutaminase B) and other is intracellular (Glutaminase A)[43]. The glutaminases from different sources differ in their stability to pH, temperature, molecular weight, structure etc[44]. Kidney type glutaminase is used to treat cancer. Colon cancer can be treated by liver type glutaminase[45].

Enzymes have been distributed among plants, animals, yeast, fungi and bacteria[46, 47]. Of all the sources microorganism have the ability to synthesize the products with in limited time[20]. Therapeutic enzymes derived from microorganism are incompatible with human immune system. Hence, there is a need to develop L-Glutaminase which is compatible with immune system[48]. Endophytes are the microorganism which lives within host for part of life[49]. Endophytes are major sources in producing bioactive compounds[50]. Enzymes from endophytes of medicinal plants can minimize the side effects[51]. Most of the enzymes used in industries are of microbial origin[17, 18]. Microorganisms are highly diversified and it is easy to perform modelling and optimization studies[52]. L-Glutaminases from terrestrial microorganisms and marine microorganism has been reported. Genetic manipulation of microorganism for large scale

production is easy, hence microorganisms are preferred in industries[53]. The occurrence of GSL1 and GSL2 has been reported and very well studied in *Aspergillus sps* [54]. The production studies of L-Glutaminase from *Aspergillus sojae* have been increased by mutation and protoplast fusion techniques[55].

In the current research, production studies were carried out using randomly mutated *Bacillus stratosphericus*. Traditional optimization of the media components were performed by varying one media parameter at a time to identify the factors influencing and how they are interacting. Plackett-Burman followed by Central Composite Design was employed. To find the global optimal solution with in the data supplied genetic algorithm studies were performed. From a set of population genetic algorithm selects the best fit value by Darwin's natural selection.

#### MATERIALS AND METHODOLOGIES

Genetic Algorithm (GA) is used to find the global optimal solution of the data sets within the desired space[56]. Optimization (discontinuous, mixed-integer and non-linear objective functions) which cannot be solved by statistical methods can be solved by employing GA[57, 58]. Natural selection proposed by Darwin was employed to find the global optimal solution[59]. From the given data sets the parents are selected randomly. Application of genetic operators to the population results in new generation by using survival of fittest phenomena. The population at each step was evaluated and selected based on the best fitness value to attain global optimal solution[60]. The advantage of using GA over classical algorithm is – GA selects a population of points where as classical algorithm uses single point of data at a time[61]. The three main rules used to study GA are as follows[62]:

1. Random selection rule to choose parent
2. Crossover rules to create new generation having best fit value.
3. Mutation rules to create new generation with hybrid qualities by altering the genes.

#### Steps followed:

1. Creating random population



2. Choosing the parents randomly at each step to create new generations. At each iteration/generation, the fitness value of the individual was analysed and the best fit individuals are chosen to create new generation. The children at each step are produced by using genetic operators.

The genetic operators used in this study are as follows[63]:

1. Mutation
  2. Crossover
  3. Reproduction
3. Stopping the algorithm.  
Multi-objective optimization of GA was performed by using `optimtool` in MATLAB R2013a.

**Creating population:** The first step in performing GA optimization. From the given data sets the population was generated randomly[64]. Different types of population were used based on genetic operator functions used to create a population[65]. They are:

1. Double vector
2. Bit string
3. Custom

The second and third types are used when crossover and mutation functions are used for creation[66]. The population size indicates the number of individuals at each generation. Default number of individuals is:  $15 \times \text{number of variables}$ , or we can customise by creating user defined functions. Constraint dependent function was used to create population. GA starts by the initialization of population with scores of each individual within the given range. The algorithm computes the scores based on their fitness value. The parents at each generation are selected by selection function[67]. The children having the best fit value will survive for next generation. By using reproduction function the children for the next generation were created. Constraint dependent mutation creates random changes at each generation by using Gaussian or Adaptive feasible function. Crossover function helps to recombine the parents to generate new individuals[68]. Out of different types of crossover objective functions

intermediate cross over function with default ratio of 1.0 was chosen in this study. Migration specifies the moment of individuals between the subpopulations. The moment of individuals is either in forward direction or both the directions. The fit population among all the generations were chosen to get diverse characteristics. Genetic algorithm stops when it meets the stopping criteria[60]. Algorithm stops when it meets number of generations, time limit, fitness and other parameters. Plot function enables to plot different functions.

The Anova equation obtained from the RSM was used to find the global optimal solution of L-Glutaminase:

$$y_1 = (-91.74 + 0.35x(1) + 13.3x(2) + 0.66x(3) + 2.96x(4) + 0.70x(1)x(2) + 0.20x(1)x(3) + 0.086x(1)x(4) - 0.024x(2)x(3) - 0.124x(2)x(4) - 6.98E-003x(3)x(4) - 3.04x(1)^2 - 0.68x(2)^2 - 0.0112x(3)^2 - 0.025x(4)^2);$$

Where,

Y = L-Glutaminase activity

x1-Galactose(g/L)

x2-pH

x3-Temperature( $^{\circ}$ C)

x4-Time(h)

The equation contains four significant input variables obtained from Plackett-Burman studies [69]. Low and high limits for the variables were shown in Table 1:

## RESULTS:

The data sets obtained in the RSM experiments were further optimized by employing GA. GA divides the given data sets into population based on the survival of fittest. Four variables (Table 1) were chosen to optimize the production of L-Glutaminase from novel Mutant *Bacillus sps*. The quadratic equation obtained from statistical method is used as fitness function to perform GA optimization. Default parameters of `optimtool` in MATLAB were chosen to study L-Glutaminase production. The process of optimization was



repeated until best results are obtained. The estimated L-Glutaminase activity from GA was 26.57IU/mL (Table 2). At same conditions experiments were performed and the L-Glutaminase activity estimated was 27.8 IU/mL (Table 2). Best function value at each generation w.r.t iterations was shown in Figure 1 and the fitness achieved was 0.0376 at iteration number 51. Selection plot explains which parents are contributing towards next generation (Figure 7). The number of mutation (Red), cross-over (Blue) and elite (Black) individuals are represented in Figure 5. The current best individuals among the input variables are plotted in Figure 4. The average distance between the individuals at each generation are represented in Figure 2. From range plot (Figure 3) the maximum minimum and average fitness of each generation can be observed. Score histogram was shown in Figure 6. The stopping criteria at 51 iteration where maximum fitness achieved was shown in Figure 8.

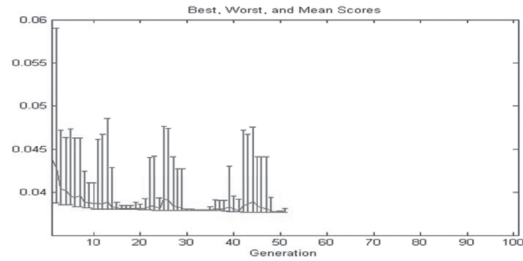


Figure 3. Best, worst and mean scores of individuals w.r.t generations

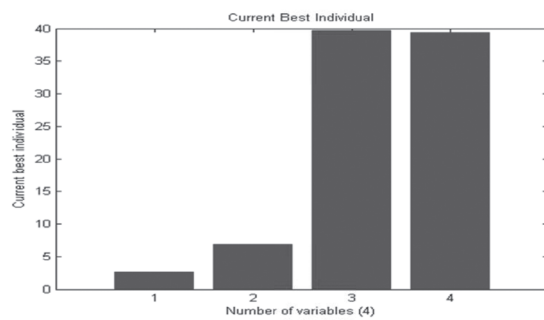


Figure 4. Current Best individual among parameters chosen for optimization

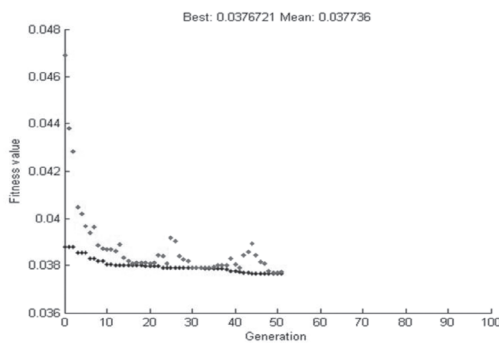


Figure 1. Best fitness of individual w.r.t generations

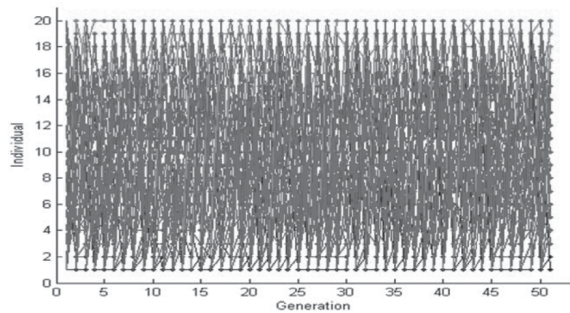


Figure 5. Genealogy of individuals

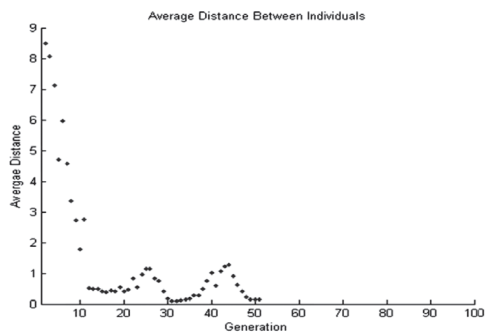


Figure 2. Average Distance between individuals

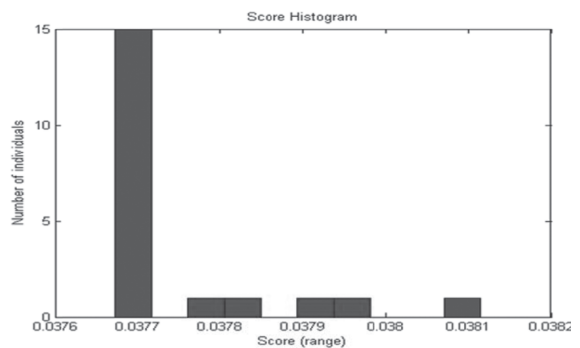


Figure 6. Score histogram

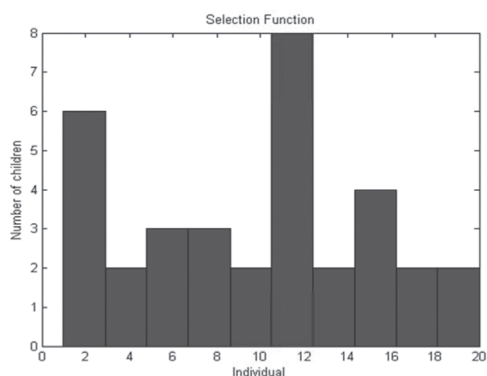


Figure 7. Selection functions of individuals as parents

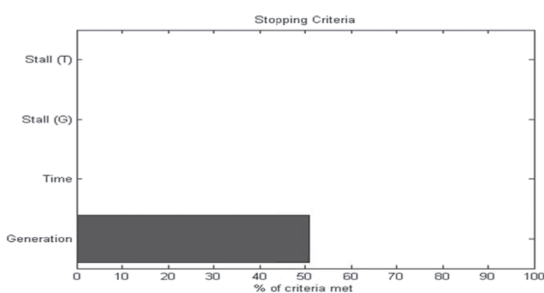


Figure 8. Stopping criteria w.r.t to generation, time and stall

**CONCLUSION:**

Global optimization was performed on significant variables ( Galactose (1-3 g/L), Time (24-48 h), Temperature (20-40 °C) and pH (5.0-9.0) ) obtained from statistical method to increase the production of L-Glutaminase. GA is an evolutionary algorithm which finds the global optimal solution with in the data space. The quadratic equation obtained from the RSM was used as objective function. The best optimal solution was obtained after 51 iterations. L- Glutaminase was found to be 27.8 IU/mL at Galactose (2.61 g/L), Time (40 h), pH(6.8) and Temperature (40 °C). The obtained solution from GA is higher in comparison with RSM. It was observed that L-Glutaminase activity was enhanced by performing GA optimization studies.

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**Table 1.** Parameters chosen for Optimization

Parameter	Lower Bound	Upper Bound
Galactose(g/L)	1 g/L	3 g/L
pH	5.0	9.0
Temperature (°C)	20 °C	40 °C
Time (h)	24h	48h

**Table 2.** GA optimized solution

Galactose (g/L)	pH	Time (h)	Temperature (°C)	L-Glutaminase optimized value from GA (IU/mL)	L-Glutaminase Experimental (IU/mL)
2.61	6.80	40	40	26.57	27.8 ± 0.35

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## Antibacterial study of the seaweed *Ulva fasciata*

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### ABSTRACT

The **marine environment** supplies many kinds of habitats that support **marine** life. **Marine** life depends in some way on the saltwater that is in the sea (the term **marine** comes from the Latin mare, meaning sea or **ocean**). Marine organisms are potentially prolific sources of highly bioactive secondary metabolites that might represent useful leads in the development of new pharmaceutical agents. The principal use of seaweeds are as a source of human food as well as a source of gums (phycocolloids). Phycocolloids like agar agar, alginic acid and carrageenan are primary constituents of brown and red algal cell walls and are widely used in industry. The antibacterial activity of the Acetone, Methanol and Ethanol extracts of the marine algae *Ulva fasciata* from Ennore Beach near Chennai (coast of TamilNadu) were tested in-vitro against a panel of Gram positive and Gram negative bacterial strains. In this study, agar well diffusion test technique was followed. The result exhibited antibacterial activity of the algal extracts against both Gram positive and Gram negative bacteria on selective media.

**Keywords :** Marine Environment , Seaweeds , *Ulva fasciata*, Antibacterial Activity and Agar Diffusion Test.

### INTRODUCTION

A seaweed may belong to one of several groups of multicellular algae : the red algae , green algae and brown algae. As these three groups do

not have a common multicellular ancestor, the seaweed are in a polyphyletic group. In addition, some tuft – forming blue green algae (Cyanobacteria) are sometimes considered to be seaweed. Seaweed has a variety of purposes, for which it is farmed or foraged from the wild. At the beginning of 2011, Indonesia produced 3 million tonnes of seaweed and surpassed the Philippines as the world's largest seaweed producer. Seaweed extract is also used in some diet pills (**Hayato Maeda et al/2005**). Other seaweed pills exploit the effect as gastric banding, expanding in the stomach to make the body feel fuller (**Elena Gorgan, 2009**). Other seaweed may be used as fertilizer, compost for landscaping, or a means of combating beach erosion through burial in beach dunes. Seaweed is also under consideration as a potential source of Bioethanol.

*Ulva fasciata* also known as *Limu palahalaha* and sea lettuce, is a common green alga that is used for consumption in many parts of the world. High nutrients and freshwater are often indicated by its presence. *Ulva fasciata* is commonly found on intertidal rocks, in tidepools, and on reef flats (**Litter D.S. and Mark 2000**). It is often abundant in areas of fresh water runoff and high in nutrients such as near the mouth of the streams and runoff pipes. *Ulva fasciata* or sea lettuce is commonly found in areas where nutrients are high, wave forces low and herbivory reduced. It is tolerant of stressful conditions, and its presence often indicates freshwater input or pollution. It is the first macroalgae to colonize newly opened substrate in intertidal areas with high nutrients.

Their opportunistic success can be attributed to their simple morphologies and fecundity. In *Ulva* species, between 20 and 60 percent of their overall biomass can be allocated monthly to reproduction. The alga's reproductive success is partly due to the reproductive cell's photosynthetic ability (Beach et al 1995). The zoospore's and gametes ability to photosynthesize subsidizes their motility and rapid growth once attached to the substrate. Reproductive cells of *Ulva fasciata* have similar photosynthesis rates to adult vegetative cells with higher respiration rates. Ecologically successful green alga are potentially invasive. Coastal waters near harbors, industrial complexes and residential areas with nutrient rich and or fresh water input often have blooms of *Ulva* species that coat ship's hulls, cover pilings and shorelines, and restrict outflow pipes. Thus *U.fasciata* is classified as a marine fouling organism, and studies in control and eradication are presently underway.

#### TEST ORGANISMS

*Escherichia coli* are bacteria found in the environment, foods and intestines of people and animals. Antibiotic – resistant *E.coli* pass on the genes responsible for antibiotic resistance to other species of bacteria through a process called horizontal gene transfer. *E.coli* bacteria often carry multiple drug resistance plasmids, and under stress, readily transfer those plasmids to other species. Mixing of the species in the intestine allows *E.coli* to accept and transfer plasmids from and to other bacteria. Thus *E.coli* and other *Enterobacteria* are important reservoirs of transferable antibiotic resistance (Salyers AA, Gupta A, Wang Y 2004).

*Staphylococcus aureus* is a Gram positive, round shaped bacterium that is a member of the formicetes and it is a member of the normal flora of the body, frequently found in the nose, respiratory tract and on the skin. It is often positive for catalase and nitrate reduction and is a facultative anaerobe that can grow without the need for oxygen. Pathogenic strains often promote infections by producing virulence factors such as potent protein toxins, and the expression of a cell

surface protein that binds and inactivates antibodies (Masalha M, Borovol I et.al 2001).

*Pseudomonas aeruginosa* is a common Gram – negative, rod – shaped bacterium that can cause disease in plants and animals, including humans. It is a multidrug resistant pathogen recognized for its ubiquity its intrinsically advanced antibiotic resistance mechanism and its association with serious illness – hospital acquired infection such as ventilator – associated pneumonia and various sepsis syndromes. The organism is considered opportunistic as serious infection often occurs during existing diseases or conditions. It is also found in the immune-compromised but infect immunocompetent (Hoiby et al, 2010).

*Klebsiella pneumonia* can cause destructive changes to human and animal lungs if inhaled, specifically to the alveoli resulting in bloody sputum. It naturally occurs in the soil, and about 30% of strains can fix nitrogen in anaerobic conditions. *Klebsiella* can cause infection in urinary tract, lower biliary tract and surgical wound sites. The use of antibiotics can be a factor that increases the risk of nosocomial infection with *Klebsiella* bacteria. Sepsis and septic shock can follow entry of bacteria into the blood (Cheli Riggs et al 2001, Sreenivasa Rao, P. and K.S.Parekh, 1981 and Sreenivasa Rao P. and Karmakar S.M (1988).

#### MATERIALS AND METHODOLOGY

In the present antibacterial study the marine algae *Ulva fasciata* was collected from the coast around Ennore Beach in Chennai, Tamil Nadu. The collected algae sample was identified by algal experts and was rinsed with water to remove epiphytes and necrotic parts. It was then rinsed again with sterile water to remove any associated debris. The algae after rinsing were dried carefully in shade under room temperature for 10 days and then immediately subjected to extraction. The algae after drying were weighed and then chopped and finely powered using a clean motor and pistle. The finely powered sample was weighed and 5 grams of sample was dissolved in various organic solvents, such as 80% Ethanol, Methanol and Acetone. It was kept for 48 hours at room

temperature and mixed at regular intervals. After 48 hours the sample dissolved in each solvent was filtered using Whatman filter paper to separate the filtrate for further use in antimicrobial testing of algal samples. Pure cultures of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae* were used as the test microorganism for antibacterial testing. Each bacterial strain was maintained in a nutrient agar slant. Slant of all the four microorganisms were prepared in nutrient agar media at a pH-7.2, and kept for incubation at 37°C for 24 hours. A nutrient agar slant without any bacterial strain was maintained as control. From the 24 hours incubated nutrient agar slant of each test organism a loop full of the microorganism was inoculated in nutrient broth at pH-7.4 so as to activate the bacterial strains used as test organisms. The broths were kept for incubation at 37°C for 24 hours so that the microorganism can grow till the log phase. A nutrient broth was maintained as a control without inoculating the test organisms. Antibacterial activity was assayed using the agar well diffusion test technique. For comparing the antibacterial activity of the isolated seaweed extracts with the therapeutic action of a number of known broad spectrum antibiotics, Antibiotic Disc Diffusion Test was done.

Standard antibiotics disc which were used are as follows

ü Nalidixic Acid	N30-30mcg/disc
ü Oxicillin	O10-10 mcg/disc
ü Bacitracin	B10-10Units/disc
ü Streptomycin	S10-10mcg/disc
ü Erythromycin	E10-10 mcg/disc
ü Chloramphenicol	C10-10mcg/disc

#### CONFIRMATION TEST

Screening of the algal extract was done for testing the antibacterial activity against the test microorganisms. It is done by allowing test organisms to grow in the respective selective media. In this confirmation test, agar well diffusion technique was done to obtain a sure result exhibiting the antibacterial activity of the seaweed extracts.

#### RESULTS

The seaweed sample was collected and the extract was tested against a range of microorganisms (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae*) for the presence of the antibacterial activity. In the present study, it is observed that methanol and ethanol were the best organic solution for the effective antibacterial material from the algae species used. The result exhibited by acetone was less than that exhibited by ethanol and methanol. The best halo-zone was produced in the ethanol extract of *Ulva fasciata*. The percentage of antibacterial activity observed for *Ulva fasciata* was highest. The experiment showed that the gram positive bacterial strain used as test organism was less effective compared to the gram negative bacterial strains. Among all the three gram negative bacterial strains, *Pseudomonas aeruginosa* and *Escherichia coli* were noted as the best Halo zone producers (Tables 1 – 11).

In the antibiotic disc diffusion test the best result were seen in *Escherichia coli* and *Staphylococcus aureus* plates. The zone of inhibition was 26mm and above 26mm in Ampicillin, Nalidixic acid and Chloramphenicol indicates very active antibacterial activity. In the ethanol and methanol extract of *Ulva fasciata* the measurement of zone of inhibition was above 26 mm which indicates that this extract can be used as an effective antibacterial agent against human pathogenic bacteria causing diseases. The Zone of inhibition of Bacitracin disc of *Escherichia coli*, Oxacillin disc, Nalidixic acid disc of *Pseudomonas aeruginosa*, Gentamycin disc, Ampicillin disc of *Klebsiella pneumoniae* were 10 mm and less than 10 mm indicates their least activity against the antibiotic.

#### CONCLUSION

*Ulva fasciata* or sea lettuce is commonly found in areas where nutrients are high, wave forces low and herbivory reduced. It is tolerant of stressful conditions, and its presence often indicates

**Table 1 : Activity of extract of the marine algae against the test bacterial strains**

Marine algae	<i>E.coli</i> (zone of inhibition in mm)	<i>P.aeruginosa</i> (zone of inhibition in mm)	<i>S.aureus</i> (zone of inhibition in mm)	<i>K.pneumoniae</i> (zone of inhibition in mm)
<i>Ulva fasciata</i>	<b>Good Result</b>	Average Result	Average Result	Average Result

**Table 2 : Zone of inhibition (in mm) of acetone, ethanol and methanol extract for green algae *Ulva fasciata* in MHA media**

Concentration of <i>Ulva</i> extract (in microlitre)	<i>E.coli</i> (zone of inhibition in mm)			<i>P.aeruginosa</i> (zone of inhibition in mm)			<i>S.aureus</i> (zone of inhibition in mm)			<i>K.pneumoniae</i> (zone of inhibition in mm)		
	Act	Eth	Met	Act	Eth	Met	Act	Eth	Met	Act	Eth	Met
100	-	16	17	13	13	-	8	8	14	-	-	-
150	-	-	18	15	15	-	11	10	-	-	-	-
200	-	-	20	15	17	-	15	12	-	-	-	-

Act - Acetone extract, Eth - Ethanol extract, Met - Methanol extract

**Table 3 : Zone of inhibition (in mm) of acetone, ethanol and methanol extract for green algae *Ulva fasciata* in Selective Media**

Concentration of <i>Ulva</i> extract (in microlitre)	<i>E.coli</i> (zone of inhibition in mm)			<i>P.aeruginosa</i> (zone of inhibition in mm)			<i>S.aureus</i> (zone of inhibition in mm)			<i>K.pneumoniae</i> (zone of inhibition in mm)		
	Act	Eth	Met	Act	Eth	Met	Act	Eth	Met	Act	Eth	Met
100	18	17	23	-	-	15	-	-	-	-	11	15
150	23	29	30	-	-	15	-	22	16	-	13	21
200	25	35	39	-	12	16	-	30	20	-	-	25

**Table 4 : Zone of inhibition for *Escherichia coli* in Disc Diffusion Test in MHA media**

Names of the Antibiotic discs used (mcg/disc)	Zone of inhibition (in mm)
Nalidixic Acid N30	26
Gentamycin G10	16
Ampicillin A10	19
Chloramphenicol C30	21
Bacitracin B10	10
Oxacillin Ox1	-

**Table 5 : Zone of inhibition for *Escherichia coli* in Disc Diffusion Test in Selective media**

Names of the Antibiotic discs used (mcg/disc)	Zone of inhibition (in mm)
Nalidixic Acid N30	23
Gentamycin G10	15
Ampicillin A10	23
Chloramphenicol C30	22
Bacitracin B10	8
Oxacillin Ox1	-



**Table 6 : Zone of inhibition for *Pseudomonas aeruginosa* in Disc Diffusion Test in MHA media**

Names of the Antibiotic discs used (mcg/disc)	Zone of inhibition (in mm)
Nalidixic Acid N30	6
Gentamycin G10	18
Ampicillin A10	-
Chloramphenicol C30	12
Bacitracin B10	-
Oxacillin Ox1	-

**Table 7 : Zone of inhibition for *Pseudomonas aeruginosa* in Disc Diffusion Test in Selective media**

Names of the Antibiotic discs used(mcg/disc)	Zone of inhibition(in mm)
Nalidixic Acid N30	5
Gentamycin G10	15
Ampicillin A10	-
Chloramphenicol C30	21
Bacitracin B10	9
Oxacillin Ox1	3

**Table 8 : Zone of inhibition for *Staphylococcus aureus* in Disc Diffusion Test in MHA media**

Names of the Antibiotic discs used (mcg/disc)	Zone of inhibition (in mm)
Nalidixic Acid N30	19
Gentamycin G10	25
Ampicillin A10	11
Chloramphenicol C30	20
Bacitracin B10	7
Oxacillin Ox1	-

**Table 9 : Zone of inhibition for *Staphylococcus aureus* in Disc Diffusion Test in Selective media**

Names of the Antibiotic discs used (mcg/disc)	Zone of inhibition (in mm)
Nalidixic Acid N30	22
Gentamycin G10	21
Ampicillin A10	35
Chloramphenicol C30	30
Bacitracin B10	15
Oxacillin Ox1	20

**Table 10 : Zone of inhibition for *Klebsiella pneumoniae* in Disc Diffusion Test in MHA media**

Names of the Antibiotic discs used (mcg/disc)	Zone of inhibition (in mm)
Nalidixic Acid N30	15
Gentamycin G10	9
Ampicillin A10	8
Chloramphenicol C30	20
Bacitracin B10	-
Oxacillin Ox1	-

**Table 11 : Zone of inhibition for *Klebsiella pneumoniae* in Disc Diffusion Test in Selective media**

Names of the Antibiotic discs used (mcg/disc)	Zone of inhibition (in mm)
Nalidixic Acid N30	20
Gentamycin G10	18
Ampicillin A10	-
Chloramphenicol C30	25
Bacitracin B10	-
Oxacillin Ox1	-



freshwater input or pollution. It is the first macroalgae to colonize newly opened substrate in intertidal areas with high nutrients. Their opportunistic success can be attributed to their simple morphologies and fecundity. Reproductive cells of *Ulva fasciata* have similar photosynthesis rates to adult vegetative cells with higher respiration rates. Ecologically successful green algae are potentially invasive. Coastal waters near harbors, industrial complexes and residential areas with nutrient rich and or fresh water input often have blooms of *Ulva* species that coat ship's hulls, cover pilings and shorelines, and restrict outflow pipes. The present research findings can form a pilot study to explore and discover the potential use of them in drug designing in the field of Pharmacogenomics.

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## Characterization of Bacteriocin Producing Probiotic Properties of *Enterococcus casseliflavus* MI001 Isolated from Curd Sample

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### Abstract

The identification of bacteriocin with a wide activity spectrum as a consequence of bacterial infections and spoilage microorganisms addresses an important aspect of food safety. In this study, the potential bacteriocin-producing bacterium was preliminarily confirmed as *Enterococcus* species and the isolate was identified as *Enterococcus casseliflavus* MI001. The probiotic properties of *E. casseliflavus* MI001 was studied for acid and bile tolerance tests. In addition, the aggregation and co aggregation ability of the strain to protect the host from colonization was studied. Further, the strain was also found antibiotic susceptible against commonly available antibiotics.

**Key words:** Bacteriocin; Probiotic; *Enterococcus casseliflavus*; Antimicrobial activity.

### Introduction

Bacteriocins are gene-encoded ribosomally synthesized peptides produced by lactic acid bacteria which are inhibitory to other gram positive bacteria (1). Bacteriocins have varying inhibitory spectra, as well as its antagonistic activity is of significant interest for their use in food preservation. Considering consumers growing interest in natural products, reduction in intensity of physical treatments, satisfying the consumer needs for foods is the one possible way to replace the chemical preservatives (2). Humans have been using lactic acid bacteria for fermentation and preservation of various food products for major

health benefits. Lactic acid bacteria produces several metabolites which includes antimicrobials and flavoring agents that augment the stability and aroma of the food products (3). Enterococci are lactic acid bacteria (LAB) represent an important component of the bacterial flora of fermented foods as well as dairy products manufactured from milk (4). One special benefit of enterococci is production of antimicrobial compounds called as enterocins. Enterocins have become attractive in recent years as natural preservatives of food because they have broad spectrum of activity against food borne pathogens such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *Escherichia coli* (5). Great attention towards bacteriocins have gained due to their potent inhibitory effect, safe use for humans, stability and various modes of action and no resistance towards bacteriocins guarantee the safety of foods (6,7). Therefore, studies regarding the bacteriocinogenic potential of strains are showing increasing attention for natural way of food preservation. A few strains of enterococci also showed probiotic effect which help in colonization as well as antimicrobial effect against the pathogens reside in the gastrointestinal tract of the host. Thus, the search for bacteriocins producing probiotic organisms has been extended. In this study the bacteriocin producing strain was isolated from fermented milk and its probiotic property was evaluated.

## Materials and methods

**Proteolytic nature of bacteriocin:** Proteolytic enzymes such as trypsin, proteinase K, papain and bromelain were used to evaluate the peptide nature of the cell free supernatant. Enzymes were added to cell free supernatant at a final concentration of 1.0 mg/ml. The inhibitory activity against the selected indicator organisms was tested after incubation for 2 h at 37 °C. The cell free supernatant without the enzyme served as control (8).

## Morphological, physiological and Biochemical characterization of the bacterium:

The morphological and physiological parameters of selected isolate (SC1) were studied using MRS broth at different temperatures between 10 and 45 °C, pH 2.5 and 10.5 and salt concentration 2 and 20% (9). Various biochemical tests were performed to evaluate the characteristic profile of the selected isolate (10).

## In vitro Probiotic properties of bacteriocin producing strain

**Acid and bile tolerance test :** For acid and bile tolerance tests, overnight grown culture of selected strain centrifuged at 12,000 rpm for 15 min. The pellet was washed and resuspended in 10 ml of PBS (pH 7.0). Then, 1% (v/v) suspension was inoculated into modified MRS broth adjusted to pH 2, 3, 4 and 7 (control), and another set with different concentrations of bile salt i.e., 0.1%, 0.25%, 0.5% and 1.0% and were incubated at 37 °C for 4 h to determine the number of viable cells (11). *L. fermentum* was used as a control.

**Antibiotic susceptibility test :** The antibiotic susceptibility test was carried out according to Pisano et al. (12). The overnight grown culture of *Enterococcus* and *L. fermentum* was inoculated as a lawn on Muller Hinton agar plates. Various antibiotic discs such as Ampicillin (10mcg), Erythromycin (15mcg), Kanamycin (30mcg), Streptomycin (10mcg), Chloramphenicol (30 mcg), Penicillin (10 units), Amoxicillin (30mcg), Tetracycline (10mcg) and Ceftriaxone (30 mcg) were placed on the surface of agar. Plates were incubated at 37 °C for overnight and zone of

inhibition was observed. The results were expressed in terms of sensitive or resistance to antibiotics.

## Auto aggregation assay and Co-aggregation assay :

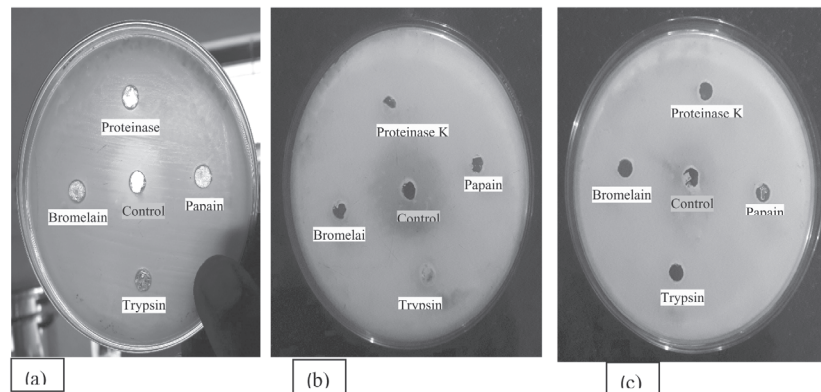
*Enterococcus casseliflavus* and *L. fermentum* (control) were grown at 37°C in MRS broth and pellet was collected by centrifugation. The bacterial suspensions were made by suspending pellet in PBS (pH 7.0) and incubated at 37 °C for 24 hrs. At different time intervals (2, 4, 6, 12 and 24 hrs) absorbance values were taken at 600 nm and auto aggregation percentage was measured (13, 14). For co-aggregation assay, absorbance (OD at 600 nm) of the suspensions was adjusted to 0.16±0.05 nm to standardize the concentration of all organisms. 1.0 ml cell suspensions was mixed with 1.0 ml of various suspensions of indicator organisms as mentioned in Table 1. The percentage co-aggregation ability was measured after 4 hrs of incubation at 37 °C (13).

## Results and discussion

The cell free supernatant (CFS) treated with proteases was also tested for antimicrobial activity and found no zone of inhibition (Figure 1) which depicts that the CFS containing bacteriocin is proteinaceous in nature. In a recent study, the bacteriocin produced by *Enterococcus faecalis* 478 treated with protease reported the proteinaceous nature of cell free supernatant (6).

## Morphological, physiological and biochemical characterization of Enterococcus casseliflavus :

The morphological features of isolate SC1 were found to be cocci shape, gram-positive and catalase negative and showed good growth in MRS broth over wide range of temperature, pH and salt concentrations between 15 and 45 °C, pH 4.5 and 8.5 and salt concentration 2 and 10%. The morphological and physiological features were represented in table 1. It was reported that the most of *Enterococci* are tolerant to extreme environmental conditions such as temperature, pH and salt concentration (15, 16). The biochemical profile of the isolate SC1 and fermentation of various sugars such as glucose,



**Fig. 1.** Screening of CFS against proteolytic enzymes to confirm proteolitic nature of bacteriocin against (a) *P.aeuriginosa* (b) *S.aureus* and (c) *E.coli*. CFS= cell free supernatant.

mannose, fructose, galactose, lactose, maltose, ribose and sucrose were represented in table 2. Earlier, a similar report on the biochemical profile of the *Enterococcus* species was studied and compared with that of other species in the same genus (17). Based on the characteristic features it was observed that the newly isolated strain SC1 was grouped under the genus *Enterococcus* (18). In the previous studies many authors reported that the *Enterococcal* strains had been used as probiotics as starter cultures, preservation of foods and dairy products formulation (19-21).

#### **In-vitro probiotic characteristics**

##### **Acid tolerance and Bile salt tolerance**

Survival of probiotic bacteria under stressful conditions in *in-vitro* had been studied over the last decade. The beneficial effects of novel probiotics are strain dependent (22). Many studies have reported that *in-vitro* and *in-vivo* studies vary from strain to strain such that a few probiotic strains adhere better to small intestine, while others adhere specifically to large intestine. The acidic conditions of stomach digestive enzymes work well at low pH and they are the influencing factors for the survival conditions of bacteria which enter through the oral route. The primary characteristic features of probiotic are acid and bile tolerance. In the present study, isolated strain, *E. casseliflavus* MI001 showed tolerance at pH

2.0 for 2 hrs and was observed a little increase in number of viable cells at pH 3.0 during the course of incubation for 4 hrs, which further suggested that the strain can tolerate and remain viable at acidic pH 3.0 (Fig. 2a). In case of control strain *L. fermentum*, the viable cell count was increased at pH 3.0 after 4 hrs of incubation (Fig. 2b) (23). Based on the results represented in figures 2(a) & 2(b) the bacterium *E. casseliflavus* MI001 can tolerate even at low pH conditions in the stomach. Tolerance to bile salts is a desirable property of the probiotic bacteria for colonization and metabolic activity in the intestine. The concentration of human bile ranges from 0.3% to 0.5% and it reduces the survival ability of the bacteria in the intestine. The bile salt tolerance pattern of *E. casseliflavus* MI001 and *L. fermentum* was represented in figure 3 (a) & (b). It was observed that there was an increase of viable cell count ranged from 0.1% to 1.0% bile concentration for *E. casseliflavus* MI001. In recent study by Garcia et al [30] the *L. fermentum* UCO-979C strain showed resistance to 2% bile salts. Hossieni et al (23) also reported that the enterococcal species tolerated 2% of bile salt concentration.

**Antibiotic susceptibility :** Antibiotic susceptibility is considered to be one of the key safety aspects for probiotic strains which lack antibiotic resistance. The selected isolate MI001



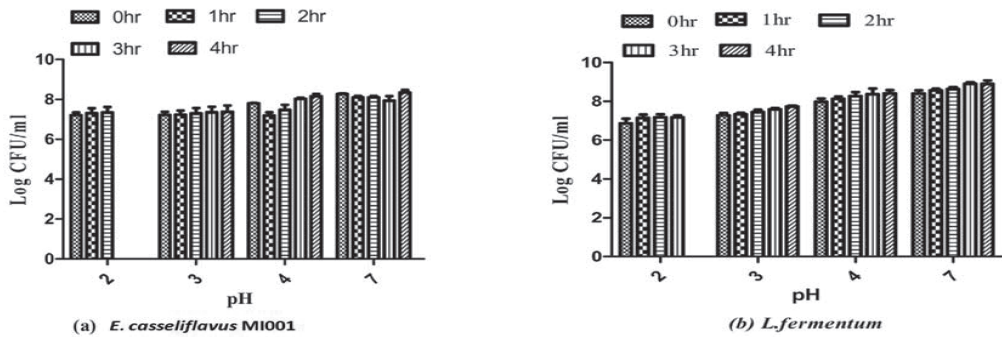


Fig. 2. Acid tolerance of (a) *E. casseliflavus* MI001 and (b) *L. fermentum* after exposure to acidic conditions (pH 2.0, 3.0, 4.0 and 7.0) during 4 hrs incubation.

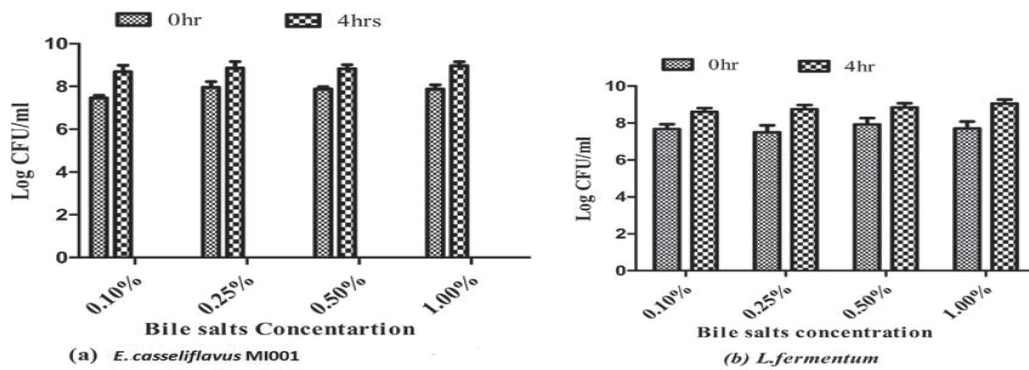


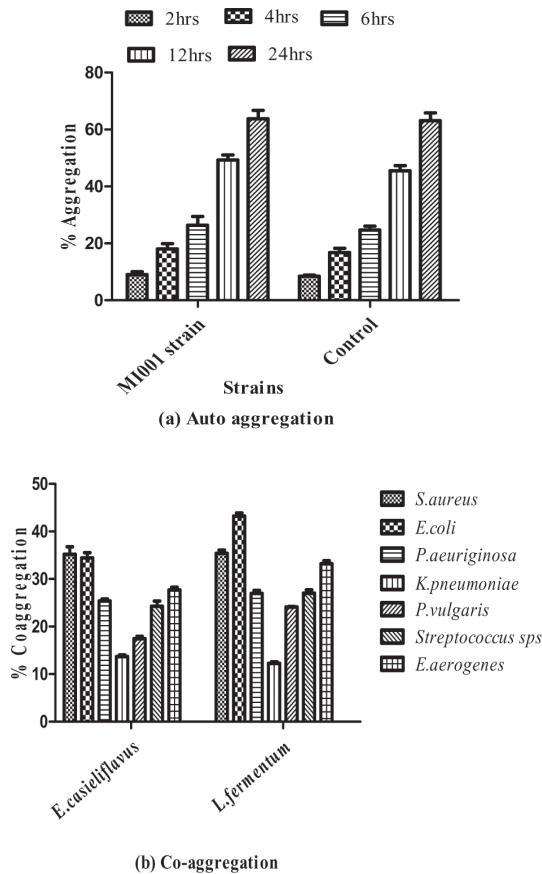
Fig. 3. Tolerance of (a) *E. casseliflavus* and (b) *L. fermentum* to bile salt concentrations varying from 0.1% to 1.0% after 4 hrs incubation.

Table 3 - Antibiotic susceptibility test for *E. casseliflavus* and *L. fermentum*.

Antibiotic	Concentration of antibiotic (mcg/units)	<i>Enterococcus casseliflavus</i>	<i>L. fermentum</i>
Ampicillin	10	S	S
Erythromycin	15	S	S
Kanamycin	30	S	S
Streptomycin	10	S	S
Chloramphenicol	30	S	S
Penicillin	10	S	R
Amoxicillin	30	S	S
Tetracyclin	10	S	S
Ceftriaxone	30	R	R

S =Susceptible; R= Resistance





**Fig. 4.** Comparison of (a) Auto-aggregation (b) Co-aggregation ability of *E. casseliflavus* and *L. fermentum*

and *L. fermentum* were subjected to various antibiotics represented in table 3. The present study demonstrated that the strain *E. casseliflavus* showed susceptibility to all antibiotics. This indicated that the *Enterococcus casseliflavus* has low pathogenic potential and it was found strain specific. In the previous study also among all *Enterococcus* species, *Enterococcus casseliflavus* infections were rarely reported (24). The control strain *L. fermentum* also reported resistance to both penicillin and ceftriaxone. Gracia et al (25) reported that the strain *L. fermentum* developed resistance to penicillin antibiotic. High level antibiotic resistance was reported for hospital isolates of

**Table 1** - Morphological and physiological characterization of the strain, *Enterococcus casseliflavus* M1001.

Characteristic feature	<i>Enterococcus casseliflavus</i>
<b>Morphological features</b>	
Cell form	Coccus
Gram staining	Positive
Spore staining	Spore former
<b>Physiological features</b>	
Growth at different Temperatures	
10°C	-
15°C	+
25°C	+
30°C	+
35°C	+
37°C	+
45°C	+
<b>Growth at different pH</b>	
2.5	+
4.5	+
6.5	+
8.5	-
10.5	-
<b>Growth at different salt concentration</b>	
2%	+
4%	+
6%	+
8%	+
10%	+
20%	-

**Table 2-** Biochemical characterization of *Enterococcus casseliflavus* MI001

Biochemical characteristic feature	<i>Enterococcus casseliflavus</i>
Catalase production	-
Indole test	+
Methyl red test	+
Voges-proskauer test	-
Citrate utilization test	-
Starch hydrolysis	+
Lipid hydrolysis	-
Urea hydrolysis	-
<b>Carbohydrate utilization profiles</b>	
Sucrose	+
Mannose	+
Fructose	+
Lactose	+
Rhamnose	-
Xylose	+
Dextrose	+
Mannitol	+
Sorbitol	-
Maltose	+

*Enterococcus* species and this resistance varied at species level (26). Identifying the non-expressed safety risk factors such as resistance genes is a criterion for evaluating the probiotic potential of the bacteria (27).

**Auto-aggregation and Co-aggregation :**

Aggregation is a reversible phenomenon of gathering of bacterial cells which belong to the same strain and it is a prerequisite for an organism considered to be a probiotic. Self aggregating bacteria form biofilms to protect the host from colonization by pathogenic organisms. In this study the aggregation ability of *E. casseliflavus* showed 63.7% followed by control strain *L. fermentum* 63.1% represented in figure 4(a). The aggregation ability is because of proteins present in the culture supernatant and aggregation substances and polysaccharides like EPS (28). In previous study reported by Todorov et al (29)

that the *Enterococcus* species *E.mundtii* ST4V showed 41.34% aggregation. Further, the work reported by Katarina et al (30) showed AggE is an aggregate protein present in the *Enterococcus faecium* BGG09-28 responsible for aggregation and biofilm formation. Various degree of co-aggregation was observed when *E. casseliflavus* and control were paired with indicator organism's represented in figure 4 (b). *E. casseliflavus* showed co-aggregation ability with all strains and less towards *K. pneumoniae* (13.6%). Among all, the highest activity was observed for *S. aureus* (35.2%) and *E. coli* (34.4%). The control strain *L.fermentum* showed highest coaggregation ability with *E. coli* (43.2%). The ability of the bacteria to co-aggregate with pathogens enables them to form a protective barrier which prevents the colonization by pathogenic strains (11).

**Conclusion**

The potential bacteriocin producing bacterium was isolated from household curd sample and was identified as *Enterococcus casseliflavus* MI001 strain through biochemical and molecular characterization. Further, the probiotic characterization studies showed that the identified strain did not show any pathogenic properties and found the ability for bacteriocin production. Furthermore, the bacteriocin production, aggregation and co-aggregation abilities help the strain for establishment and competition in the gastrointestinal tract. Thus, *E. casseliflavus* MI001 was found as a promising alternative strain for commercial applications and production of probiotics.

**Conflict of interest :** None declared

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## Diphtheria: An overview of diagnosis and therapeutic applications

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### Abstract

Diphtheria is a dreadful disease caused by *Corynebacteria diphtheria* by secretion of diphtheria toxin by the presence of bacteriophage tox gene. It is one of the contagious diseases with a mortality rate of 5% to 10% worldwide. The disease resurgence in recent years alarmed the world as the numbers of cases are increasing in adult population due to low immune response towards diphtheria. Expanded diphtheria vaccination programs have been introduced in developing countries for more coverage and administration of booster doses to increase the immunity, though sporadic cases of *Corynebacteria diphtheria* are observed worldwide causing harmful effects. Emphasis was focused on the epidemically circulating *Corynebacteria diphtheria* for signs and symptoms of rapid diagnosis and treatment. Shift in the age group distribution of diphtheria and changes in the epidemiology of developing countries in the vaccine era led to strengthening of laboratory diagnosis. Rapid methods were developed for reliable and simple diagnosis in treatment of disease. Here, we describe the current laboratory approaches leading to diagnosis of diphtheria and prophylaxis.

**Key words:** Monoclonal antibodies, Diphtheria, Laboratory diagnosis.

### Introduction:

Diphtheria is a contagious disease that causes the mortality worldwide (1). Mass vaccination and awareness of the disease was brought control over diphtheria infection. However,

outbreak of diphtheria in the late 1980's in Soviet Union caused panic and alerted the world, once which was considered a neglected disease(2). Since then few incidences are being noticed in endemic countries. From well developed nations to developing countries, the rate of infection was a serious concern as its prevalence was found to be more in children below five years of age and in adults with lower immunity (3, 4) and spread of diphtheria in different countries as shown in Table 1.

The diphtheria epidemics that have occurred over recent decades made the clinicians, laboratory scientists and epidemiologists in different parts of the world to recall old lessons and develop new methods for microbiological diagnosis, prevention, control and treatment of diphtheria (5,6). In developing countries, in which the incidence of diphtheria remains relatively high and vaccination coverage continues to be insufficient, laboratory support in the light of deficiencies. Hence it is essential to comprise accurate, rapid, and economical and easily performed diagnostic tests for diphtheria cases caused both by *C. ulcerans* and *C. diphtheria*.

Diphtheria is caused by an exotoxin released by the gram positive bacteria *Corynebacterium diphtheria* (7). The toxicity of the bacteria is caused by the expression of tox gene in the chromosome and not by the presence of inheritance but by the phage movement into the bacteria (8). The presence of non-toxigenic strains does not cause outbreak but the infection may be severe and fatal



which includes septic arthritis and osteomyelitis (9) and involves the antibiotic treatment if the patient is symptomatic (10, 11, 12).

*Corynebacterium* species are gram positive, non-motile rods, often with clubbed ends, occurring singly or in pairs and their size is in between 2-6  $\mu\text{m}$  in length and 0.5  $\mu\text{m}$  in diameter. They group together and forms a "V", "palisades", or "Chinese letters"(13). The potentially toxigenic *Corynebacterium* comprises of *C. diphtheriae*, *C. pseudotuberculosis* and *C. ulcerans*. *C. diphtheriae* consists of four bio- variants as gravis, mitis, intermedius and belfanti. The presence of *C. intermedius* was found to be sparse than other types (14) and the bio- variants are being identified based on their growth and colony morphology (15).

**Laboratory diagnosis:** There is a need for rapid diagnosis for *C. diphtheria*, where clinical awareness is less, physicians were not properly experienced and proper diagnosis delayed due to lack of facilities and negligence of disease. Moreover, in patients with milder symptoms (pharyngitis without "pathognomonic" pseudo-membranous or with cutaneous diphtheria), establishment of the definite diagnosis might be achieved only by the detection of a toxigenic *Corynebacterium* species. Different tests are being performed for screening and identification of *Corynebacterium spp.* include gram staining, blood agar culture, primary media containing tellurite, pyrazinamidase activity, selective media containing cysteinase, urease hydrolysis, nitrate reduction, fermentation of Hiss serum water sugars and biochemical characterization using API Coryne kits (Biomerieux). Other assays used to detect toxin production includes the passive hemagglutination, *in vivo* test, and Vero cell based assay. Molecular epidemiology of *C. diphtheriae* includes ribotyping, PCR typing, pulsed field gel electrophoresis, single stranded conformational polymorphisms (SSCP) of the tox gene, and amplified fragment length polymorphisms (AFLP)(6).

Molecular techniques like PCR helps in detecting the presence of tox-bearing *C. diphtheria*

(16, 17) and also *C. ulcerans* strains which are unable to produce active diphtheria toxin (18). The first step in diagnosis of *cornyobacteria* is the cultivation of microorganisms in the respective blood agar or tellurite media (19). Tellurite media acts as a selective or differential media for the growth of colonies of *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* with grey/black colonies surrounded by brown hallow after overnight incubation. Some strains of *C. diphtheria* are sensitive to potassium tellurite.

Many conventional biochemical tests are available for the identification of *C. diphtheria* like reduction of nitrate, hydrolysis of urea, pyrazinamidase activity and fermentation tests like glucose, sucrose, maltose and urea. Most potential bacteria tend to be pyrazinamidase negative, nitrate positive (Except belfanti) and cysteinase positive and ferment glucose, maltose and starch(Figure 1).

**Enzyme Linked Immunosorbent Assay (ELISA):** ELISA is the method of choice for measuring the serum anti- toxin levels and in identification of classification of antibodies (20). This method is a simple, cost effective and ease to perform and it can be automated. ELISA assays are being used frequently for the detection of diphtheria toxin A and B and its sensitivity and specificity levels were found to be higher (21). Direct ELISA tests are highly repeatable and easy to perform (22, 23). These methods can be used to measure the presence of bacteria or the toxin and also used for checking protective titer and potency in immunized guinea pigs. When the antibody level is above 0.1 IU/ml, the results of the ELISA test correlate well with the *in vivo* neutralization test carried out in guinea-pigs (24) and the neutralization test performed in tissue culture (23).

**Elek Test:** The *in vitro* method for detection of diphtheria toxigenicity is the Elek immunoprecipitation test which indicates presence of a biologically active protein (25, 26). As *in vivo* test was considered as the standard method for toxicity testing as described previously

(27). The limitations with the in-vivo test involving usage of animals, time taking and high costing made the replacement with in-vitro test. Basically, this test is performed with the *corynebacterial* colonies, plated on agar in a 90 degree angle towards either a trench cut into the agar containing immune serum (28). Subsequently, diphtheria toxin produced by toxigenic *corynebacteria* and antitoxin diffuses into the medium and finally interacts with each other. After 24 to 48 h of incubation, diphtheria toxin and antitoxin immune complex precipitates become visible to the naked eye, the precipitin lines were described as fine "moustache-like streaks" or "arrow-heads" virtually marks the toxigenic strains(29). The position of the precipitin lines and consequently the readability of the test depend on the initial concentration and the diffusion velocity of the two reacting substances. Therefore, issues influencing both the sensitivity and the specificity have been experimentally addressed in several studies aiming to optimize the original protocols like type of medium and its components, distance between colony streaks and anti-toxin source density of the bacterial inoculum, agar thickness, anti-toxin quality and concentration, time needed to obtain a definite and reliable result, etc. Modifications were made in the media and methodology to overcome the limitations in the test and improved the accuracy(30). Modified Elek test with optimized distance between the disc and the anti-toxin reduced the time for the formation of precipitin bands. The time of test result was reduced from 48 hours to 16 hours and several colonies can be screened. These advantages of the Elek test made the implementation around the world as a diagnostic tool (31,32).

**Polymerase chain reaction (PCR):** Genotyping using PCR is having several advantages as it is simple, rapid and easy to interpret the results and a widely used technique worldwide (16). However, it does not indicate the secretion of tox gene which acts as an adjunct for disease diagnosis. An Elek test is a time consuming and is not used worldwide due to lower incidence of diphtheria and lack of expertise and availability of anti-toxin. The

detection of tox gene in *Cornybacteria* by PCR method was validated in correlation with the biological methods and the results were in complete agreement as reported earlier (33). The results are provided in few hours that can help to initiate further preventive measures. The PCR were found to be not recommended as a single test for toxigenicity as it depicts the presence of toxin gene but not the toxin expression. Though some isolates possess the toxin gene and are unable to express the protein as described earlier (34, 35, 36).

**Real time polymerase chain reaction (RT-PCR):** Quick and reliable tests are needed for diagnosis of the disease and real time PCR is more sensitive test with high precision. It is one of the rapid method with high accuracy and specific when compared to the conventional PCR. This can detect upto 2 CFU and exhibits 10-fold greater sensitivity than the traditional PCR results (37). Many methods of PCR were switched to real time PCR as it has several advantages over it and is a reliable tool where the PCR is unable to detect. Thus rapid identification of domestic and imported cases in lower time leads to its usage in place of existing standard PCR (38). Only lower amount of DNA two or three copies is sufficient to detect diphtheria tox gene (33). Having higher sensitivity of 25 times than PCR, Real time PCR is more advantageous for the recognition of diphtheria tox gene when Elek test failed at lower sensitive levels (39). It can be used to detect and differentiate *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis* and fragments of their toxin gene instead of multiple PCR assays. The presence of tox gene can be identified by the test but the expression of tox gene can be diagnosed by Elek test. Hence it acts as an adjunct tool in the diagnosis of diphtheria.

**Enzyme Immunoassay:** The limitations associated with the PCR technique, EIA (Enzyme immunoassay) is being performed for the diagnosis of diphtheria. This method uses the equine polyclonal as a capture and monoclonal antibody conjugated with alkaline phosphatase as a detection which is specific to fragment A of diphtheria toxin (40). It is an easy, rapid and

sensitive method for detection of diphtheria toxin and correlates well with Elek test. It is one of the simple method (Ex. Tellurite agar and Tinsdale agar) for the determination of diphtheria toxin (41).

**Schick test:** This is an old method used as a standard procedure for estimating the diphtheria antitoxin serum levels or immunity to diphtheria (42). It is a simple test to differentiate the candidate from susceptible to those who have protective levels by administering lower dose of diphtheria toxin. This test was performed by giving an intradermal injection and susceptible patients develops an erythema. It is a frequently used test and results can be interpreted within 24 to 36 h. Minimum lethal dose of diphtheria toxin was injected intradermal to forearm to humans for their response (43). If the inflammation is observed and with no anti-toxin levels and persists for 3 to 4 days is an indication of positive reaction. A control test is arranged on the other arm with heat inactivated diphtheria toxin. This is a simple test and correlates with the serum anti-toxin levels. The major disadvantages of this test involves an intradermal injection at two sites on the arm and the inflammation will persist for 24 to 36 hours. The protective antibody titers in humans were usually tested by Schick test instead of direct measurement of anti-diphtheria antibodies in serum (44, 45).

**In vitro neutralization test:** *In-vitro* methods are specific, reproducible and sensitive (46) when compared to *in vivo* tests. The detection level for active diphtheria toxin is estimated to be about 10 to 100-fold higher in *in vitro* cytotoxicity assays (47). *In-vivo* diphtheria toxin detection is the conventional "gold standard" for toxigenicity testing and laborious, depends on animal usage like rabbits and or guinea-pigs (16, 48, and 49). International regulatory authorities and animal ethical committees are recommending the replacement of existing *in vivo* tests with *in-vitro* tests which are more specific and sensitive (50, 51).

WHO emphasized the use of Vero cell assay for testing the potency of diphtheria toxoid

(52). *In vitro* cytotoxicity assay allows the detection of active diphtheria toxin in which Vero cells were added to the sera which was incubated with diphtheria toxin. The assay helps in detecting the presence of low traces of diphtheria toxin in bulk diphtheria toxoid samples used for vaccine preparation. Change in color from red to yellow is the identification of neutralization. Cell types commonly used for cytotoxicity testing are Vero cells (31), human heteroploid HeLa cells, and to a lesser degree Chinese hamster ovary (CHO) cells and rabbit kidney cells.

**Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS):** MALDI-TOF MS is a new technology for species identification based on the protein composition of microbial cells (53). MALDI-TOF MS technique is useful for final species differentiation of clinically relevant *Corynebacteria*, and also as a screening tool for *Corynebacterial* colonies for toxigenicity testing. It can also distinguish closely related *Corynebacterium* colonies as reported previously (54). This new technology helps in identification and evaluation of toxigenic *Corynebacterium*. Moreover, this technique is fast, reliable and easily diagnosed method that helps in quick management and protection.

**Typing Methods for *C. diphtheria*:** Typing techniques are useful in identifying a disease causing bacteria during a local outbreak by exploring the strain identity of different isolates and also for monitoring of clonal similarity and structure of endemically or epidemically spreading strains. Distinguishing between epidemic, endemic or imported cases can help to timely implement and reconsider adequate preventive measures by public health authorities.

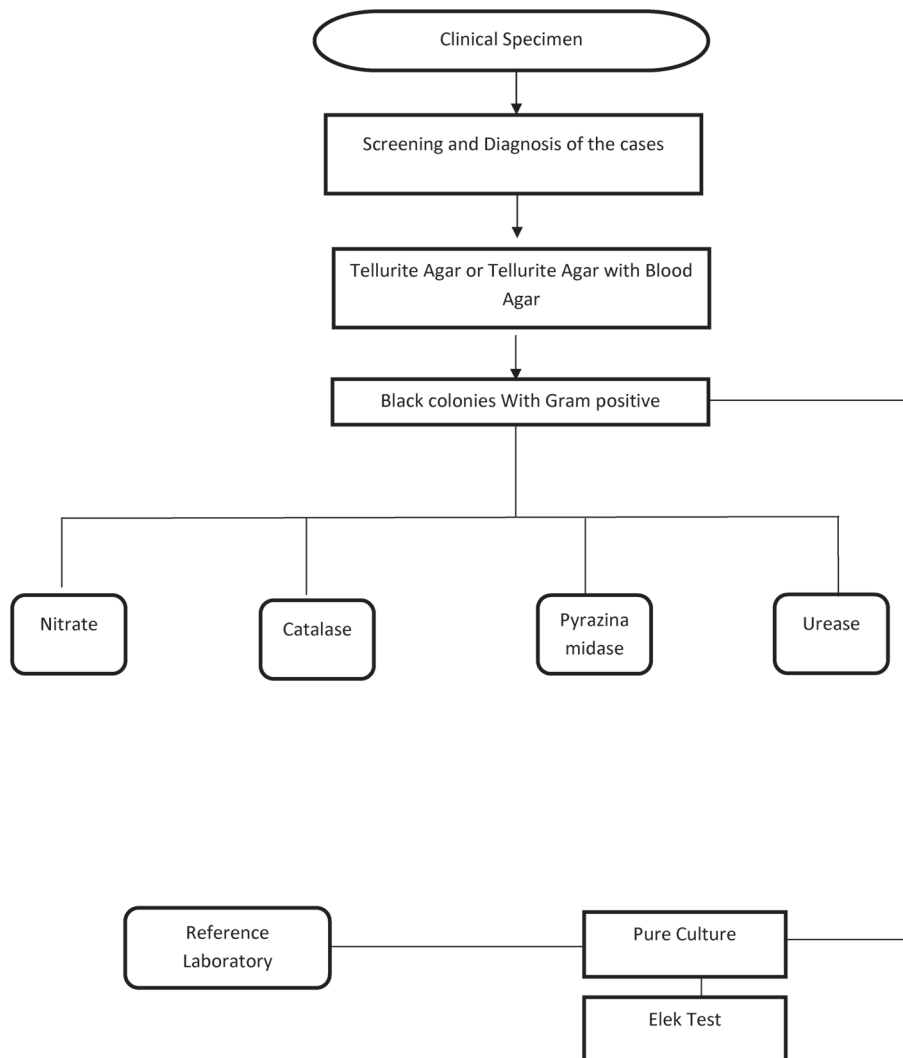
Molecular typing techniques are better for the serotyping, a phage typing and for epidemiological surveillance of diphtheria outbreaks and long-term persistence of *C. diphtheriae* strains in the population. Ribotyping technique for the typing of *C. diphtheriae* strains is laborious and performed with radiolabelled

riboprobes. Pulsed-field gel electrophoresis (PFGE), on the other hand, is the method of choice for typing most of the bacteria. Molecular typing techniques, including amplified fragment length polymorphisms (AFLP) (6), random amplified polymorphic DNA (RAPD) (55,56), multilocus enzyme electrophoresis (MEE) (57), spoligotyping (58) and pulsed-field gel electrophoresis (PFGE)

(59) shown significant interspecies genetic diversity.

**Antibodies and its application:** The fatality of the diphtheria was rapid and its incidence is being increased due to negligence of disease. Delay in the diagnosis causes more fatal and may lead to death. The current treatment involves the application of diphtheria anti-toxin and with the

Figure 1: Algorithm for Diphtheria Diagnosis



Diphtheria: An overview of diagnosis and therapeutic applications

**Table 1:** Countries where diphtheria circulates in the population

Africa:	Algeria, Angola, Egypt, Niger, Nigeria, Sudan and sub-Saharan countries
Americas:	Bolivia, Brazil, Colombia, Dominican Republic, Ecuador, Haiti and Paraguay
Asia/South Pacific:	Afghanistan, Bangladesh, Bhutan, Burma (Myanmar), Cambodia, China, India, Indonesia, Laos, Malaysia, Mongolia, Nepal, Pakistan, Papua New Guinea, Philippines, Thailand and Vietnam
Middle East Europe:	Iran, Iraq, Saudi Arabia, Syria, Turkey, Yemen, Albania, Russia and countries of the former Soviet Union
(Source: CDC, 2008)	

anti-microbial therapy to prevent the secretion of toxin by *Cornybacteria* diphtheria. Emil von Behring won the first Nobel Prize for medicine in 1901 for his work on “Serum Therapy in Therapeutics and Medical Science” where he noted the importance of early use of diphtheria serum in order to achieve successful “detoxication of the bacillus poison”. Equine immunoglobulins collected from the hyperimmunized animals were used as an alternative for drugs in treating diphtheria (60). The protective role of anti-sera against the toxin was documented by Behring in the late nineteenth century and the use of diphtheria antiserum raised in horses to treat humans (61). The diphtheria anti-toxin (DAT) binds to circulating toxin and blocks it from binding to the tissue and not the toxin which already attached to tissue. The equine polyclonal anti-serum which is used as DAT causes serum sickness or allergic reactions to the recipient (62). Several countries stopped manufacturing their own DAT supplies following the introduction of mass vaccination in 1940s/50s and the consequent decline in diphtheria cases. The reasons behind decreased supply of DAT are probably multifactorial, reduced demand, purity standards and high regulatory requirements for the safe manufacture of blood-derived products (63). Another strategy is to use the serum from the patients who were vaccinated or recovered from diphtheria. It has less side

effects than horse serum and can be used for treatment in future (64).

Neutralizing monoclonal antibodies (mAbs) represent a promising alternative to polyclonal products and countries would benefit greatly from their replacement (65). The use of mAbs could circumvent limitations arising from the production of antiserum, including limited supply, high manufacturing cost, risk of hypersensitivity reactions associated with equine sera and potential risk of contamination in blood-derived products and public averse towards the horse serum (66). The discovery of potent neutralizing antibodies against the diphtheria toxin holds promising as a potential therapeutics. Several human monoclonal antibodies developed were in pre-clinical stages (67).

Monoclonal antibodies have potential in diagnostic assays and wide usage in determining the toxin in clinical isolates by fluorescence Tag or coupled with HRPO. An alternative and more rapid phenotypic method to detect diphtheria toxin were available in the late 1990’s (68). However, production of low economic profitability diagnostic kits for diphtheria toxin like immunochromatic strips development were halted. It would thus be fortuitous if a similar robust and simple diagnosis tool could be revisited for the phenotypic detection of diphtheria toxin. Other immunological assays



including agglutination (69,70,71), counterimmuno-electrophoresis (72), dot immunobinding methods (73) are being used to detect DT from *Corynebacterium* isolates or from clinical samples.

#### Conclusion:

Diphtheria is an endemic and communicable disease in humans and the large vaccination has led to dramatic decrease in the spread of the disease. Globalization with rapid development and change in the climate, more endemic in tropical or temperate zones, poor hygienic conditions had caused spread of disease. Currently major incidence is being reported in adults and child population. Negligence in the diagnosis causing a severe implication with spread of the disease and most of the under developed countries are lacking proper healthcare infrastructure to eradicate the disease. Circulation of diphtheria throughout the world remains a threat to the developing and emerging countries and need to be vaccinated with booster doses with proper coverage in children and adults. The success in preventing the disease would be possible with high level of clinical awareness of diphtheria, proper identification and strengthening the laboratories with rapid detection tools.

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## **A review on chloro substituted marine natural product, chemical examination and biological activity.**

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### **Abstract**

The present review describes the marine chlorosubstituted natural products and their pharmacological properties *viz.* biological activity, anti-bacterial, antitumor, anti-parasitic, anti-fungal, anti-viral, anti-inflammatory, antioxidant and enzymatic activity. Typical methods of isolation of marine compounds are applied by extensive chromatographic techniques to isolate these compounds which yield in milligram quantities. Further the characterization and structure determination involves spectrometric techniques like FT-IR,  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$ , NOE, HMBC and etc. Although biological activity is assessed for most compounds by extracts a practical drug or lead molecule is yet emerge.

### **Introduction**

Till date more than 5000 halogenated natural products are found in sea water. In nature sea water is saline and rich in chloride, bromide and iodide ions. The plants marine organism, insects, mammals and biosynthetic natural processes produce halogenated marine natural products. The number of known organohalogenes has increased dramatically since 1968. Halogenated molecules a natural product in which The carbon-halogen bond is formed by enzymatic, thermal and other natural process. These natural processes constantly occur in the oceans, marine organisms soil atmosphere and terrestrial plants. Chlorinated hydrocarbons, phenols, and other

chlorinated compounds including dioxins and Chlorofluorocarbons (CFCs) found in the atmosphere due to industrial pollution, smog exhaust fumes or due to pesticides and industrial chemicals. We found these chloro compounds contaminates in atmosphere, air, water, soil and in our food compounds as contaminates are mostly carcinogenic.

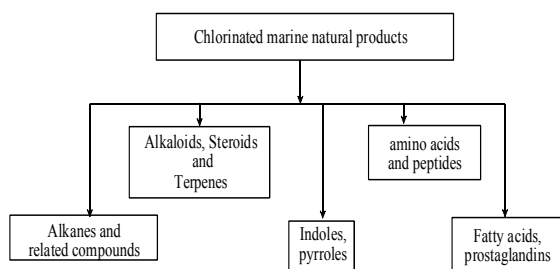
The halogen compounds alter the physical properties including electronic and steric effects determine the affinity and selectivity of interactions with biological targets. Natural halogenated compounds possess a variety of antibacterial, antifungal, antiviral, anti-inflammatory, antiproliferative, cytotoxic, antifouling, antifedant, ichthyotoxic, and insecticidal activities and are widely used in medicine and agriculture.

Secondary metabolites from marine organisms greatly differ from those of terrestrial sources both in structure and bio-activity. Chloro metabolites are the most predominant modification found in nature, followed by bromo metabolite, while iodination and fluorination are very less in nature. The functions of chloro metabolites have distinct physiological or biochemical roles, for example 2,6-dichlorophenol as a sex-pheromone, while 4-chloroindolyl-3-acetic acid is a plant growth hormone. Several chloro metabolites in marine organism have a defensive role and a number of chlorometabolites isolated from bacteria and fungi have antibiotic activity, for example

chloramphenicol and chlortetracycline. All chlorine containing organic molecules are xenobiotic that chloride does not participate in biological processes. Later, researchers have revealed that chloride plays an active role in complex biogeochemical cycle. The present review focuses on few chlorinated molecules and their biological activities found in marine natural products.

### Occurrences

Naturally marine chlorinated compounds occur in different class of organic compounds which exhibit various promising biological activities such as antibacterial, antitumor, antiparasitic, antifungal, antiviral, anti-inflammatory, antioxidant, and enzymatic activity. The nature of class of compounds observed is alkaloids, steroids, terpenes, indoles, pyrroles, aminoacids, peptides, aliphatic and aromatic compounds etc as shown in Figure 1 below.

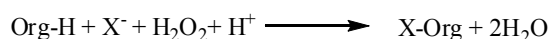


**Fig 1.** Classification of marine natural products

Compared to terrestrial plants, the occurrence of chloro compounds in oceans and marine organisms are high due to saline nature of sea water.

### Biogenesis of marine natural products – the role of Haloperoxidases enzyme

Marine organisms having haloperoxidase enzymes that can catalyses the oxidation of halide ion (Cl<sup>-</sup>, Br<sup>-</sup> and I<sup>-</sup>) by hydrogen peroxide that results in the halogenation of organic compounds.



Generally compounds generated by marine haloperoxidases are having huge importance in

biological activity which include anti-fungal, anti-bacterial, antineoplastic, anti viral (anti-HIV), anti-inflammatory and other activities.

The first chloroperoxidase (CPO) enzyme was described caldariomyces-producing fungus *Caldariomyces fumago* which serves as the proto typical heme-iron peroxidase. CPO was dichlorinated to activate carbon of 1, 3 cyclopentanedione and halogenate other rich carbons having β- carbon dimedone and 3, 5 positions of the amino acid tyrosine. CPO also catalyzes a host related reactions including oxidation of iodide to iodine, sulfoxidation of dialkyl sulfides, P-450 like insertion of oxygen into C-H.

CPO can utilize halide ions (chloride, bromide and iodide) and catalyse the corresponding carbon halogen bond formation in the suitable acceptor molecules like β-keto acids, cyclic-β-di ketones and substituted phenols. CPO catalyzed halogenation of amino acid like tyrosine which followed by measuring the decrease in fluorescence of tyrosine associated with the formation of mono and dihalogenated tyrosine. Sea water – saline water is rich in chloride, bromide and iodide ions but disadvantages may be that the enzyme (CPO) has its maximal halogenating activity at around pH 3.0, readily gets inactivated by elevated temperature and high concentrations of peroxides.

Haloperoxidases have been discovered from all classes of marine algae, the activity of haloperoxidases has been detected in many other species of algae (1) and in other marine organisms and they classified into two classes viz. (i) vanadium bromoperoxidase (V-BrPO), a non-heme enzyme and (ii) FeHeme bromoperoxidase (FeHeme-BrPO). In the case of marine snail *Murex truncuzus*, a marine haloperoxidase has been isolated, but the vanadium or FeHeme content has not been determined (2). Peroxidases are found to occur in the thyroid gland of mammals (3). The enzymes like chloroperoxidase, HRP and vanadium peroxidase are capable of chlorinating phenol ethers and phenols in the presence of chloride (4,5). The utilization of halide-peroxidase-hydrogen peroxide chemical system by humans

and other mammals in an electrifying development to generate active halogen (HOCl or HOBr) in order to destroy microorganism.

### Simple alkanes and its derivatives

Chloromethane are found abundant on our planet, produced by marine algae, giant kelp, wood rotting fungus, the ice plant, cultivated mushrooms, the pencil cedar, the evergreen cypress, several forms of fungi and phytoplankton. Plants take chlorine as Cl<sup>-</sup> ion from soil, wood and minerals, and thus plays an important role in plants, including in photosynthesis, osmotic adjustment and suppression of plant disease and their combustion inevitably leads to the formation of organochlorine compounds. However, high concentrations of chloride ion cannot only reduce the yield but also cause toxicity problems in crops due to accumulation of chloride ion in the leaves. Chloro methane can also be produced from eruption of volcanoes such as CH<sub>3</sub>Cl (6), CHCl<sub>3</sub> (7), CCl<sub>4</sub> (7), CH<sub>2</sub>ClBr (8), CH<sub>2</sub>ClI (8), CHBrCl<sub>2</sub> (9).

Marine algae not only produce simple one to two carbon halogens, but also produce halogenated alcohols, ketones, carboxylic acids, amides, aldehydes, epoxides and alkenes which have been isolated and characterized. Some algae secrete halo compounds as antipredator chemicals. Examples are secretion of *in vivo* form, chloroform and bromoform.

### Alkaloids

Alkaloids are cyclic organic compounds containing nitrogen in negative oxidation state which are distributed in plants and marine organisms. They are the secondary metabolites, and the presence of nitrogen atom in their structure confers biological activity to an exceptionally large group of this class of compound. A variety of marine sources including sponges, tunicates, halo corals, red algae, acorn worms, and symbiotic bacteria have been shown to generate alkaloids, which represent the largest number and most complicated of the marine alkaloids. Alkaloids of marine algae are relatively rare, when compared to the terrestrial plant alkaloids. Therefore research on marine drugs has been largely focused on finding new drugs for cancer treatment, but unfortunately the alkaloids are found in marine algae have not been approved as a practical medicine. Chart-I shows some marine alkaloids (compounds 1-5) and biological activity in Table-1.

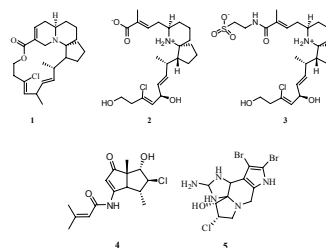


Chart I: Marine halo alkaloids

Table 1: Biological activity of extracts of some selective halogenated marine sponges and etc.

C.No.	Isolation	Activity
1.	Marine sponge <i>Halichondria okadai</i> Kadota (10)	Selectively inhibits the induced expression of VCAM1 with an IC <sub>50</sub> of 7 mg/mL.
2, 3	Okinawan bivalve <i>Pinna muricata</i> (11)	Inhibitory activity toward cytosolic phospholipase A2
4	Culture extracts of a marine-derived <i>Streptomyces</i> sp. (12)	Cytotoxicity against L1210 cells with IC <sub>50</sub> value of 2.5 µg/mL.
5	Marine sponge <i>Axinella brevistyla</i> (13)	Cytotoxicity against L1210 cells with IC <sub>50</sub> values of 1.1, 0.66, and 2.5 µg/mL respectively



**Fig. 2** Marine resources (algae, cyanobacteria, invertebrates)

### Steroids

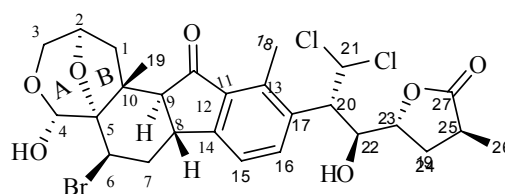
Steroids are a class of organic compounds which are characterized by a molecular structure ranging from C18-C30 carbon atoms arranged in four rings as a cyclopentano perhydro phenanthrene nucleus. It includes vast number of naturally occurring organic compounds like steroids, bile acids, sex hormones, adreno-cortical hormones, cardiac glycosides, sapogenins and some alkaloids. The steroids like bile acids, sex hormones and adrenocortical hormones have a number of functions in human physiology and are of immense biological importance. Cholesterol is the most important steroid from where remaining steroids are derived.

Halogenated steroids represent a group of natural molecules are also found in algae, marine invertebrates, marine sponges, corals, stars, and seaweeds (14-18) as shown in figure 2. Marine organisms produce steroids having chlorine, bromine or iodine atoms in the skeleton core. Clionastatins are strong cytotoxic chlorinated steroids which are isolated from burrowing sponge *Cliona nigricans* (19). Chlorohydrins are biosynthetic products of corresponding epoxides. Other related chlorinated steroids compounds and their activities are described below (Chart-II). Biological activity is given in table 2.

Marine natural products in general and halogenated compounds in particular are found

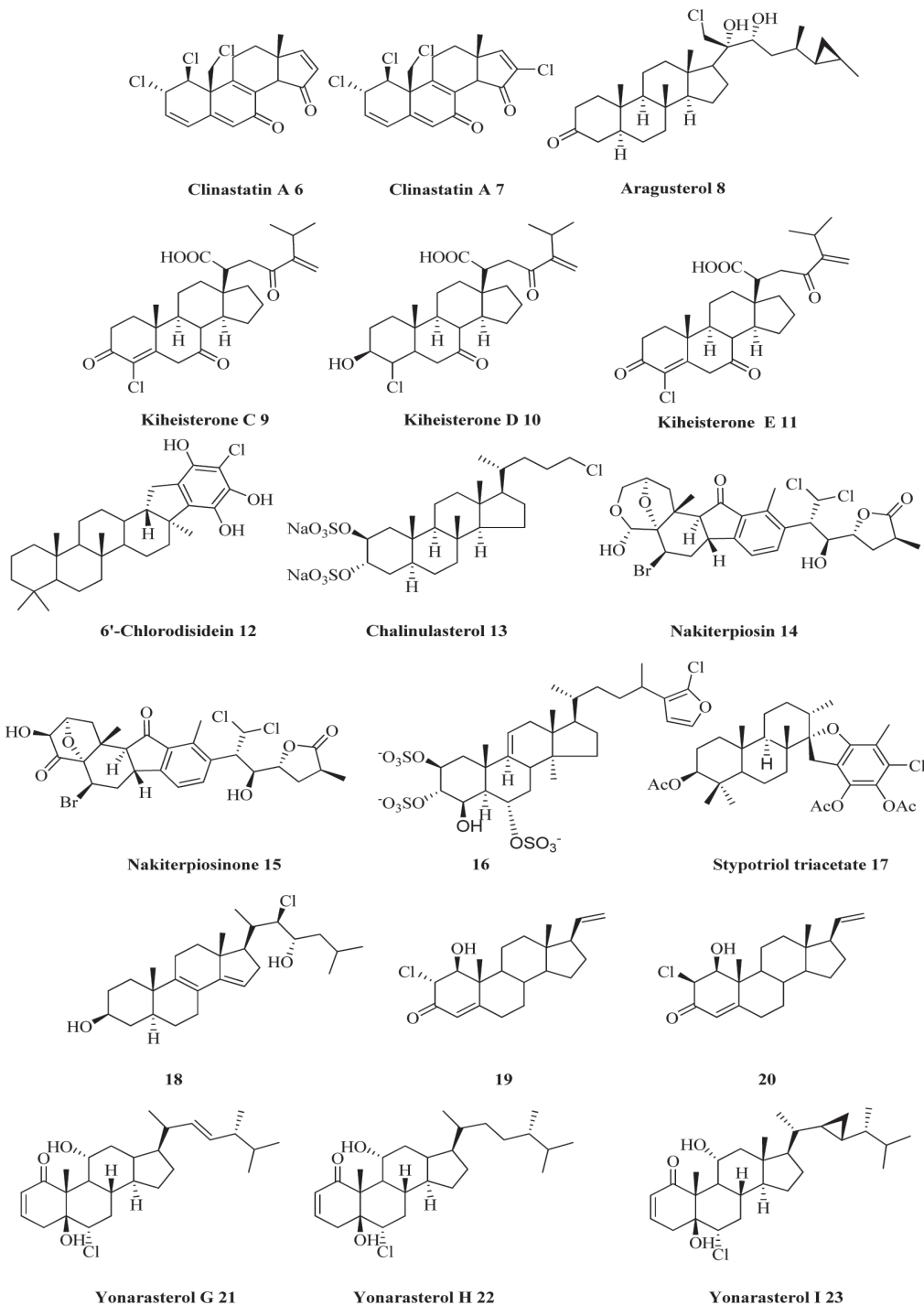
usually in milligrams quantities. Their isolation requires extensive chromatographic separation. A typical method of isolation is described in figure 3 and biological accounts in Table 2. Although the yields are poor (less than a milligram) but it is possible to determine their structures based on advanced techniques of spectrometry (FT-IR, HPLC, UV, HPLC/MS,  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$ , 2D-NMR, NOE, DEPT etc.)

### Structure determination of Nakiterpiosin 14



**Nakiterpiosin 14**

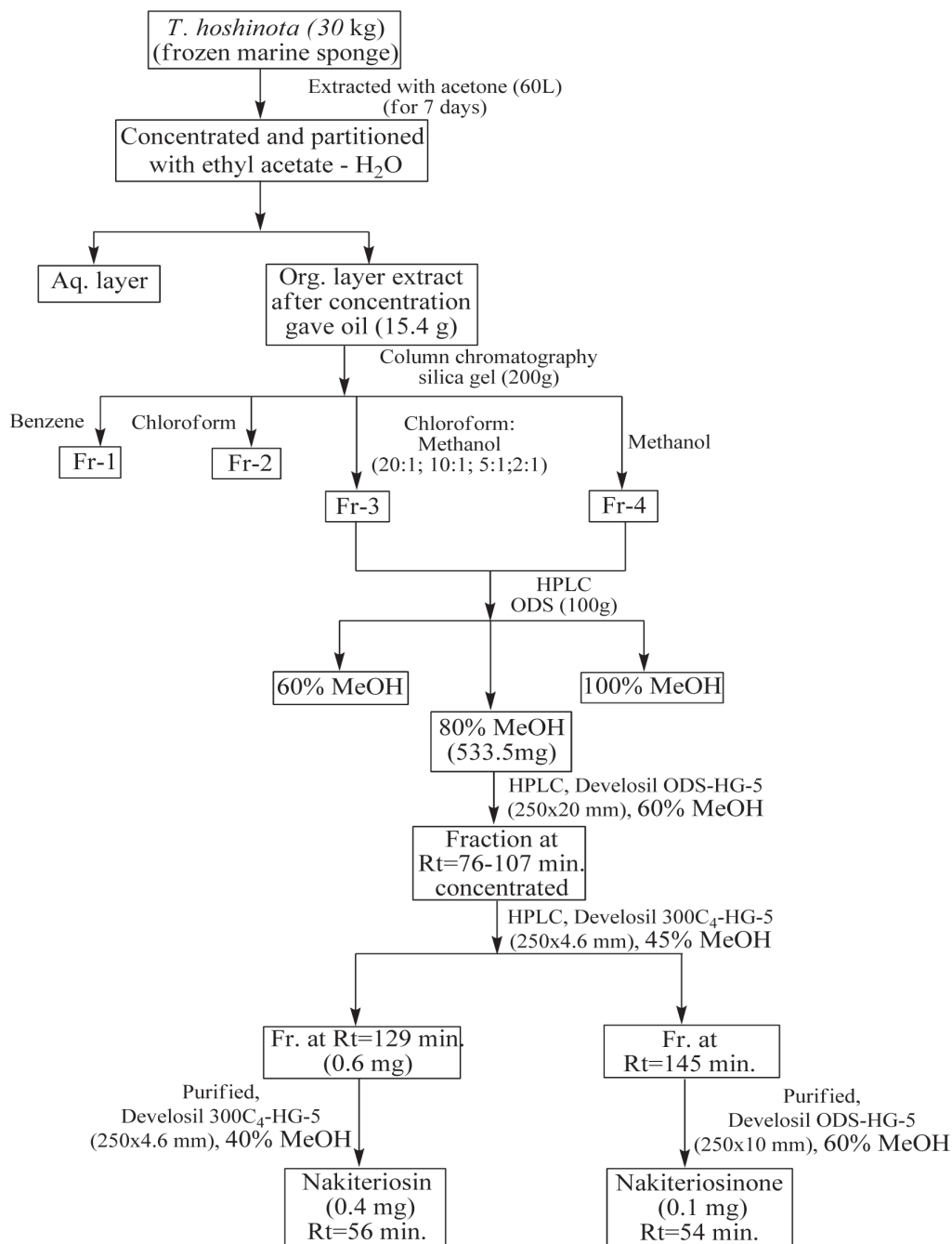
Nakiterpiosin **14** was isolated from the marine sponge of *T. hoshinota* and shows strong P388 growth inhibition. The ESI-MS spectrum. The ESI-MS mass spectrum indicated that the molecular formula  $\text{C}_{27}\text{H}_{31}\text{BrCl}_2\text{O}_7$ . A detailed analysis of  $^1\text{H NMR}$ ,  $^{13}\text{C NMR}$  and HMQC spectra showed that compound **14** having three methyl groups, four methylene groups, 12 methine, eight carbons and two hydroxyl protons. The  $^1\text{H NMR}$  spectrum of compound **14** showed the presence of 1,2,3,4-tetrasubstituted benzene ring at  $\delta$  7.33 and 7.89 ppm and coupling constant  $^3J_{\text{H}15-16} = 8.2$  Hz. An analysis of COSY and HOHAHA spectra of compound **14** permitted for partial structure C1-C2, C6-C9, C15-C16 and C20-C26. HMBC techniques were utilized to link these partial structures through cross peaks due to  $^2J_{\text{CH}}$ ,  $^3J_{\text{CH}}$  long range coupling with quaternary carbons. The HMBC peaks at H-26/C27 and H-25/C27 suggested that connectivity between C25 and C27. The connections of aromatic partial structure were given by the HMBC peaks at H-18/C12, H-18/C13, H-18/C17, H-16/C13, H-16/C14, H-15/C17 and H-15/C12. The HMBC peaks, H-19/C1, H-19/C5, H-19/C9, H19/C10, H-6/C5, H-6/C10 and H-2/C5 indicated a 5,6 bicyclic (B, C) ring and B, C-bicyclic ring was linked to the A-ring to make 6,5,6 tricyclic



**Chart II:** Halosteroids isolated from marine source

A review on chloro substituted marine natural product





**Fig 3:** Scheme for the isolation of marine natural products from the sponge of *T. hoshinota* (24a)

ring as suggested by the cross peaks H-2/C3, H-3/C1, H-3/C2, H-3/C4, H-4/C3 and H-4/C5. The HMBC cross peaks between C9 and C11 and H-16/C20, H-20/C16 and H-22/C17 suggested the connectivity between C17 and C20. The allylic coupling (H-8 and H-15) and HMBC cross peaks at H-15/C8 and H-8/C14 suggested connectivity between C8 and C14. The connectivity between C11 and C12 was suggested by the chemical shift

of C11, which is considered to be  $\alpha$ ,  $\beta$ -unsaturated ketone. The presence of signals at  $\delta$  75.2 ( $C^{13}$  NMR) and 6.32 ppm ( $H^1$  NMR) indicates dichloromethyl group and bromine having methane carbons was considered to be C6 because of its reasonable chemical shifts  $\delta$  51.8 ( $C^{13}$  NMR) and 4.70 ppm ( $H^1$  NMR).

From  $^3J_{H-H}$  coupling and NOE correlations from NOESY data give relative stereochemistry

**Table 2:** Biological activity of extracts of halogenated steroids

C. No.	Source	Biological activity
6&7	Burrowing sponge <i>Clina nigricans</i> (19)	Cytotoxic
8	Okinawan marine sponge of the genus <i>Xestospongia</i> sp. (20)	Strongly inhibited the proliferation of KB Cells at $IC_{50}$ 0.041 $\mu$ g/ml, and potent in vivo antitumor activity against L1210 Lukemia in mice.
9-11	Sponge <i>Strongylacidon</i> sp. (21)	Cytotoxic
12	Marine sponge <i>Disidea pallescens</i> (22)	Potent cytotoxicity against mouse lymphocytic leukemia cell (P388)
13	Caribbean sponge <i>Chalinu moliba</i> (23)	No significant activity
14&15	Okinawan sponge <i>Terpios hoshinota</i> (24)	Strong P388 growth inhibition
16	Marine sponge <i>Topsentia</i> sp. (25)	Effective inhibitor of endo-1,3- $\beta$ -D-glucanase
17	Brown algae <i>Styopodium flabelliforme</i> (26)	A light range of antifeedant and anti-inflammatory activity
18	Starfish <i>Echilaser sepositus</i> (27)	-----
19 & 20	Pacific octocoral <i>Carijoa multiflora</i> (28)	Increase the conversion of testosterone (T) to DHT producing androgen activity. The compound stimulated T conversion, suggesting a potential application in the treatment of androgen-dependent diseases
21-23	Okinawan soft coral, <i>Clavularia vidis</i> (29)	Activity against human colorectal adenocarcinoma cells (DLD-1, $IC_{50}$ 3,3,50 and 0.02 $\mu$ g/ml respectively) and also against human T lymphocyte leukemia cells (MLOT-4, $IC_{50}$ 2.5,3,10 and 0.01 $\mu$ g/ml respectively)

of compound **14**. The coupling constant of  $^3J_{H_8-H_9} = 9.3$  Hz and NOESY cross peaks at 19-Me/H-8 show that 19-Me at C10 was in axial position. The configuration at C4 and C6 based on NOE cross peaks 19-Me/H-4 and H-4/H. The coupling constant of  $^3J_{H_{20}-H_{22}} = 3.8$  Hz and  $^3J_{H_{22}-H_{23}} = 8.0$  Hz indicates that H-20 and H-22 were located in a *gauche* and in *anti*. The alkyl chain of compound **14** have zigzag conformation, the relative stereochemistry at C20, C22 and C23 were 20R\*, 22S\* and 23R\* and NOE cross peaks 18-Me/H-20, H-16/H-21, H-22/H-24b, H-23/H-24a and H-24a/H-25 indicated that the relative stereochemistries at C22, C23 and C25 were 22S\*, 23R\* and 25R\*. The absolute of stereo chemistry of compound **14** at C4 and C22 established as 4R and 22S was determined by modified Mosher's method.

### Terpenes (Terpenoids)

Terpenoids are derivatives of terpenes which are unsaturated hydrocarbons having general formula  $(C_5H_8)_n$ , where *n* is number of isoprene (2-methyl, 1,3-butadiene) units and they are arranged in head to tail fashion. Terpenes are classified into many categories based on number of isoprene residues present in the structure. There

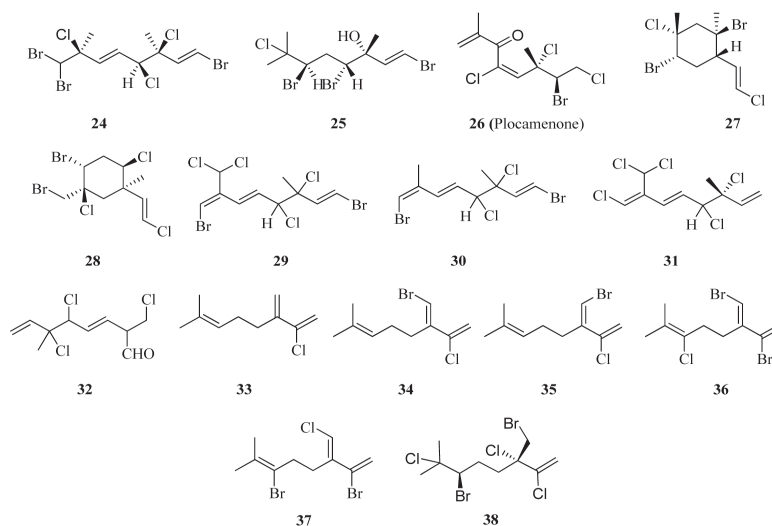
are mono terpenes (2 isoprene units), sesquiterpenes (3 isoprene units), diterpenes (4 isoprene units), triterpenes (6 isoprene units), tetraterpenes (8 isoprene units) and polyterpenes (number of isoprene units).

Terpenoids are not only isolated from plants but also obtained from marine sources like algae, sponge, corals and etc. The halogens in sea water appear which are fundamentally involved in terpene biosynthesis and also in the production of halogen containing acetogenins and phenolic compounds. Secondary metabolism in the marine environment lies in terpene biosynthesis and common metabolites are found in marine both plants and animals. Marine environment lies largely in the production of higher molecular weight terpenes. The mechanism of the biogenesis of halo marine natural products involving CPO is explained above. Interestingly, algae used bromide more frequently for organohalogen production. However, chlorine occurs in higher concentrations than bromine in sea water. Chlorinated or brominated marine natural products are important compounds in algae than iodinated compounds. First halogenated marine terpene was isolated in the year 1963. The more important biosynthesis of marine terpene is found in the involvement of halogens, particularly

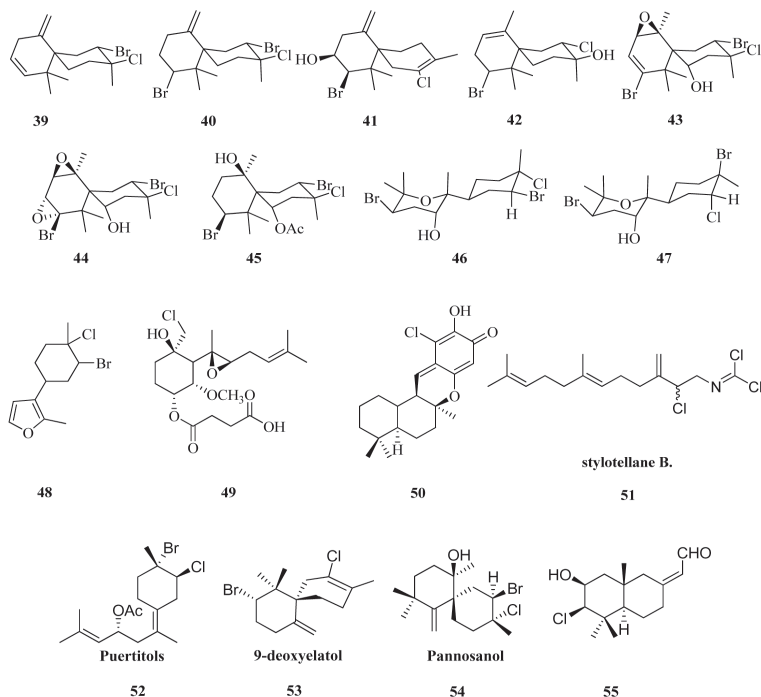
**Table 3:** Biological activity of the extracts of some selective halogenated mono terpenes

C.No	Source	Biological activity
24	Red alga <i>Plocamium costatum</i> (30)	Very weak antimicrobial and antialgal As per P388 murine leukemia cell assay it is showing cytotoxic activity. <i>Bacillus subtilis</i> , <i>Candida albicans</i> and <i>Cladosporium resinae</i> are showing inhibitory activity.
26	<i>Plocamium angustum</i> (31)	
27	Sea hare <i>Aplysia dacylomela</i> (32)	Cytotoxic toward HeLa cells with IC <sub>50</sub> values of 4.5 µg/mL and 1 µg/mL.
28	Sea hare <i>Aplysia dacylomela</i> (32)	Weakly cytotoxic toward Lu1 (human lung cancer; IC <sub>50</sub> 12.9 µg/mL), KB (human oral epidermoid carcinoma; IC <sub>50</sub> 13.3 µg/mL), and ZR-75-1 (hormone dependent human breast cancer; IC <sub>50</sub> 7.8 µg/mL) cells.
31	Sea hare <i>Aplysia dacylomela</i> (32)	cytotoxicity toward the cell lines HM02 (gastric carcinoma), HEP G2 (liver carcinoma), and MCF 7 (breast carcinoma) at concentrations of 17 µg/mL and lower.
38	Red algae <i>Portieria Hornemannii</i> (33)	<i>in vitro</i> human tumor cell lime screening panel, brain tumor, renal, and colon tumor cell lines

bromine, in the primary production of terpenes and through cyclization reaction yields bromo terpenes that can be further chlorinated, or brominated etc. Chlorinated and brominated monoterpenes (Compounds **24-38**) from *Rhodophyta* (Compounds 24 to 31) and some other marine resources (compounds **32 and 38**) are given in chart III.



**Chart III:** Halogenated mono terpenes found in *Rhodophyta* and other marine resources



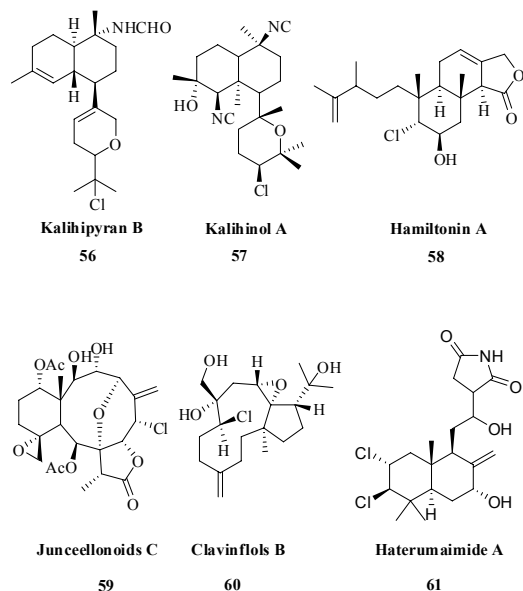
**Chart IV:** Some chlorinated sesquiterpenes

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**Table 4:** Biological activity of the extracts of some selective halogenated sesquiterpenes

C.No.	Source	Biological Activity
49	marine-derived strain of <i>Penicillium</i> (34)	strong inhibitory activity against an osteosarcoma cell line
50	Verongida sponge (35)	Strongly inhibits the effect of cholesteryl ester transfer protein (CETP; IC <sub>50</sub> ) 0.3 μM
51	Indo-pacific sponge <i>Pseudaxinyssa pitys</i> (36)	Weak P388 activity
52	Red algae of the genera <i>Laurencia</i> (37)	Antimicrobial activity against <i>Escherichia coli</i> and <i>Bacillus megaterium</i> (bacteria)
53	Marine red alga <i>Laurencia mariannensis</i> (38)	Antibacterial activity against <i>Staphylococcus aureus</i> and <i>Escherichia coli</i> , as well as antifungal activity against <i>Candida albicans</i> and <i>Aspergillus niger</i> using standard agar diffusion tests.
54	Red algal genus <i>Laurencia</i> (39)	Antibacterial activities against marine bacteria
55	Sponge <i>Stylotella aurantium</i> (40)	Showed cytotoxicity with a range of IC <sub>50</sub> values of 0.1-1 μg/mL against several tumor cell lines

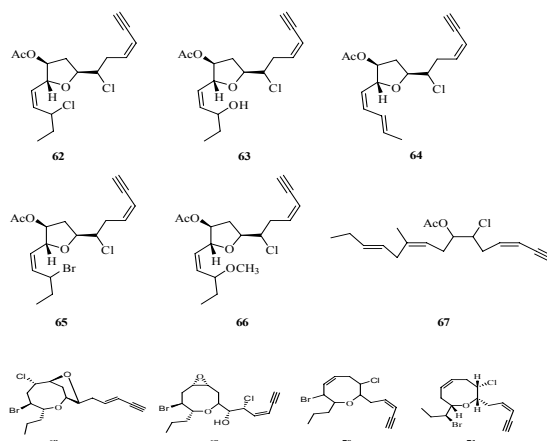
Chlorine containing diterpenes isolated from soft corals given below compounds (**56-61**) (references as given from 41-45). (Chart V and some biological activity in table 5).



**Chart V:** Halogenated diterpenes

### Acetogenin

Acetogenins are non terpenoids compounds, C<sub>15</sub>-acetogenins are a class of compounds that originate in the polyketide pathway. Mainly these compounds are present in marine red algae, *Laurencia*, are rich source of halogenated compounds. (Compounds **62 to 71** in Chart VI and biological activity in table 6).



**Chart VI:** Some halo acetogenin from marine sources



**Table 5:** Extract from some marine sources having diterpenes and its biological activity

C. No.	Source	Activity
56	Marine sponge <i>Acanthella cavernosa</i> (46a)	Antifouling activity against larvae of the barnacle <i>Balanus amphitrite</i> .
57	Marine sponge <i>Acanthella sp.</i> (46a,b)	<i>In-vitro</i> activity against <i>Bacillus subtilis</i> , <i>Staphylococcus aureus</i> , and <i>Candida albicans</i> .
58	Nudibranch <i>Chromodoris hamiltoni</i> (47)	<i>In-vitro</i> toxicity towards human pathogens
59	Coral <i>Junceella fragilis</i> (48)	Mild cytotoxicity against human galactophore carcinoma (MDA-mB-231 and MCF-7) cells at the concentration of 100 µM.
60	Soft coral <i>Clavularia inflata</i> (49)	Cytotoxicity against human oral epidermoid carcinoma (KB) cells
61	<i>Caledonia ascidian Lissoclinum voeltzkowii</i> <i>Michaelson (Urochordata)</i> (50)	Strong cytotoxic activity has been determined on human carcinoma KB cells (IC <sub>50</sub> : 14 µg/ml) and P388 leukemia cells (IC <sub>50</sub> : 1 µg/ml).

**Table 6:** Extract from halo acetogenins and biological activity

C. No	Isolation	Activity
62 & 67	<i>Laurencia glandulifera</i> (51)	Cytotoxicity towards the HT-29, MCF-7, PC-3, HeLa and A431.
68-70	Red Alga <i>Laurencia</i> (52)	—
71	Red seaweed <i>Laurencia pinnatifida</i> (53)	—

### Indoles

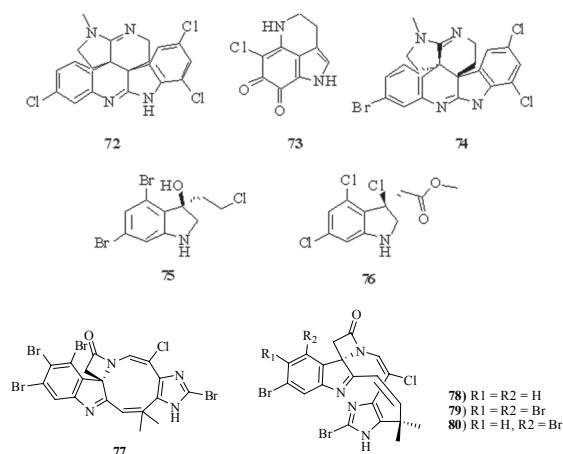
Indoles are “cyclic organic compounds containing nitrogen in a negative oxidation state which are distributed in living organisms”. They are secondary metabolites, the presence of nitrogen atom in their structure confers biological activity to an exceptionally large number of compounds. Indoles are also isolated from genetically engineered marine derived organisms. The indole nucleus is an important ring system for pharmaceutical development and it has been termed a “privileged structure”. It is frequently

associated with action on G-protein coupled receptors and in particular modulation of neuronal signal transmission through receptors for serotonin (5-hydroxytryptamine, 5-HT). The presence of Indole nucleus effects glycine gated chloride channel receptors and human protein tyrosine phosphatase-1B and also associated with CXCR4 (C-X-C chemokine receptor type 4) chemokine receptor, Na<sup>+</sup>/K<sup>+</sup>-ATPase, nitric oxide synthase, α-secretase, protein kinase C-β, butyrylcholinesterase, and acetylcholinesterase, and also it shows valuable action on cytotoxic, antineoplastic, antibacterial, antifungal, antiinsecticidal, and antiplasmodial activities have been detected.

A variety of marine sources including sponges, tunicates, red algae, acorn worms, and symbiotic bacteria have been shown to generate indole alkaloids, which represent the largest number and most complicated of the marine indoles.

An unusual tetra halogenated indoles was isolated from a fraction of a semipurified extract obtained from the red alga *Rhodophyllis membranacea* by Peter T. Northcote in 2016. Antifungal activity was observed in *R membranacea*

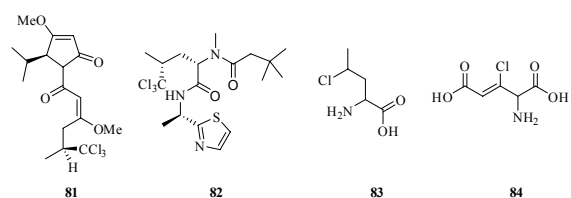
and *Saccharomyces cerevisiae* by performing cell proliferation assay. Antifungal activity was exhibited by these species due to the presence of tetra halogenated indoles. The indole compounds isolated from marine sources are given below compounds (**72 to 80**) and biological activity in Table 7.



**Chart VII:** Indoles from marine sources

### Amino acids and Peptides

Few naturally occurring amino acids and peptides such as *Dysidea herbacea* produces trichloromethyl metabolites, which contain dysidin (**81**), dysidenin (**82**) (references 60-64) in marine sponge. Several chlorinated amino acids and peptides from *Streptomyces* and *Pseudomonas* spp. possesses potent antibacterial properties. *Streptomyces griseosporus* yields  $\gamma$ -chloronorvline (**83**) (reference 65) and another *Streptomyces viridogenes* produced compound **84**. (Chart VIII)



**Chart VIII:** Halo amino acids and peptides from marine sources

### Fatty acids

Halogenated fatty acids are produced from microorganisms like algae, marine invertebrates, higher plants and some animals in which chlorinated fatty acids are major constituents. The

**Table 7:** Biological activity of the extracts of some selective halogenated indoles

C. No.	Isolation	Activity
72	Ascidian <i>Perophora namei</i> (54)	cytotoxicity, (HCT116, IC <sub>50</sub> ) 60 $\mu$ M
73	Marine sponge <i>Zyzzya fuliginosa</i> (55)	low cytotoxicity against cancer cell lines (IC <sub>50</sub> = >50 mM)
75	Floridina marine bryozoan <i>Amathia convolute</i> (56)	The tumor cell line HL-60
76	Tunicate <i>Cynthia savignyi</i> (57)	antifungal activity against two tomato pathogenic fungi, <i>Botrytis cinerea</i> and <i>Verticillium albo atrum</i> , antibacterial activity against some gram (+) and gram (-) bacteria and cytotoxicity against <i>Artemia salina</i> larva
77	Bryozoan <i>Chartella papyracea</i> (58)	No significant activity
78-80	Bryozoan <i>Chartella papyracea</i> (59)	No significant activity

organohalogen compounds are present mainly in fish, molluscs, some invertebrates and seaweeds. Chlorinated fatty acids, 9-chloro-10-hydroxypalmitic acid, 10-chloro-9-hydroxypalmitic acid, 9-chloro-10-hydroxystearic acid, 10-chloro-9-hydroxystearic acid, 11-chloro-12-hydroxystearic acid, and 12-chloro-11-hydroxystearic acid are obtained from jellyfish lipids, for the first time identified in invertebrates. Tetrachloro fatty acids have been obtained from blue mussel *Mytilus edulis*, collected from the southern parts of the Baltic sea (66).

### Prostaglandins

Marine Prostaglandins were first time discovered in a gorgonian and isolated from red algae and invertebrates. Punaglandins (**85-90**) were isolated from *Telestoa rissei* an octocoral algae which showed anti-tumor and anti-cancer activity. The compound **87** inhibits L1210 leukemia cell proliferation with an  $IC_{50}$  value of 0.02  $\mu\text{g/mL}$ . It had 15 fold greater activities than clavulone (67). (Chart IX, Compounds **85** to **95** are given below).

Prostanoids exemplified by clavulones are rich source of marine prostaglandins which are obtained from Okinawan soft coral *Clavularia viridis*. Halogenated prostanoids such as chlorovulones are having stronger anti proliferative activity against tumor cells, which is also exhibiting leukemia cell proliferation.

Compound **91** is exhibited strong antiproliferative activity against tumor cells compared to remaining compounds. (Chart IX).

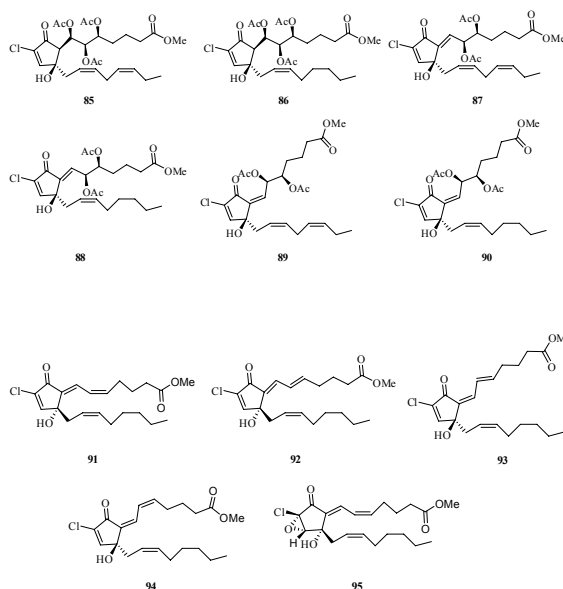


Chart IX: Halo prostaglandins from marine sources

### Pyrroles

Pyrrrole is a privileged scaffold which associated with nature of biological activities. There exist several natural chlorinated and brominated pyrroles. The pyrrolomycin B produces from the soil microbe *Streptomyces* Sp. (Compounds **96** to **98**) Chart X.

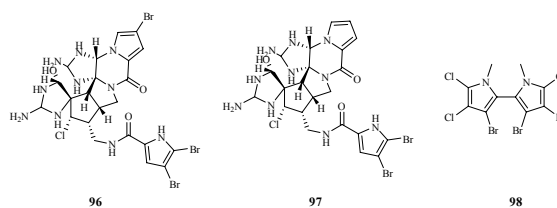


Chart X: Halogenated pyrroles

Table 9: Isolation & biological activity of pyrroles from marine sponge

C.No.	Isolation	Activity
1.	Okinawan marine sponge <i>Hymeniacidon</i> sp. (68)	Inhibitory activity against cyclin dependent kinase 4 (cdk4)
2.	Marine sponge of <i>Stylotella aurantium</i> (69)	Inhibit the moulting of cyprid larvae of barnacles.

### Phenols

Simple chlorophenol (2-chlorophenol, 3-chlorophenol, 4-chlorophenol) were isolated from fishes, invertebrates and algae. The presences of chlorinated phenols are due to natural product degradation, industrial activities and agriculture activities. (Chart XI and biological activity in Table 10)

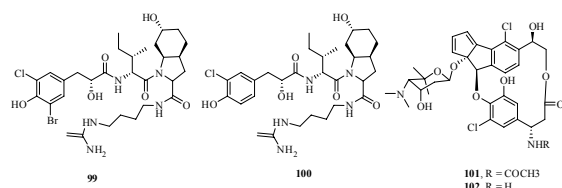


Chart XI: Chlorinated phenols

Although extensive biological studies were carried out as given in tables, a practical drug or

a lead molecule for further synthetic studies is yet to emerge.

### Conclusion

The marine natural products containing organohalogen compounds exhibit antifungal, antibiotic, cytotoxicity and other potentially valuable widespread biological activities. The utilization of more advanced isolation techniques and recent analytical methods in isolation and structure elucidation of these chlorinated marine natural products.

### Acknowledgement

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Table 10: Biological activity of chlorinated phenols isolated from marine resources

C.No.	Isolation	Activity
99	Cyanobacterium <i>Microcystis aeruginosa</i> (70)	Serine-protease inhibitor activity
100.	Cyanobacterium <i>Microcystis</i> sp. strain BHU006 (71)	Inhibit the activity of the proteolytic enzyme trypsin with IC <sub>50</sub> 's of 4.3 and 4.1µM.
101	<i>Nocardiopsi</i> (72)	A potent inhibitor of TNF-R-induced NFκB activation

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## NEWS ITEM

### **IIT Mandi finds malarial drug that inhibits Zika virus**

Researchers at Indian Institute of Technology (IIT) Mandi have found a drug hydroxychloroquine or HCQ, that is already being used for treating malaria to be effective in inhibiting Zika virus growth and replication. Also, the drug was able to significantly reduce viral load in placental cells. Zika virus is known to damage and kill the placental cells (which act as a barrier to protect the developing foetus from disease-causing organisms) leading to foetal infection. The drug might therefore help in preventing vertical transmission of Zika virus from the placenta to the foetus. Since the HCQ drug is already approved for use in pregnancy, positive results in human trials will mean that it can be given to pregnant women infected with Zika to reduce the chances of vertical transmission. Some foetuses infected with Zika virus are born with a small head (microcephaly). As the Zika virus protease structure is already available in the literature, a team led by Dr. Rajanish Giri from IIT Mandi identified the druggable site on the protease enzyme. The Zika protease enzyme is a good target for drug action as inhibition of the protease stops Zika virus growth. The team then screened FDA-approved drugs and identified five compounds based on their binding to the active site of the enzyme. The malarial drug HCQ was one of the five compounds selected. Inhibiting the protease activity leads to stoppage of virus replication and survival. This will eventually result in reducing the viral load, says Ankur Kumar from IIT Mandi and first author of a paper published in the journal ACS Omega. The HCQ drug acts on Zika virus through multiple mechanisms. We exploited the autophagy pathway to reduce the viral load in placental cells. The drug also inhibits the protease enzyme activity to limit its growth, says Prof. Mysorekar, who is a co-author of the latest paper. Besides autophagy, the drug inhibits Zika infection of placental cells by binding to the enzyme. The drug modulating the autophagy process is important in placental

cells, while inhibiting the protease enzyme activity could help stop the growth and kill the virus in other sites of the body, says Prof. Mysorekar.

### **The potentiality of twin drugs for TB augmented by Diabetic drugs**

A protein essential for the formation of biofilm by TB-causing bacteria (*Mycobacterium tuberculosis*) has been finally identified by a multi-institutional team from Delhi's Jamia Hamdard, National Institute of Pathology and Indian Institute of Technology (IIT) Delhi. Biofilm formed by TB bacteria acts as a physical barrier to drugs thereby protecting the microorganisms. The researchers also found two FDA-approved drugs used for treating diabetes and suppressing the immune responses were able to disrupt biofilm formation by the bacteria. When the existing drugs were used along with first-line anti-TB drugs, the dosage required to kill the bacteria was drastically reduced by over 50%. To validate the promising results obtained in the lab, the team has already initiated trials on mice. The protein (peptidyl-prolyl isomerase-B or PpiB) belongs to a class of cyclophilins that facilitates protein folding. In other bacteria, the cyclophilin class of proteins is known to be involved in protein folding and biofilm formation. But its role in biofilm formation by TB bacteria was not known so far. Since bio-safety level 3 lab is required to work with TB bacteria, the researchers initially used the non-pathogenic *M. smegmatis* to understand the role of TB bacteria protein PpiB in biofilm formation. The researchers inserted the PpiB gene of TB bacteria into *M. smegmatis* and tested its ability to form biofilm. *M. smegmatis* bacteria generally produce less of biofilm in response to stress. But when we added the PpiB gene into *M. smegmatis*, the biofilm production was 1.5-fold higher, says Ashutosh Kumar from JH-Institute of Molecular Medicine, Jamia Hamdard, New Delhi and first author of a paper published in the journal npj Biofilms and Microbiomes. Dr. Kumar is currently at the Department of Microbiology at Tripura



Central University. We validated the binding property through in vitro studies. Both the drugs as well as gallium nanoparticles were able to bind very well with the purified form of PpiB protein, says Anwar Alam from the Kusuma School of Biological Sciences at IIT Delhi and another first author of the paper. Recombinant *M. smegmatis* expressing TB bacteria PpiB protein were cultured in the presence of FDA-approved drugs and gallium nanoparticles to check for biofilm formation. There was reduced biofilm formation by recombinant *M. smegmatis* in the presence of the drugs and gallium nanoparticles, Dr. Alam says.

### **Antarctic glacier's giant cavity hints rapid melting**

NASA scientists have discovered a gigantic cavity, almost 300 metres tall, growing at the bottom of the Thwaites Glacier in West Antarctica, indicating rapid decay of the ice sheet and acceleration in global sea levels due to climate change. The findings, published in the journal *Science Advances*, highlight the need for detailed observations of Antarctic glaciers' undersides in calculating how fast sea levels will rise in response to warming. Researchers expected to find some gaps between ice and bedrock at Thwaites' bottom where ocean water could flow in and melt the glacier from below, NASA said in a statement. The size and explosive growth rate of the newfound hole, however, surprised them. It is big enough to have contained 14 billion tonnes of ice, and most of that ice melted over the last three years. We have suspected for years that Thwaites was not tightly attached to the bedrock beneath it, said Eric Rignot of the University of California, Irvine in the U.S. Thanks to a new generation of satellites, we can finally see the detail," said Mr. Rignot, who is also associated with NASA's Jet Propulsion Laboratory (JPL). The researchers also used data from a constellation of Italian and German spaceborne synthetic aperture radars. The cavity was revealed by ice-penetrating radar in NASA's Operation IceBridge, an airborne campaign beginning in 2010 that studies connections between the polar regions and the global

climate. These very high-resolution data can be processed by a technique called radar interferometry to reveal how the ground surface below has moved between images. (The size of) a cavity under a glacier plays an important role in melting. As more heat and water get under the glacier, it melts faster, said Pietro Milillo of JPL. Even with this accelerating retreat, however, melt rates on this side of the glacier are lower than on the western side, they said.

### **Microsat – R: Military satellite successfully launched by ISRO**

ISRO scientists broke into celebration at the mission control centre here, about 130 km from Chennai, as the Microsat-R was released in a 274-km polar sun synchronous orbit, marking another success story for the space agency. Former ISRO chairmen Krishnaswamy Kasturirangan and AS Kiran Kumar were among those who witnessed the launch. The fourth stage of the rocket with co-passenger Kalamsat, a students' payload, would now be moved to a higher circular orbit, around 450 kms from earth, so as to establish an orbital platform for carrying out experiments. The ISRO said it would take about 90 minutes for the fourth stage to reach the desired orbit. Microsat-R, an imaging satellite, is meant for military purposes, but the ISRO did not give any details about it. Built at a cost of around Rs 12 lakh, the Kalamsat is an experimental satellite for studying the communication system of nano satellites, which can be useful in many fields, predominantly disaster management. The PSLV C44 is the first launch for the country's space agency in 2019. Contributed by college students and the members of a Chennai-based organisation — Space Kidz India — Kalamsat is the first to use PS4 (the fourth stage of the vehicle) as a platform to orbit around the earth. We have been working on the project for over six years now. These students are from various backgrounds and the youngest one is studying B.Sc Physics, Space Kidz India CEO Srimathy Kesan told PTI. Kesan said Kalamsat was the lightest ever satellite to be launched by India.

### **Silk scaffold developed by IIT Guwahati researchers that aids in regeneration of bones**

A scaffold made of silk composite functionalised with copper-doped bioactive glass to facilitate faster bone regeneration has been developed by researchers at Indian Institute of Technology (IIT) Guwahati. The scaffold seeded with stem cells was found to differentiate into bone cells, facilitate growth of blood vessels and successfully integrate the newly formed bone cells with the native bone. The researchers were able to replicate the results in rabbits using functionalised non-mulberry silk composite. Rabbits with scaffolds implanted at the site of bone injury showed successful growth of bone cells and integration with the native bone at the end of three months. Commercially available synthetic grafts have a failure rate of about 25% and 30-60% complication rates. This is due to slower bonding with native bone and poor blood vessel growth. The team led by Prof. Biman Mandal from the Department of Bioscience and Bioengineering at IIT Guwahati developed the silk composite by adding chopped silk fibre to liquid silk. Unlike pure silk, the silk composite has greater strength. The addition of bioglass further enhanced the strength of the composite. Besides other kinds, both mulberry and non-mulberry silk composites were tested. The non-mulberry silk composite was found to be superior in all respects. The RGD sequence in non-mulberry silk is a cell binding site and helps in better cell attachment and proliferation. As a result, more stem cells get attached to the composite leading to better bone tissue formation with time. Bone cells prefer rough surfaces and the scaffold mimics the native bone surface architecture, says Prof. Mandal. Bioglass also helps in stem differentiation. We found stem cells differentiating into bone cells with the formation of extracellular matrix similar to natural bone, he says. The doped copper plays a crucial role in stabilising the gene responsible for blood vessel formation. The gene, in turn, regulates the downstream angiogenic factors thus helping blood vessel formation. The non-mulberry silk material will be replaced completely in a few years.

Since bone healing is slow, the silk material should not degrade quickly, Prof. Mandal says. The researchers tested the potential of the composites in repairing bone defects in rabbits and found more than 80% bone formation at the end of 30 days. In the rabbits, the scaffolds promoted new bone tissue formation and growth of blood vessels. The resorbable nature of the scaffolds enabled them to degrade inside the body while being replaced with viable bone tissue in the small focal sized bone defects. No remnants of the scaffold were seen, says Joseph Christakiran Moses from the institute's Department of Bioscience and Bioengineering and first author of a paper published in the journal *Advanced Healthcare Materials*.

### **Researcher manufactured Cellulose derived eco friendly napkin**

Preethi Ramadoss, a research scholar from the Department of Crystal Growth Centre of Anna University, has come up with a biodegradable sanitary napkin that does not have any plastics and can degrade within a month. It is made of cellulose derivatives. It has turmeric, vetiver and neem and lemon extracts. It has been tested against common pathogens responsible for bacterial vaginosis in women, explained the scholar, who has developed this as part of her Ph.D. thesis. The university has applied for a patent for the product. The materials used in this super-thin, less than 3-mm thick napkin absorb water up to 1700% of its own weight when tested in CIPET as well the university's own laboratories. The raw materials used are naturally occurring polysaccharides and polymers derived from plants, Ms. Ramadoss, who began work in 2015 July, explained. Napkins and diapers available in the market contain plastics and take a long time to degrade. They contain cellulose pulp that is recycled from waste paper and are bleached with chlorine that emits dioxins. They also contain a hydrophobic covering sheet which is made of polypropylene (plastic derivative) that induces skin rashes in most cases. Apart from this, a lot of wood pulp is also required for non-gel napkins/diapers. D. Arivuoli Dakshinamoothy, her Ph.D. guide said the project had funding from the

Department of Science and Technology for women scientists.

### **Study suggests that suicidal behaviour in some whales aggravated by sonar waves**

Scientists have long known that some beaked whales beach themselves and die in agony after exposure to naval sonar, and now they know why: the giant sea mammals suffer decompression sickness, just like scuba divers. The explanation was laid out on Wednesday by 21 experts in the Royal Society journal *Proceedings B*. Evolution has turned whales into perfectly calibrated diving machines. The heart rate slows, blood flow is restricted, oxygen is conserved. So how could they wind up with nitrogen bubbles poisoning its veins, like a scuba novice rising too quickly to the surface? In the presence of sonar they are stressed and swim vigorously away from the sound source, changing their diving pattern, lead author Yara Bernaldo de Quiros said. The stress response, in other words, overrides the diving response, which makes the animals accumulate nitrogen, she added. It's like an adrenalin shot. Outwardly, the whales showed no signs of disease or damage: they had normal body weight, and no skin lesions or infections. Internally, nitrogen gas bubbles filled the veins, and their brains were ravaged by haemorrhaging. Autopsies also revealed damage to other organs, as well as to the spinal cord and central nervous system. As with altitude sickness, reactions — in humans, and probably in whales — to nitrogen bubbles in the blood vary in type and intensity. A 2003 study in *Nature* on the link between sonar and whale deaths led Spain to ban naval exercises around the Canary Islands in 2004.

### **IIT Delhi researchers developed drug laden microparticles to combat Parkinson's disease**

Parkinson's disease, which affects the central nervous system of the body currently does not have an effective cure. The dopamine (neurotransmitter chemical) deficiency caused by the disease can, however, be overcome by providing drugs which are capable of crossing the blood-brain barrier. But most of the time only 1%

of the drug (levodopa) reaches the brain after being orally taken. Using dual drugs (levodopa with carbidopa) have increased the fraction of drug reaching the brain, but low half-life has posed challenges. Also, continuous intake of levodopa has in some cases caused serious side effects such as LID (Levodopa-induced dyskinesia). With a long list of problems staring at Parkinson's disease management, now researchers from Indian Institute of Technology, Delhi have tasted success. They have fabricated disc-shaped microparticles, merely 15 micrometres in size, made up of two compartments for carrying dual drugs without having drug-drug interactions. The particles are made entirely of biodegradable and biocompatible polymers. The polymers used are also FDA-approved and are currently in use as drug carriers. We tuned them according to our need. We made disc-shaped particles as they have a superior ability to attach to the intestinal lining, thereby increasing the bioavailability of the drug, crucial for oral drug delivery system says Mr. Ashok Parthipan, first author of the paper published in *Journal of Material Sciences*. Ashok completed his M.Tech degree from the Department of Materials Science and Engineering (DSME) (formerly Centre for Polymer Science and Engineering), IIT Delhi. Disc-shaped bicompartamental particles made of mucoadhesive polymers can also prolong the gastric residence time which helps in providing sustained release of dual drugs in the gastrointestinal tract. The researchers simulated an environment similar to our digestive system and found that major release of the drugs took place in the stomach and intestine. The drugs get absorbed in the small intestine area and then travel via blood to the blood-brain barrier. Now, carbidopa acts as a helping hand and allows levedopa to cross the crucial barrier, reach the target zone in the brain and effectively manage the dopamine deficiency. Also, more than 80% of the drug was released within five hours in the simulated gastric fluid, which is highly beneficial from a pharmacological point of view.

**Highly economical medicines produced by GM chickens** : Genetically modified chickens

that produce human proteins in their eggs can offer a cost-effective method of manufacturing drugs widely used for treating cancer and other diseases, a study has found. The research, which initially focused on producing high quality proteins for use in scientific research, found the drugs work at least as well as the same proteins produced using existing methods. According to researchers from the University of Edinburgh in the U.K., high quantities of the proteins can be recovered from each egg using a simple purification system and there are no adverse effects on the chickens themselves, which lay eggs as normal. The findings, published in the journal BMC Biotechnology, provide sound evidence for using chickens as a cheap method of producing high quality drugs for use in research studies and, potentially one day, in patients. Eggs are already used for growing viruses that are used as vaccines, such as the flu jab. This new approach is different because the therapeutic proteins are encoded in the chicken's DNA and produced as part of the egg white. The team have initially focused on two proteins that are essential to the immune system and have therapeutic potential — a human protein called IFN $\alpha$ 2a, which has powerful antiviral and anti-cancer effects, and the human and pig versions of a protein called macrophage-CSF, which is being developed as a therapy that stimulates damaged tissues to repair themselves. Just three eggs were enough to produce a clinically relevant dose of the drug. As chickens can lay up to 300 eggs per year, researchers say their approach could be more cost-effective than other production methods for some important drugs. We are not yet producing medicines for people, but this study shows that chickens are commercially viable for producing proteins suitable for drug discovery studies and other applications in biotechnology, said Helen Sang, a professor at University of Edinburgh in the U.K.. Protein-based drugs, which include antibody therapies such as Avastin and Herceptin, are widely used for treating cancer and other diseases.

## POST DOC OPPORTUNITIES

### **Indian Institute of Science: Raman Post-Doc**

The Indian Institute of Science (IISc) has been recognised as an Institution of Eminence (IoE) by the Government of India. As a part of the IoE initiative, IISc has created the Raman Post-Doc Program, a highly selective Post-Doc program with 50 positions. The Institute invites applications for intensely motivated individuals with an established record of high quality research, for the positions of Raman Post-Docs. Overseas Citizens of India (OCI), Persons of Indian Origin (PIO), and foreign nationals are also eligible to apply. Refer web site: [iisc.ac.in](http://iisc.ac.in)

### **Indian Institute of Science Education and Research, Bhopal Post-Doctoral Fellow Positions**

Indian Institute of Science Education and Research Bhopal invites applications for Institute Post-Doctoral Fellow (PDF) positions in the following Departments: Biological Sciences [hod\\_bio@iiserb.ac.in](mailto:hod_bio@iiserb.ac.in) Chemistry [hod\\_chm@iiserb.ac.in](mailto:hod_chm@iiserb.ac.in). Refer web site: [iiserb.ac.in](http://iiserb.ac.in)

### **General Electric Company, Bengaluru, Karnataka: Postdoctoral Research Fellow**

The GEHC Life Sciences Postdoctoral Research Fellowship is for researchers who are in the early stages of their research careers. This industrial Fellowship will provide the opportunity for a talented researcher to establish a convergence of cell biology and computational analytics to empirically predict cell culture performance. Refer web site: [ge.com](http://ge.com)

### **Indian Institute of Science Bengaluru, Karnataka Post Doc and Research Associate**

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