ISOLATION, BIOCHEMICAL CHARACTERISATION AND ANTI MICROBIAL STUDIES ON P.VULGARIS, S.AUREUS, E.COLI, S.CEREVISIAE AND C.ALBICANS

Eswari beeram[1*], Bukke Suman[2], K.Yoga priyanka[3], U. Bharath[4], Bhargavi[5]

Abstract:
Development of multidrug resistant strains of bacteria and fungi is a major problem faced by the mankind and development of plant anti microbial that show broad spectrum against bacteria and fungi is necessary now a days. In the antimicrobial assay carried for *E.coli, P.vulgaris, S.aureus*, and fungal strains *S.cerevisiae* and *C.albicans* with aqueous extracts of *Tamarindus indica*, *Curcuma longa* and *Citrus limon* (Lemon) by using Streptomycin as reference antibiotic for bacteria and pencillin as reference antibiotic for fungal strains. *Citrus limon* (Lemon) peel extract showed highest activity against *S.cerevisiae and C.albicans* but T.indica also showed highest activity against *S.cerevisiae* but not proven to be more effective for *C.albicans, E.coli* and *P.vulgaris*. None of the plant extracts proven to be effective against *S.aureus* but streptomycin is more effective against *S.aureus* by 48 hrs of incubation.

Keywords: *E.coli, P.vulgaris, S.cerevisiae, C.albicans, S.aureus, Curcuma longa, Tamarindus indica and Citrus limon (Lemon).*

[1*]Assistant Professor, School of liberal Arts and Sciences, Mohan Babu University, A.P, India.
Email: eshu.sonu@gmail.com
[2] PDF, Department of Biochemistry, Sri Venkateswara University, A.P., India.
[3]Msc, Department of Chemical sciences, Sree Vidyanikethan Degree College, A.P, India.
[4]Msc, Department of Chemical sciences, Sree Vidyanikethan Degree College, A.P, India.
[5]Msc, Department of Chemical sciences, Sree Vidyanikethan Degree College, A.P, India.

***Corresponding Author:** Eswari beeram
*Assistant Professor, School of liberal Arts and Sciences, Mohan Babu University, A.P, India.
Email: eshu.sonu@gmail.com

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Introduction:

Proteus spp. Bacteria was first described by Gustav Hauser in 1885 who obsevered intensive swarming growth of the bacteria. Currently the genus is divided in to *Proteus vulgaris, P.mirabilis, P. penneri, P. hauseri* and three unnamed 4,5,6 spp.The genus *Proteus* is a gram negative rod, facultatively anaerobic opportunistic bacteria causing urinary tract infections, Nosocomial infections and respiratory tract infections. *Proteus vulgaris* is indole negative, sucrose utlisation positive and citrate utilisation positive organism having flagella and secretes proteases, hemolysin and ureases.

Proteus spp. can be resistant naturally to antibiotics such as benzylpencillin, oxacillin, tetracycline, macrolides and can acquire resistance to ampicillin through plasmid mediated beta lactamases and chromosomal expression of beta lactamases [1][2]. Recent reports on Proteus spp. proven to confer resistance to third generation cephalosporins through expression of extended - spectrum beta lactamases (ESBLs). ESBLs generally wont hydrolyse cephalomycins and carbapenems, however resistance to carbapenems is found to be observed in Proteus spp [3].

Staphylococcus aureus is a major bacterial pathogen that causes wide variety of clinical manifestations and infections both related to community acquired and hospital Acquired. It has become difficult to treat *Staphylococcus* due to development of multidrug resistant strains such as MRSA (Methicillin-Resistant *Staphylococcus aureus*).[4][5] *S. aureus* normally resides on the skin and mucus membranes surronding the nasal cavity and it will not cause the infection in healthy individuals unless it enters in to systemic circulation. The entry is through direct contact and transmission by various other methods.

Staphylococcus aureus is Gram-positive bacteria that stains purple by gram staining and facultative anaerobe and can grow at temperature ranging between 18^0c to 40^0C. S.aureus divides in random plane and appears as group like grapes. Typical biochemical identification tests include catalase positive, Novobiocin sensitive, Indole positive, Citrate utilisation test positive and coagulase positive. S.*aureus* causes major bacterial infections like infective endocarditis, Bacteremia, skin and soft tissue infections like impetigo, carbuncles, furnuncles, meningitis, toxic shock syndrome and septic shock [6] [7] [8]. *S.aureus* escape the host defense mechanisms by formation of antiphagocytic capsule, biofilm formation, inhibit chemotaxis of leukocytes and sequestration from immune defense mechanism [9] [10] [11].

Escherichia coli (*E. coli*) is a gram-negative bacillus capable of causing intestinal illness and extra intestinal illness in humans [12]. Hundreds of *E.coli* strains has been identified that can cause mild to severe infections like renal failure and septic shock. *E.coli* can evade the host defense and development of antibiotic resistant strains has become a major concern. *E.coli* cause infections when it gets translocates from the intestinal tissue and urinary tract is the most common site of infection and hospitalisation after pneumonia [13]. *E.coli* can cause common acquired pneumonia but ventilator acquired pneumonia is more common. *E.coli* bacteremia is one of the systemic infection caused by the organism other than the urinary tract infections.

S.cerevisiae is a unicellular fungi found in small numbers compared to other fungal strains *Candida* and *Pichia*. Despite of its small number *S.cerevisiae* has its stable presence in wasp community and is considered as one of the model organism for genetical studies [14]. S. cerevisiae is generally considered as nonpathogenic safe organism but recent findings on opportunistic infections of *S.cerevisiae* causing vaginitis in healthy patients, cutaneous infections to systemic infections and infections to essential organs has been identified especially in immuno compromised individuals [15] [16] [17].

Candida albicans is a polymorphic fungus included as a member of the normal human microbe that resides as a lifelong harmless commensal in the oral cavity. Under certain circumstances *Candida* can cause opportunistic infections ranging from superficial infections of the skin to systemic infections majorly in HIV affected patients. Several factors secreted by the fungus majorly contribute to the pathogenic potential of the organism and mediate adhesion and invasion in to host tissues. Secretion of hydrolases, fungal dimorphism [18], Phenotypic switching, biofilm formation are the major attributes responsible for the pathogenicity of the fungus[18].

Candida constitute around 70% of the total microbiotome in the oral cavity of the human and responsible for oral candidiasis [19]. The medical impact of C.albicans depends on its ability to form biofilm, a closely packed group of cells. Biofilms formed on medical implant devices including catheters, pacemakers, dentures and prosthetic joints acts as source of transmission of microbe to healthy individuals.

Biofilms are instrinsically resistant to anti fungal therapeutics, host immune system defenses and other environmental variations making Biofilm mediated infections a significant clinical challenge [20] and use of plant compound that can kill and resist biofilm formation in fungus can be effective method of controlling candidiasis infections in mankind.

Methodology:

Isolation of Gram negative bacteria:

Isolation of *E.coli* and *P.vulgaris*:

Fecal contaminated water was collected from the washrooms of the MBU and subjected to serial dilution and 0.1 ml of serially diluted sample from 10^{-5} and 10^{-6} tubes are plated on macconkey agar and maintained at 37^0c in a incubator for 24 hrs and a control plate was maintained for each study. After the required growth, biochemical tests are carried through IMVIC tests and the bacteria is observed for morphological appearance and characterized based on either lactose fermenting or non lactose fermenting and as gram positive or negative using Gram staining and the microscopic results are recorded. E.coli will form pink colour colonies on Macconkey agar.

P. vulgaris was isolated from the fecal water sample collected from the wash rooms, serially diluted and 0.1ml of serially diluted sample from 10^{-5} and 10^{-6} tubes are plated on Macconkey agar + Nacl sterile medium and maintained at 37^0c in a incubator for 24 hrs and a control medium plate is maintained for each study. Biochemical studies was performed using IMVIC tests and the colonies are observed for morphological appearance and characterized as either gram positive or gram negative by gram staining procedure and the microscopic results are recorded.

Isolation of *Staphylococcus aureus*:

Nasal secretions are collected from the patient with cold and throat infection using sterile swabs and the swab is aseptically transferred to the test tube 1 of serial dilution and the solution is properly mixed with sterile micro pippette and serially diluted. 0.1 ml of serially diluted sample from 10^{-5} and 10^{-6} tubes are used for plating on Mannitol salt agar plates and maintained at 37^0c in a incubator for 24 hrs. Control medium plate was maintained for each study and the colonies are studied for morphological features and characterized as either gram positive or gram negative using gram staining procedure and biochemical metabolic studies are carried out through IMVIC tests.

Isolation of Fungi:

Isolation of *Saccharomycetes cervisiae*:

1g of curd sample is serially diluted and 0.1ml of serially diluted sample from 10^{-5} and 10^{-6} tubes are plated on Potato dextrose agar and maintained in a incubator at 37^0c for 48 hrs. After the required growth the organism was identified using Methylene blue staining procedure. Briefly, a loop of culture is smeared over the glass slide, heat fixed and flooded with methylene blue stain and incubated for 2 min. After the time period the slide is washed under running tap water, air dried and observed under Bright field light microscope and the microscopic results are recorded.

Isolation of *C.albicans*:[21]

Fresh urine sample was collected and 1ml of urine was transferred to 10 ml of distilled water and the sample is serially diluted. 0.1 ml of serially diluted sample from 10^{-5} and 10^{-6} tubes are plated on Potato dextrose agar and maintained at 37^0c in incubator for 48 hrs and the organism hyphae was identified by lactophenol cotton blue staining and biochemical methods after 48 hrs and 72hrs.

Similar procedure is followed for the sample collected by mouth goggling using distilled water.

Lactophenol cotton blue staining:

Loop of culture is transferred to drop of lactophenol cotton blue stain placed over the slide and subjected to smearing for spreading of mycelium and a cover slip is placed over the smear avoiding air bubbles and observed under 10 X objective in a bright field light microscope and the microscopic images are captured.

Biochemical tests:[21]

2% solutions of sucrose, Maltose, Starch and glucose are prepared in boiling test tubes and the discs are overlaid with sugar solution after placing them on inoculated plates and examined for growth around the discs after 48 hrs and 72 hrs of plating. Glucose is used as positive control. Paper discs are prepared by punching machine and the discs are sterilised using autoclave after wrapping in a paper.

IMVIC tests:[22]

Tryptone broth was prepared and 10 ml of broth is transferred to each tube and inoculated with cultures of *Proteus vulgaris, E.coli and staphylococcus aureus* and incubated for 24 hrs. After 24 hrs kovac's reagent is added to each tube and observed for appearance of red color.

Citrate utilisation test was performed by using Simmons citrate agar medium and the agar slants are prepared and inoculated with *Proteus,*

Staphylococcus and E.coli and incubated for 24 hrs in a incubator and observed for the color change from green to blue.Control agar slant is maintained. *E.coli* and *Staphylococcus aureus* are both indole positive and citrate utilisation test positive where as *Proteus vulgaris* is indole negative and citrate utilisation test positive.

Anti microbial Assay:

From the cultured plates the inoculate is used to grow the *P.vulgaris, E.coli, S.aureus, S.cerevisiae and C.albican*s in liquid broth and used for antimicrobial assay. Nutrient broth is used to grow the bacterial cultures and czapekdox broth is prepared by mixing individual components and used for cultivation of fungal strains and the inoculated broth is maintained at 37^0c for 24 hrs in a incubator for bacterial cultures and 48 hrs for fungal strains and after required growth 0.1 ml of the broth culture is transferred on to nutrient agar and potato dextrose agar plates, spreaded using L rods and wells are made using sterile tips and the aqueous extracts of *Tamarindus indica, Curcuma longa* and *Citrus limon* (Lemon) are added to each well and the results are recorded after 24 hrs of incubation.

Preparation of plant extracts:

Tamarindus indica leaf powder, *Curcuma longa* stick powder and *Citrus limon* (Lemon) peel powder are used for anti microbial studies. Leaves of *Tamarindus indica* is shade dried and 1g of grinded powder is added to 10 ml of distilled water and the resulting solution is used for antimicrobial studies.*Curcuma longa sticks* and *Citrus limon* (Lemon) peel is dried under the sun and 1g of grinded powder is transferred to 10ml of distilled water in to each test tube seperately and the resulting solution is used for antimicrobial studies.

Results and Discussion:

1. Isolation, Characterisation and Antimicrobial assay of *Tamarindus indica* leaf extract, *Curcuma longa* stick powder and *Citrus limon* (Lemon) peel powder on *E.coli*:

E.coli is isolated from fecal water and stained as pink rods (gram negative), control plate showed no growth both in isolation procedure and anti microbial assay. Biochemical tests are performed to confirm the positive tests of *E.coli* and found to be indole positive and citrate utilisation test positive. Growth was observed after 24hrs but required growth was observed only after 48hrs of inoculation.

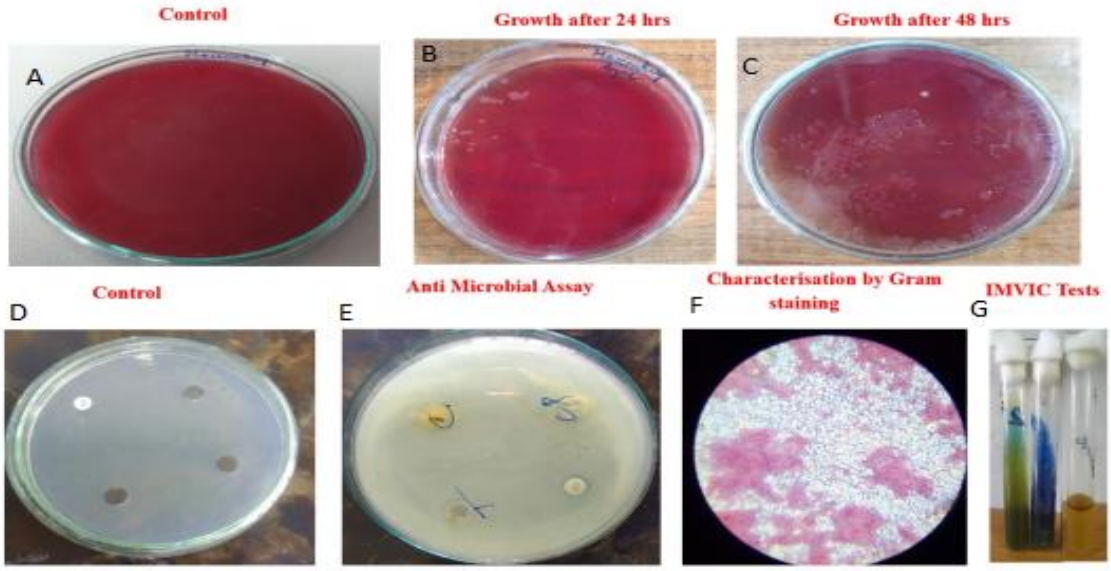

Figure:1 Isolation of *E.coli* from fecal water, Gram staining and Antimicrobial assay using aqueous extracts of *Tamarindus indica* leaf powder, *Curcuma longa* stick powder and *Citrus limon* (Lemon) peel powder. A. control plate showing no growth on Macconkey agar medium.B. Growth of E.coli after 24 hrs and E.coli is a lactose fermenter. C. Growth of E.coli after 48 hrs on Macconkey agar plate. D. Control plate showing no growth with streptomycin antibiotic disc as positive control E. Nutrient agar showing no zones of inhibition with *Tamarindus indica, Curcuma longa* and *Citrus limon* (Lemon). F. Gram staining results. G. IMVIC tests results

Antimicrobial assay was performed using aqueous extract of dried *T.indica* leaf powder, *C.longa* stick powder and *C.limon* (lemon) peel powder to the antimicrobial potential of the extracts on E.coli. Streptomycin is used as positive control. The aqueous extracts are loaded in to wells and

maintained at 37^0c for 24 hrs in an incubator. All the three extracts failed to form zones of inhibition. Aqueous extracts is proved to does not contain antimicrobial activity on E.coli. Absence of zone of inhibition with streptomycin indicates that the E.coli islated is antibiotic resistant strain.

2. Isolation, identification and Antimicrobial assay of *P.vulgaris* using *Tamarindus indica* leaf extract, *Curcuma longa* stick powder and *Citrus limon* (Lemon) peel powder:

P.vulgaris formed mucoid colonies over Macconkey agar + Nacl which selectively promotes growth of *P.vulgaris* inhibiting growth of other gram negative bacteria. *P.vulgaris* is a opportunistic bacteria and found to be indole negative and citrate utilisation test positive. *P. vulgaris* is identifed as gram negative rods with flagella at one end. *T. indica* leaf powder aqueous extract is some what effective against *P.vulgaris* compared to *C.longa* and *C. limon* extracts.

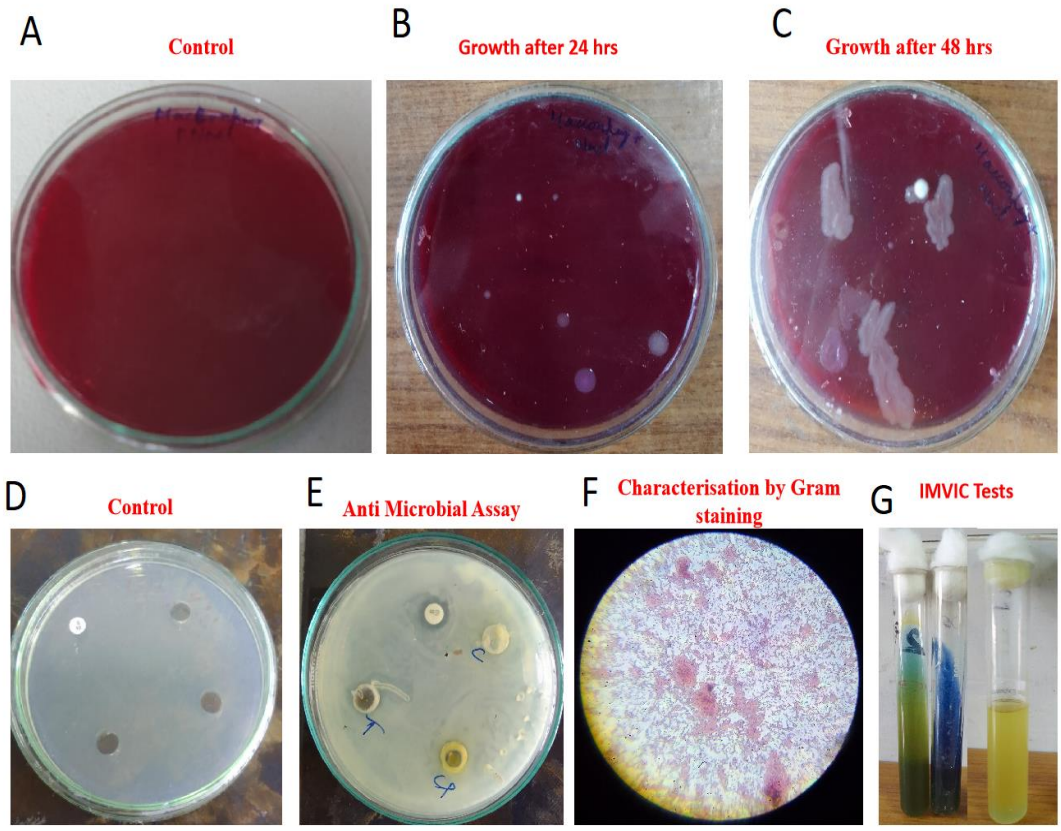

Figure: 2 Isolation, Biochemical characterisation and Antimicrobial assay of *Tamarindus indica* leaf extract, *Curcuma longa* stick powder and *Citrus limon* (Lemon) peel powder. A. Control plate with Macconkey agar + Nacl. B. Growth of *P.vulgaris* after 24 hrs of inoculation. C. *P.vulgaris* with characteristic morphological features after 48 hrs of inoculation on Macconkey + Nacl media plate. D. Control plates with streptomycin disc.E. Antimicrobial Assay of *P.vulgaris* with streptomycin as positive control.F. Gram staining showing pink rods. G. IMVIC tests Showing positive result for citrate utilisation test.

3. Isolation of *S.aureus* from nasal secretions on MSA, IMVIC tests and antimicrobial assay of *S.aureus*:

S.aureus is isolated by using nasal swab and cultured on Mannitol salt agar plate which selectively promotes growth of *S. aureus* while inhibiting growth of all other gram negative and gram positive bacteria.

Significant growth and colony formation was observed only after 48 hrs of inoculation. S.aureus stained as purple cocci in clusters or groups (Gram positive) and found to be indole positive and citrate utilisation test positive. All the three compounds (*Tamarindus indica* leaf extract, *Curcuma longa* stick powder and *Citrus limon* (Lemon)peel powder) does not have any atimicrobial activity over S.aureus but streptomycin showed significant inhibition after 48 hrs of plating.

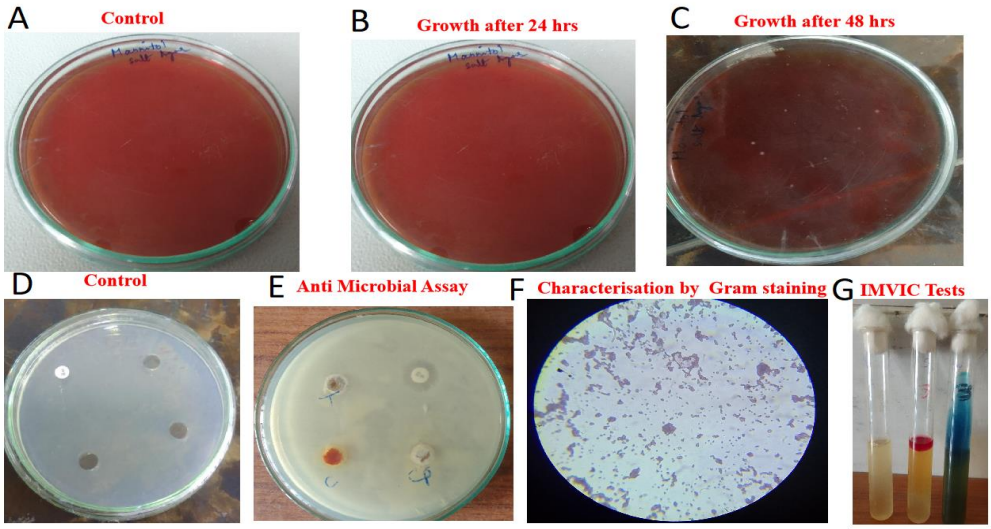

Figure: 3 Antimicrobial assay and Isolation, Selective growth and characterisation of *S.aureus* using MSA, Gram staining and IMVIC tests. A. Control plate showing no growth. B. Culture plate after 24 hrs of inoculation. C. Mannitol salt agar plate showing colonies of *S.aureus*. D. Control plate of Antimicrobial assay. E. Antimicrobial assay of *S.aureus* showing no zones of inhibition F. Gram staining showing Purple colour cocci G. IMVIc tests with control tube for Indole test.

4. Isolation, Antimicrobial assay and characterisation of Fungal strain *S.cerevisiae* using Methylene blue staining:

S. Cerevisiae was isolated from the curd sample and visible growth was obtained after 48 hrs of inoculation and stained as blue colour with methylene blue. Antimicrobial assay was carried out using inoculum from liquid broth and among the three compounds *C.limon (lemon)* peel powder extract was highly effective in killing the S.cerevisiae followed by *T.indica* leaf extract and *C. longa* extract does not showed any zone of inhibition and proved to be ineffective in killing fungi.

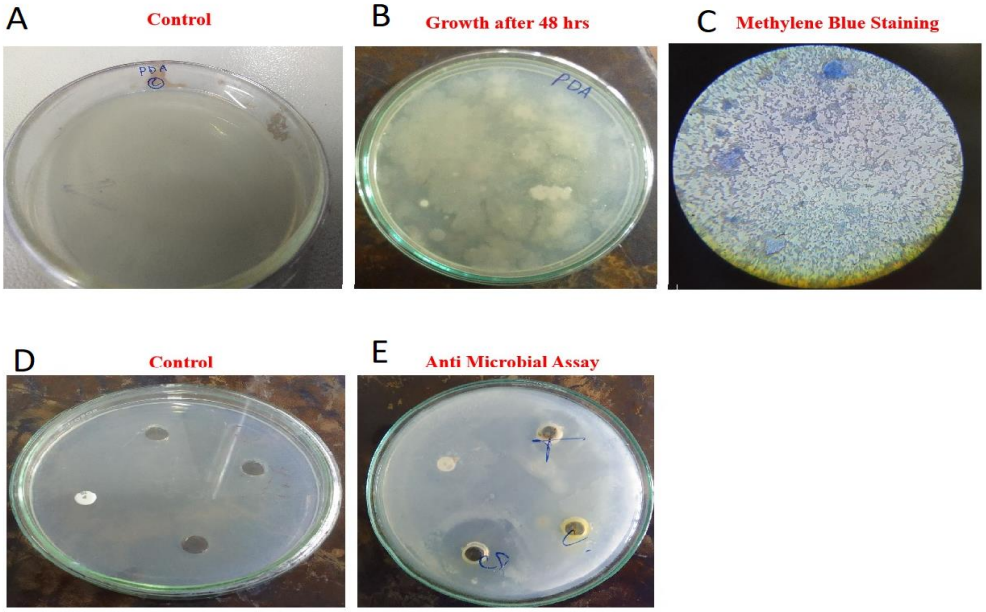

Figure: 4 Isolation, Identification of *S.cerevisiae* by methylene blue staining and Antimicrobial assay using *Tamarindus indica* leaf extract, *Curcuma longa* stick powder and *Citrus limon* (Lemon) peel powder. A. Control plate. B. *S. cerevisiae* growth on Potato Dextrose Agar plate after 48 hrs of inoculation. C. Appearance of S.cerevisiae over slide at 10X objective lens after staining with methylene blue. D. Control plate with pencillin disc. E. Antimicrobial assay showing zones of inhibition with *C. limon* (Lemon) and *T. Indica* aqueous extracts.

5. Isolation of *C.albicans* from mouth and urine samples using PDA and Antimicrobial assay of *C. albicans*:

C. *albicans* is isolated from the Mouth and urinary samples using serial dilution and plating on Potato Dextrose Agar. Lactophenol cotton blue stain was used for staining of hyphae and germ tube formation was observed after 5 days of inoculation. Antimicrobial assay with aqueous extracts of *T. indica*, *C.longa* and *C.limon* peel resulted in zone of inhibition with *C.limon* peel and is effective over candidiasis over pencillin G. C. albicans is isolated from mouth sample with negligible contamination and used as source of inoculum for performing antimicrobial assay.

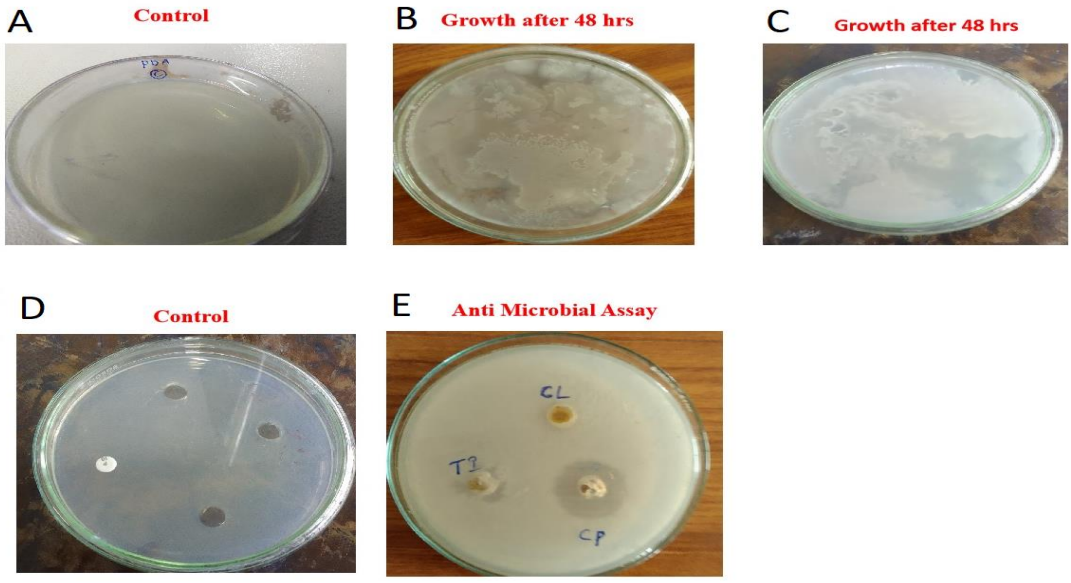

Figure:5 Isolation of *C.albicans* and Antimicrobial assay: A. Control plate for isolation. B. *C.albicans* isolated from Mouth using distilled water. C. *C.albicans* isolated from urine sample. D. Control plate of Antimicrobial assay E. Antimicrobial assay showing zone of inhibition for *C.limon*

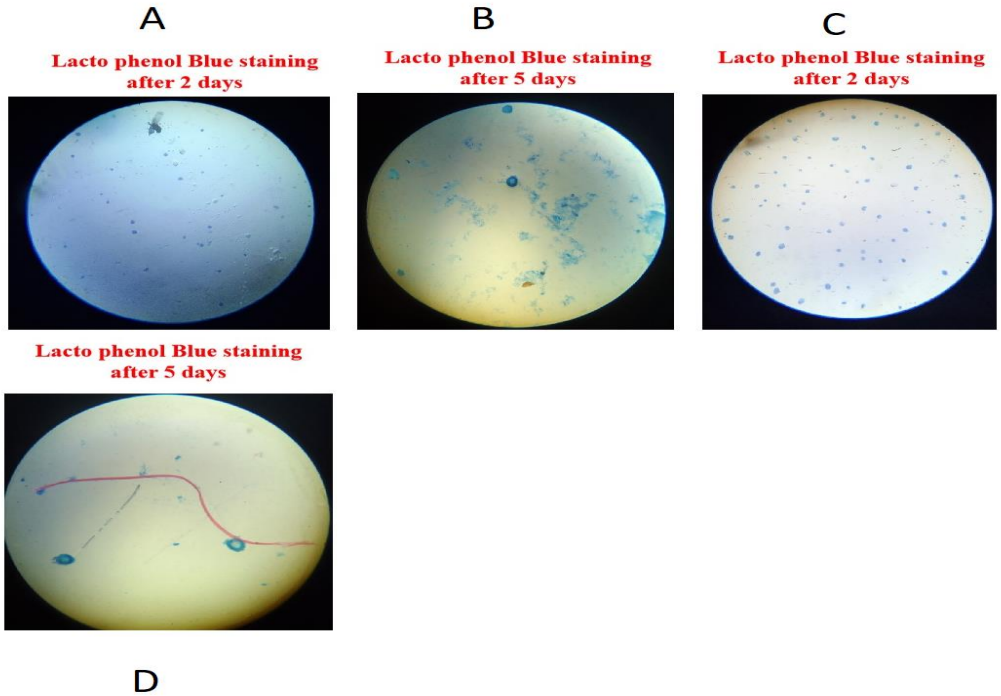

Figure: 6 Staining of Hyphae of *C.albican*s using Lactophenol cotton blue stain. A. *C. albicans* stained with lactophenol cotton blue after 48 hrs of inoculation isolated from mouth sample B. after 5 days with germ tube formation and hyphal growth. C. *C. albicans* stained with lactophenol cotton blue after 48 hrs of inoculation isolated from urine sample. D. after 5 days with germ tube tube formation and hyphal growth.

6. Biochemical tests to confirm *C.albicans*:

C.albicans showed confluent growth circles on sucrose and maltose after 72 hrs followed by glucose and little or negligible growth was observed with starch. As glucose is the readily available source of energy it is utilized immediately than sucrose and maltose resulting in less confluency around the glucose discs.

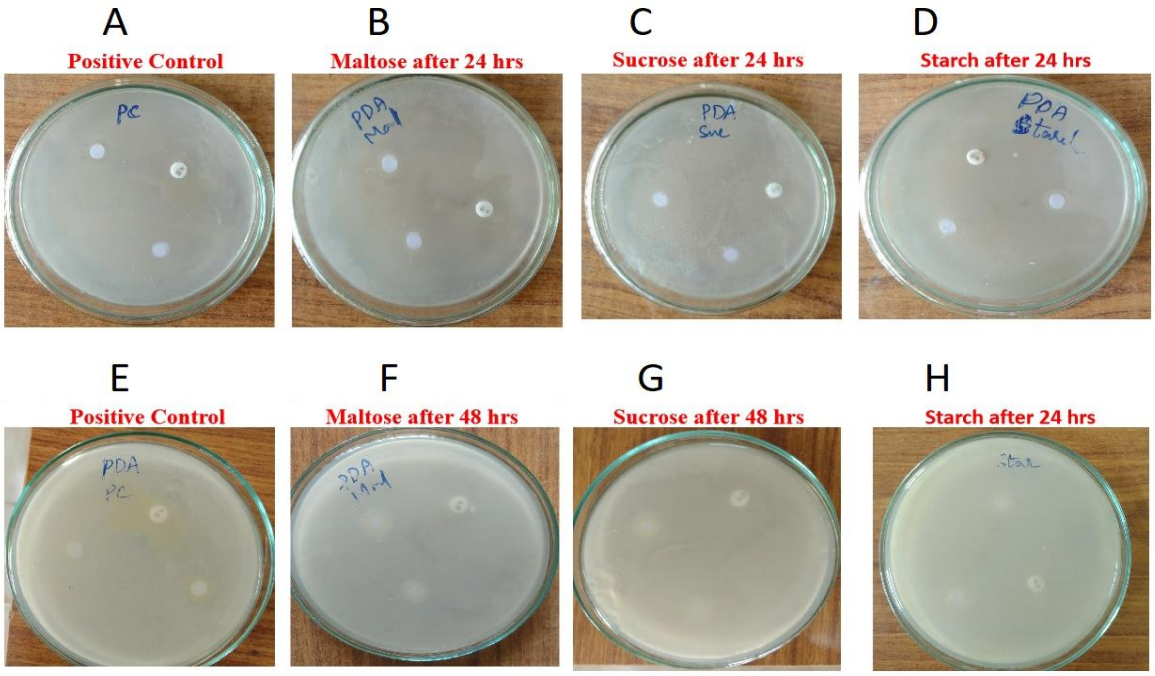

Figure: 7 Biochemical tests for identification of *C. albicans : A. Positive Control (2%Glucose solution). B.* Growth of *C.albicans* around discs overlaid with 2% maltose solution after 24 hrs. C. Growth of *C.albicans* around discs overlaid with 2% sucrose solution after 24 hrs. D. Growth of *C.albicans* around discs overlaid with 2% starch solution after 24 hrs. E, F, G,H. Growth of *C.albicans* around discs overlaid with 2% Glucose, Maltose, sucrose and starch solutions after 48 hrs.

Conclusion:

Development of Antimicrobial compound having broad spectrum activity can reduce the development of antibiotic resistant bacteria and fungi. In current study the antimicrobial compounds is having potent activity over fungal strains especially C.limon Peel on C.albicans and S.cerevisiae followed by T. indica on S.cerevisiae but not on C.albicans.

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