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Chart Review Study of Subjects Administered Amnionic Membrane for Treatment of Joint Pain

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Abstract
Context: Treatment of joint pain with injection of amnionic membrane has not been adequately studied. ***Objectives:*** Determine if patients who received cryopreserved particulate amnionic membrane (CPAM) injected into painful back and knee joints experience less back or knee pain, improve physical ability, and use less opiates and NSAIDs over a 12 week time period. ***Methods:*** Charts were reviewed for 20 consenting patients receiving CPAM, clinically available from tissue banks, injected into joints to relieve pain consistent with the clinical practice at a single center. Ten subjects had back pain, and 10 subjects had knee pain. ***Results:*** VAS pain scores improved from 7.5 to 1.1 over 12 weeks (p<0.001). WOMAC daily activity function score improved from 46 to 11over 12 weeks (p<0.001). Opiate usage decreased from 55% to 15% over 12 weeks (p<0.001). NSAID usage decreased from 80% to 10% over 12 weeks (p<0.001). Location of injection was not a significant covariate factor for any outcome. ***Conclusion:*** Thus, amnionic membrane injection into painful back and knee joints improves pain and physical function, and decreases opiate and NSAID usage for at least 12 weeks.

Key Words : *Amnion, amnionic membrane, joint pain, knee pain, back pain*

Introduction

Joint pain remains a significant debilitating problem affecting over 27 million Americans. OA is associated with ongoing inflammation, oxidative stress and activation of matrix metalloproteases (MMP) that culminate in cartilage degradation (1). Back pain accounts for 149 million work days lost, $40 billion in annual costs, 3 million ER visits costing $9.5 billion and estimated impact of $200 billion per year (2). Of knee and hip OA patients, 25% cannot perform major daily activities and 40% report fair to poor health, ranking high in disability adjusted life years with total knee replacements costing $28.5 billion (3, 4). Existing treatments for joint pain are limited to medical management, injection therapy and surgery. Medications to reduce pain are associated with significant morbidity and social concern. With 259 million opiate prescriptions, 2.1 million people suffer from substance abuse disorders, creating 1000 daily emergencies (5, 6). Opiate overdose death increased 200% from 2000 to 2016 (7). Health care providers face increasing burden and cost of chronic opiate use with increased scrutiny of monitoring patient usage and protecting against abuse.

Non-steroidal anti-inflammatory drugs (NSAIDs), are often the first line of treatment chosen by patients with chronic pain. NSAIDs are associated with gastrointestinal, hepatic, cardiovascular and anti-platelet complications, in part, due to patient overuse and concurrent use of prescribed and over-the-counter medications, producing 100,000 U.S. hospitalizations, 16,000 deaths and $2 billion in costs annually (8, 9). Steroid injections for short term pain relief can cause deleterious effects such as weight gain, exacerbating diabetes, cataracts, osteoporosis, and heighted risk of infections. Disease-Modifying

Anti-rheumatic Drugs (DMARDs), available in synthetic or biologic form, are associated with cancer, pneumonia, tuberculosis and death. Finally, surgery for chronic pain often has limited effect on pain and disability. Clearly, alternative approaches to relief of joint pain are needed.

Background on Amnionic Membrane: Amnionic membrane is clinically available from registered tissue banks globally. The form and methods for preparing amnionic membrane may vary affecting the specific contents and clinical results. Fresh amnion from healthy live births contains regenerative, anti-inflammatory, immunomodulatory and wound healing properties (10, 11). Amnionic membrane contains growth factors, cytokines, extracellular proteins and matrix metalloproteases inhibitors, including prostanoids, GDF-11, Wnt4, PGE2, IL-10, IL-1ra, HGF, VEGF, HGH, EGE, TGFâ, IL4,IGF-1and (TIMPs) (10, 12-18) that suppress cartilage damage (19, 20), stimulate endogenous chondrocyte proliferation for new cartilage (21), provide potent anti-inflammatory and anti-fibrotic effects in OA joint disease (22, 23). Of particular relevance to OA, PGE2 "reprograms" macrophages from the inflammatory M1 phenotype to the anti-inflammatory M2 phenotype (24). Amnionic membrane stimulates many metabolic processes including general protein and collagen synthesis, reducing pain, fibrosis, bacterial colonization and mediating tissue repair. Amnionic membrane is safe in humans and animals (25-29). Uses of amnionic membrane include conjunctival, pterygium, burn, chronic ulcer remodeling, as well as foot, ankle and orthopedic posterior lumbar surgery (11, 29).

Cryopreserved particulate amniotic membrane (CPAM) is cultured, particlized and cryopreserved amnionic membrane from placental tissue. Placental tissue donated by volunteers free of communicable disease undergoing caesarian section is processed to obtain amnionic membrane. The amnionic membrane is minimally manipulated under aseptic conditions, particlized and cryopreserved with DMSO, retaining much of its original matrix microstructure and cytokine profile. The cryopreserved amnionic membrane is used homologously as a protective barrier of membranous tissue placed over damaged joint tissue in patients with osteoarthritic joint pain. Patients receiving CPAM for joint pain have previously failed conservative and conventional therapies like pharmacological and physical therapy, with inadequate improvement of pain, making it medically necessary to proceed with interventional treatment. The standard procedure for therapeutic lumbar inter-laminar epidural injection with CPAM is as follows.

Background on Current Lumbar Inter-laminar Epidural Injection: After patient education and informed consent for treatment, the subject is placed into the prone position on a fluoroscopy table with moderate IV sedation. After confirming ID, site and side, the posterior lumbar region is widely prepped with Chloraprep, and sterile draped. The inter-laminar space at the level and side of the spinal lesion (confirmed by MRI and patient symptomatology) is identified with AP and oblique views fluoroscopically. The skin and underlying subcutaneous tissue are anesthetized with Lidocaine 1%. A 20 gauge, 3.5-inch Touhy needle is introduced and its direction and depth are confirmed with the AP and lateral fluoroscopic views, respectively. The needle is carefully advanced into the epidural space. Following needle placement 0.5 ml's of water-soluble contrast is injected to confirm needle position. Two 1 ml vials of CPAM at -90°C are gradually thawed at room temperature. After alcohol wipe, CPAM is drawn into a 10 cc sterile syringe containing 6 ml of sterile preservative free normal saline, injected over 30 seconds and cleared with 0.5 ml's saline. Post-procedure evaluation involves alertness, pain, stable vital signs (blood pressure, heart rate and oxygenation) and unchanged neurologic status at 15 , 30 and 60 minutes**.** After postoperative instructions, the patient is discharged when in stable condition.

Background on Ultrasound-Guided Knee Injection Utilizing The lateral Supra Patellar Approach: After patient education and consent for therapy, the patient is placed in the supine

position with the knee in extension. The lateral aspect is prepped with Betadine ×3 and sterile draped. The ultrasound probe is placed over the superior aspect of the patella to visualize the bony structures, followed by anesthetic ethyl chloride. One 1 ml vials of CPAM at -90°C is gradually thawed at room temperature. After alcohol wipe, CPAM is drawn into a 5 cc sterile syringe containing 4 ml of sterile preservative free normal saline. A 20-gauge 1.5 inch needle is directed into the suprapatellar bursa under ultrasound visualization, then the CPAM solution is injected under direct visualization. Post procedure evaluation involves alertness, pain, stable vital signs (blood pressure, heart rate and oxygenation) and unchanged neurologic status at 15 minutes, 30 minutes, and 60 minutes. After postoperative instructions, the patient is discharged when in stable condition. While CPAM injections in the knee and back are daily clinical practice at this institution, outcomes have not been previously reported.

Methods

To report the outcomes after injection of CPAM for joint pain, medical charts of 20 consenting adult subjects with joint pain, 10 back and 10 knee, previously treated with amnion at a single institution were reviewed for VAS pain scores, WOMAC daily activity function, opiate usage, and NSAID usage as well as for serious adverse events under an IRB-approved protocol.

Pain was evaluated using the Visual Analogue Scale (VAS) as assessed by the patient at baseline, 1 hour, 24 hours, 1 week, 2 weeks, 8 weeks and 12 weeks, consistent with the standard patient follow-up schedule at this institution.

Pain, stiffness and physical function were assessed using The Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) questionnaire at baseline, 2 weeks, 8 weeks and 12 weeks.

Presence of opiate and NSAID usage were recorded at baseline, 24 hours, 1 week, 2 weeks, 8 weeks and 12 weeks.

Statistical analysis was performed (SYSTAT 13) over time using a repeated measures analysis of variance. Missing data were estimated using multiple imputation for VAS and WOMAC, and "last value carried forward" for opiate and NSAID use. Location of injection, back or knee, was a covariate in the repeated measures analysis. A *p*-value of 0.05 was prospectively determined to represent significance.

Results

Medical records for 20 patients provided substantially complete data regarding demographics and outcomes. Mean age was 61.1 ±11.6 years (range 44-82). Of 10 back subjects, age was 65 ±14 years, range 46-81 years, 6 were males. Of 10 knees subjects, 8 left and 6 right, 4 were bilateral treatments, age was 57 ±6 years, range 44-65 years, 7 were male.

VAS-measured pain improved from 7.475 to 1.002 over 12 weeks ($p<0.001$) (Fig. 1). For back subjects, VAS-measured pain improved from 7.700 to 1.550. For knee subjects, VAS-measured pain improved from 7.250 to 0.553.

WOMAC-measured physical function scores improved from 46.0 to 10.8 over 12 weeks ($p<0.001$) (Fig. 2). For back subjects, WOMAC improved from 48.800 to 14.990. For knee subjects, WOMAC improved from 43.200 to 6.611.

Opiate use decreased from 55% to 15% over 12 weeks ($p<0.001$) (Fig. 3). For back subjects, opiate use deceased from 70% to 30%. For knee subjects, opiate use decreased from 40% to 0%.

NSAID use decreased from 80% to 10% over 12 weeks ($p<0.001$) (Fig. 4). For back subjects, NSAID use decreased from 60% to 10%. For knee subjects, NSAID use decreased from 100% to 0%. Location of injection was not a significant covariate factor for any outcome.

No serious adverse events were reported throughout 12 weeks. All four outcomes depict a consistent result with significant improvement. Extended follow-up averaged 360 days (179), range 122-601. Between 12 weeks and 6 months, one patient at 4 months reinjured his knee playing

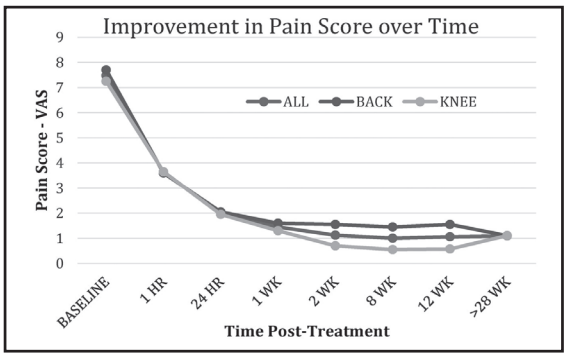

Fig. 1. Visual Analogue Scale (VAS) scores after treatment.

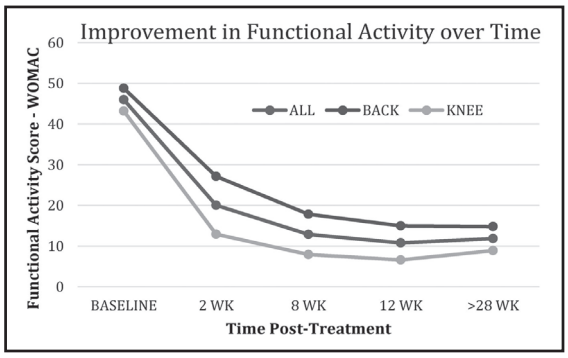

Fig. 2. The Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) scores after treatment.

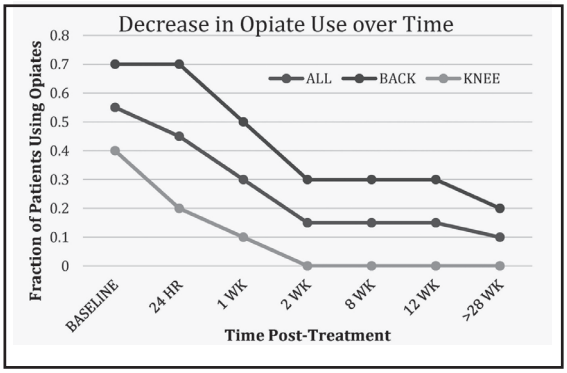

Fig. 3. Opiate use after treatment

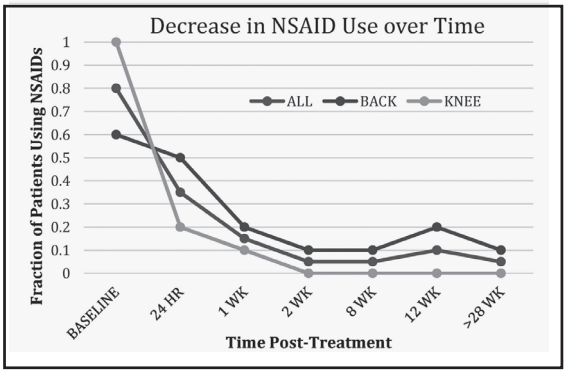

Fig. 4. NSAID use after treatment

tennis and was reinjected at 5 months. One patient at 6 months broke her toe walking her dog with temporary knee pain that resolved in 2 days without intervention. One patient with degenerative disc disease and lumbar spondylosis reported return of pain and was reinjected at 9 months. One patient with facet osteoarthritis and referred pain in the left lower extremity after 10 months requested another injection.Late interventions were without complication.

Discussion

Chart review of 20 patients with joint pain revealed the clinical benefits of injecting CPAM, whether back pain or knee pain. Improvement in pain, physical function, opiate use and NSAID use began promptly after treatment and was sustained over at least 12 week period, in most cases extending to 6 months or greater. Given that opiate use is CDC national epidemic secondary to dependency, overdose and abuse, leading to reluctance in prescribing and difficulty in managing patients on opiates, CPAM offers an important alternative (7).

Prior to treatment, patients were physically deconditioned secondary to their pain, complicating recovery and functional restoration. Therefore, patients were counseled on diet and gradual return to physical activity as critical adjunctive measures to achieving improvement in daily living metrics.

A dose of CPAM costs more than steroid; however, if pain relief from a CPAM injection

extends beyond 12 weeks, CPAM requires fewer procedures than steroids based upon Medicare utilization guidelines, e.g. 3 injections within a 6 month period. Since the costs of the interventional procedure and subsequent follow-up office visits are identical, it is plausible that the overall cost of patient management will be reduced with CPAM.

While this chart review supports the safety and effectiveness of CPAM, chart review has obvious limitations. For example, treatment was not prospectively randomized against a control depicting the current standard of care. Therefore, further research may provide additional confirmation regarding longer term outcome of pain relief, sustained functional activity, dependence on medications with known morbidity, and relative healthcare cost of treating these patients with advanced arthritic pain prior to joint replacement.

Conclusions

CPAM injection reduces pain, physical disability, opiate usage and NSAID usage in patients suffering from back and knee pain.

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Development of Monoclonal Antibodies Against Cry1Ac/Ab Protein for Designing of Sandwich ELISA to Detect BT Toxin from Cotton Seeds and Leaves

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Abstract

The design of the study is to develop monoclonal antibodies against Cry1Ac/Ab protein for designing os sandwich ELISA(hybridoma technology). Hybridoma technology was invented by Cesar Milstein and Georges J.F Kohler in the year 1975 and is an unique method used to produce identical antibodies in maximum quantities. Monoclonal antibodies were developed by immunization of Balb/C mice with Cry1Ac/Ab Protein. Titer values of mice tail bleeds were checked and the best mice with higher titer value was used for fusion. Immunized mice spleen cells were fused with Myeloma cells (SP2-O), using a polyethylene glycol (PEG) and the fused cells were incubated with HAT medium for 12 days and initially 400 positive hybridoma clones were obtained, of which 13 potential clones were selected using indirect ELISA against Cry1Ac/Ab recombinant antigen. Cross reactivity was ruled out using indriet ELSA against cry proteins such as Cry2A, Cry1F and CP4EPSPS using. Cloning was carried out twice for all 13 clones by limiting dilution factor and pure single clones were selected. The class IgG/IgM/IgA and sub classes IgG1, IgG2, IgG3 antibodies are determined by isotyping. Determination of class and subclass of an antibody is very important for selecting proper purification methods. Commercially available rapid isotyping kits were used for isotyping which provides the information of 1) IgG, IgM, IgA, IgG2a, IgG2b or IgG3 2) Light chain identification as either kappa or lambda. All pure clones were preserved in Liquid Nitrogen for future use to develop immunological kits for detection of Cry1Ac/Ab present in the plant tissue.

Keywords: *Bacillus thuringiensis*, Hybridoma Technology, Cry1Ac/Ab, HAT medium, Monoclonal antibodies, Iso-typing, genetically modified crops and Immunization.

Introduction:

The use of entamo-pathogenic microorganisms for regulating insect pests was first proposed at the end of 19th century by several pioneering scientists. The undesirable effects of chemical pests on environment and human population triggered for a change, to utilize the benefits of these biopests which are remarkably nontoxic to humans and to a large extent of non target fauna (1).

Detection of insecticidal properties of *Bacillus thuringiensis* dates back to 1901 by a Japan scientist Ishiwata (2) and was rediscovered in 1911 by Ernst Berliner in Germany. *Bacillus*

thuringiensis is a gram positive, rod shaped, aerobic soil bacterium (3,4). During sporulation it produces crystalline parasporal inclusion bodies (3) which are classified as ä- endotoxin. The toxic effects of ä- endotoxin are restricted only to insects and observed to pose no potential threat to humans (5). The larvicidal activity of *Bacillus thiringiensis* is rapid, but sustained making it an attractive agent for pest control in agriculture. Advances in Genetic engineering resulted in several transgenic plants such as tobacco, cotton, maize, tomato, potato and rice, expressing insecticidal BT proteins that can control target chewing insects (Fearing et al., 1997). When a susceptible insect ingests BT the toxin gets activated by alkaline and enzymatic condition of insect gut and leads to anorexia and death of insect (3,6).

Bacillus thiringiensis was first available as a commercial pesticide in France in 1938 and entered the United States in 1950 (7). BT cotton is the most extensively grown transgenic crop in china and India. In 2005, China grew 3.3 million hectares of BT cotton, occupying about 66% of national cotton area (8).Use of genetically modified plants for agriculture and food production is regulated by national law and novel food regulation guide lines in European countries (6) and similar approach is practiced in other countries as well.

To enforce such regulations, reliable methods for the detection and quantification of GMO in plants and food products is required. Most frequently used method is the amplification of GMO specific DNA by polymerase chain reaction (PCR). Identification of specific amplification requires performing agarose gel electrophoresis, restriction fragment length analysis, or southern blotting (9,10). Even though results are highly sensitive, excuting PCR requires well-equipped laboratory, experienced investigators to optimize results and also time consuming and difficult to quantify (11).

Immunoassay provides an alternative means for the detection and quantification of GMO based on the protein product expressed in their tissues. Different types of ELISA's have been developed to indentify the *B. thiringiensis* toxins in different crops like maize and transgenic cotton (12). Majority of these assays use polyclonal antibodies raised in rabbits (13) or goats (12) Even though polyclonal antibodies are easy to obtain, quantity of polyclonal antisera is restricted from a specific host because antisera from different hosts vary in characteristics. Also polyclonal antibodies are a mixture of immunoglobulins with many different epitope specificities of antibodies. In contrast Monoclonal antibodies are produced specifically to a single epitope of their antigen and can be produced in unlimited quantities. Therefore the aim of the study is to produce monoclonal antibodies against Cry1Ac/Ab protein from *Bacillus thuringenesis.*

Materials and methods

Animals, Chemicals and Reagemts : Balb/c mice used for immunization were obtained from National institute of Nutrition (NIN) Hyderabad, India. Lyophilized Cry1Ac/Ab Bt protein for mice immunization to develop monoclonal antibodies was provided by Amar Immunodiagnostics, Hyderabad. Cry1Ac/Ab positive cotton seed samples were purchased from the local market. Freund's complete, incomplete adjuvant, Hypoxanthine, Thymidine ,Glycine, Aminopterin, Penstrep fetal bovine serum, polyethylene glycol (PEG), Dimethyl sulfoxide (DMSO) solution and Goat anti mouse IgG labeled were purchased from Sigma Aldrich. Antibody isotyping lateral flow strip kit was purchased from Envirologix, USA. Sterile 96 well plates, T-24 plates, T25 flasks and T-75 flasks were purchased from Corning. SP2-O cells were provided by ICGEB. Ab-HRP conjugation kit(Lightning-Link Kit) purchased from Innova Bioscience.

Media and buffers : Complete growth media was prepared by adding 1% RPM I-1640, 1% Pencillin-Streptomycin (10,000 U/mL), 0.2% Sodium bicarbonate $NaHCO_3$, 0.03% L-Glutamine,0.05% 2-Mercaptoethanol (C_2H_6OS), and 10% Fetal bovine serum to final volume of one liter of reagent grade water. HAT media was prepared by adding 10 ml of Hypoxanthine 100X, 5 ml of Thymidine

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Glycine 500X, and 5 ml of Aminopterin to 800 ml of complete growth media. Saline was prepared by adding 0.85g of sodium chloride to 100 ml of reagent grade water. 100 mM phosphate buffered saline with 0.005% Tween-20. Binding or wash buffer: 20 mM Sodium Phosphate with pH 7, and the elution buffer (EB): 0.1M Glycine-HCl with pH 2.7, 1M Tris Buffer and 1N HCL

Mice Immunization : 6-7 weeks old female Balb/c mice were used for immunization. Prior to immunization 100µl control serum was collected from each mice which is also called as pre immune bleed. For primary immunization 120µg of Cry1Ac/Ab antigen in saline, was mixed with equal volume of complete Freund's adjuvant (CFA Sigma) and given intra peritoneal (IP). For the subsequent first and second boosters 120µg of Cry1Ac/Ab antigen in saline was mixed with equal volume of incomplete Freund's adjuvant (IFA Sigma) and injected intra peritoneal (IP). 10days before fusion, final boost was given without adjuvant. After 10 days of third immunization, test bleed was collected from each mice. All test bleeds were serially diluted at 1000 fold dilutions in normal saline and titer values were checked in Cry1Ac/Ab antigen coated plates by using secondary anti-mice antibody labeled with horse radish peroxidase and TMB. The best mice with highest serum antibody titer values was selected and given final booster of 25µg Cry1Ac/Ab antigen intravenously (IV).

Growing SP2-O cells : SP2-O cells were kindly provided by ICGEB and were stored at -180ºC in liquid Nitrogen. The cell were thawed at 37 ºC and grown in standard tissue complete growth (CGM) culture media. After thawing, cells were diluted in Roswell Park Memorial Institute (RPMI) 1640 media and centrifuged at 1200 rpm for 5 minutes. Supernatant was discarded pellet was carefully resuspended in 10ml of CGM and transferred to T-25 flasks (NUNC). Viability of the cells was observed under inverted microscope (Olumpus). The Flasks were incubated in CO_2 incubator with 5% CO_2 at 37 ºC temperature for for 7 days. When the cell density reaches to 1 million per ml, the cells were transferred to T-75 (NUNC) flasks with cultural conditions remain standard. Flasks were observed periodically for contamination. Cell density in the flask should not exceed more than $1x10^6$/ml . After which trypan blue a staining dye is used to check the viability of the cells. Cells are diluted at the rate of 1:2 with Trypan blue solution and number of cells are counted using Haemocytometer counting chamber. The viability of the cells are decided by dye exclusion, and viability above 95% for a cell line is considered.

Preparation of mice spleen cells : The spleen was collected aseptically from a hyper immunized Balb/c mice and chopped using a pair of autoclaved scissors, forceps and diluted in MEM minimal essential media. Single cell suspension was prepared by passing through sieve. The cells were centrifuged for 5 minutes at 1000 rpm in a table top centrifuge. Supernatant was discarded and pellet was washed with Tissue culture media, by repeating centrifugation step. Pellet was resuspended in 10 ml of RPMI solution and small aliquot was diluted at 1:10 with trypan blue solution. Total number of spleen cells were counted using haemocytometer counting chamber and the RBC present along with spleen cells are destroyed by lysis and removed by centrifugation (1200 rpm for 5 min) and the pellet was ready for fusion.

Fusion of myeloma cells with immunized mice spleen cells : Single spleen cells suspension from the immunized mouse are fused with the previously prepared myeloma cells. Fusion is accomplished by adding polyethylene glycol. PEG is a chemical substance that causes cell membranes to fuse. Mix 20 million spleen cells and 50 million myeloma cells in serum-free RPMI 1640. Centrifuge the mixture of cells at 1200rpm for 10 minutes. While the cells are centrifuging, set aside 12 ml of serum-free RPMI 1640 in sterile 50-ml tube. Keep 50% PEG and timer ready in the hood. Remove all the supernatant from the cell pellet. Overlay the pellet of cells with 0.5 ml of 50% PEG with a Pasteur pipette during 1 minute. Then add 5 ml of serum-free RPMI 1640 over 2 minutes followed by 7 ml

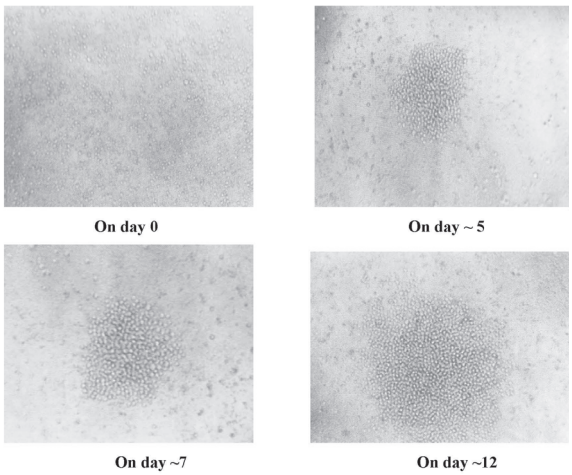

Fig.1 Microscopic view of Hybridomas in different time periods

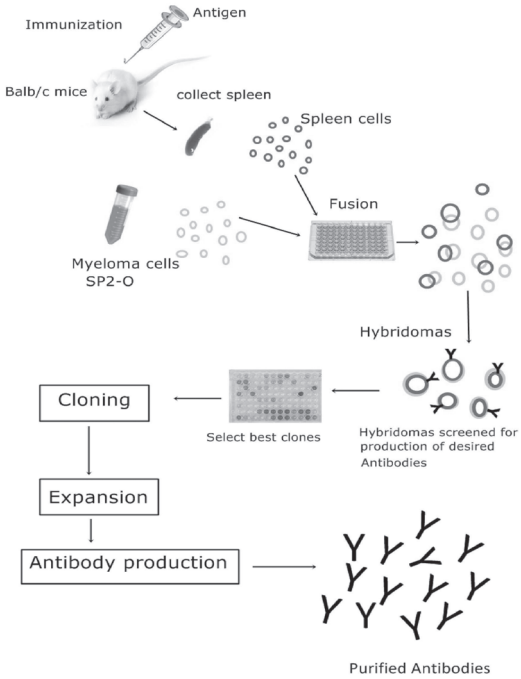

Fig.2. Schematic representation of development of monoclonal antibodies by Hybridoma technology

of serum-free RPMI 1640 over 3 minutes. Centrifuge cells at 1200rpm for 5 minutes and remove supernatant. Resuspend the pellet in HAT media. The cells are then distributed in 96 well plates containing feeder cells collected from saline peritoneal washes of normal BAlb/C mice. Feeder cells are used to supply growth factors that promote growth of the hybridoma cells (14) Fedder cells and fused cells are re-suspended in special selective media called HAT. Cells were allowed to grow for 10 days and tissue culture supernatant was collected and tested for presence of Cry1Ac/Ab antibodies.

Antibody screening: 96 well microtiter ELISA plates were coated with Cry1Ac/Ab antigen in Tris saline at 1µg/ml and incubated over night at room temperature. Plates were washed 2 times with PBS-T and blocked with blocking buffer for 1 hour at room temperature. Decanted the blocking solution, dried the wells and used for antibody screening. 100µl of Hybridoma cell culture supernatant was added to each well of microtiter plate coated with Cry1Ac/Ab antigen and incubated for 60 minutes at room temperature on a shaker, followed by washing (4 times) with PBS-T (phosphate buffered saline with tween 20). Goat anti mouse IgG-HRPO is added and incubated for 30 minutes at room temperature on a shaker followed by washing (4 times with PBS-T) and the TMB (3,3',5,5'-Tetramethylbenzidine) solution is added and incubated for 15 minutes at room temperature.

All positive clones that secrete antibodies against Cry1Ac/Ab protein develop blue color and negative clones remain colorless. Reaction was stopped by adding 1N HCL. The intensity of color was measured by reading microtiter plates at 450-620 nm in ELISA reader (Thermo scientific) and OD values were recorded and the clones with OD values of more than 3 were selected, and those were cloned twice by doing limited dilution factor.

Cloning **:** To obtain a monoclonal cell population from a mass of cells, the cells are abstained by executing a series of increasing dilutions of mother cell culture from master plate. Cloning of hybridomas by limiting dilution factor is widely used method which is fast and easy.

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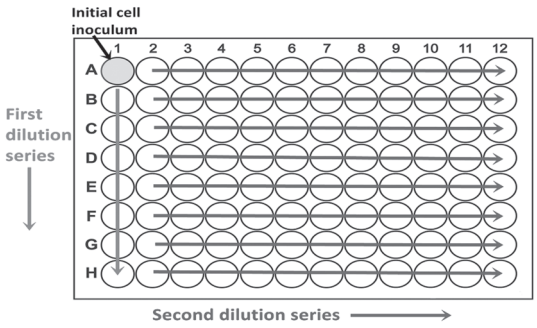

Fig. 4.2. Cloning hybridoma cell line by limiting dilution factor

96 well microtiter plates were used for cloning hybridomas. Sterile reservoir was filled with complete growth medium, using 8 channel pipette 100µl medium was added to all the wells. Approximately 2000-3000 cells of cell suspension from master fusion plate was added to well A1 using single channel pipette. Cell culture in A1 was mixed gently and transferred 100µl to B1 using same pipette, repeated 1:2 dilutions down the entire Column. 100 µl from H1 well was discarded. Then using 8 channel pipette transferred 100 µl from the wells in the first Coloumn (A1 through H1) to those in the second Coloumn (A2 through H2) using the same tips. 1:2 dilution was repeated across the entire plate. Final volume of all the wells were made up to 200 µl by adding 100 µl media to each well except (A12 to H12). All cloning plates were labelled and incubated in CO2 incubator at 37ºC and 5% CO2. Clones were visible under microscope after 4 to 5 days and were screened after 12 days. positive wells were observed under microscope and well contained single clones was marked, those single clones were expanded to T-24 well followed by T-25 flask and cloned 2nd time using Same procedure.

Production of ascites: Monoclonal antibodies can be produced in large quantities by developing acites fluid in Balb/c mice. Injected 0.2 ml of pristine per mice into the peritoneum. After 5-7 days 2 million hybridoma cells per mice were injected intra peritoneal. Tapped the ascites fluid after 2 weeks following the injection of cells, centrifuged and stored clean supernatant at -20°C. Monoclonal antibodies were purified from ascites by using Protein G column (GE health care)

ELISA Micro titer plate : Protein G purified monoclonal antibodies against Cry1Ac/Ab protein were diluted in coating buffer (100mM Tris Saline PH 7.2) at final concentration of 10µg/ml. Added 100µl of diluted antibody solution to microtiter plate, and incubated over night at room temperature. Empty the plate , tapped out the residual liquid and washed one time with 300µl wash buffer (10mM Tris saline with 0.005% Tween 20). 100µl blocking solution was added to each well and incubated 1 hour. Empty the plate , tapped out the residual liquid and packed in air tite pouch with desiccant.

Antibody labeling with horse radish peroxidase enzyme (HRPO) : Antibodies labeled with HRP using lightning link HRP conjugation kit purchased from Innova biosciences. Lightning link technology works by targeting amine groups and can be used for most biomolecules including antibodies. horse radish peroxidase (HRP) conjugation kit allows HRP conjugation to be set up in seconds, simply by adding a solution of antibody to a activated HRP ligand lyophilized powder.10µl of LL modified provided in the kit was added to 100µl (100µg)Ab to be labelled and mixed gently. Remove screw cap from the vial of lightning link mix and antibody solution was pipette directly on to the lyophilized material resuspended gently by withdrawing and redispensing the liquid twice using a pipette. Placed the cap back on the vial and incubated 3 hours. Reaction was stopped by adding 10µl of LL quencher solution and incubated 30 minutes at room temperature. Finally conjugate was resuspended in proper diluent and stored at 2-4ºC.

Protein Extraction from cotton seed and leaf samples

Extraction of protein from seed tissue : Single seed was crushed with hammer and transferred seed powder to 2ml centrifuge tube. 1ml of

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extraction buffer was added to the tube and mixed well. Sample was extracted for 5 minutes at room temperature.

Extraction of protein from leaf tissue : Two leaf punches approximately 20mg tissue was transferred to 1.5ml centrifuge tube. Tissue was ground with pestle until leaf tissue is well ground. 0.5ml of extraction buffer was added and extracted for 5 minutes sample was ready to test.

Results and Discussion:

Characterization of Cry1Ac/Ab antigen purity by SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) : The objective of the study was to immunize the mice with Cry1Ac/Ab pure antigen to develop monoclonal antibodies and to design and develop monoclonal antibody based immune assays for the detection of the Cry1Ac/Ab protein in Bt cotton. Before immunization the purity of the Cry1Ac/Ab protein was determined by SDS-PAGE. SDS results showed the presence of a single protein band with 55kd molecular weight indicating that the purity of the protein was more than 95% which is considered as high purity protein and is used as immunogen to immunize the mice.

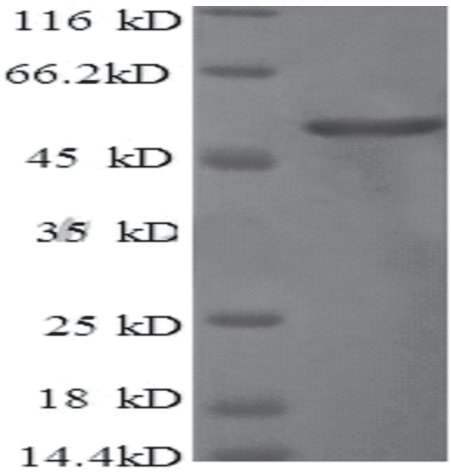

Fig. 3. Analysis of purity of Cry1Ac/Ab antigen by SDS-PAGE Lane 1: Marker. Lane 2: Cry1Ac antigen

Graphical representation of mice bleed titer values : A total of 4 mice were immunized using Cry1Ac/Ab antigen and each of the immunized animal responded differently. Mice with high titer value were selected for fusion. The titer of anti Cry1Ac/Ab specific IgG antibodies in sera of the immunized mice were monitored by collecting the mice bleed after 10 days of each immunization and antibody titer was checked against recombinant Cry1Ac/Ab antigen coated plates (Indirect ELSIA) throughout the immunization process to verify successful immunization and determine the titer values that would be useful for development of monoclonal antibodies. 100 µl of tissue culture supernatant was added Cry1Ac/Ab antigen coated plate and incubated for 60 min at room temperature. Plate was washed using PBS-T and to which 100 micro liters of secondary antibody labeled with HRPO was added and incubated for 30 minutes. Followed by washing with PBS-T. 100 micro liters of TMB substrate was added and incubated for 15 min. Reaction was stopped by adding 1N HCl. The intensity of the yellow colored product was measured at 450-620nm by reading the ELISA plate using ELISA reader. The intensity of the color is corresponded to the amount of specific antibodies in the serum of each animal. Titration curve was generated using dilution factor on X axis and O.D values on Y axis.

Antibody screening methods and preservation of positive clones : Choosing the screening method for selecting the desired Ab secreting clones is very important and critical. In this study all fusion plates were individually screened for the production of Cry Ac/Ab antigen specific antibody using indirect ELISA against Cry Ac/Ab recombinant antigen. After initial screening against recombinant antigen a total of 40 positive clones were selected with OD of more than 2.5 in ELISA. To further conform, all the 40 positive clones were further screened for the production of antibodies using indirect ELSIA against Cry Ac/Ab recombinant antigen. Based on O.D values observed in indirect ELISA, number of clones in the well, size of the clone, cultural characteristics

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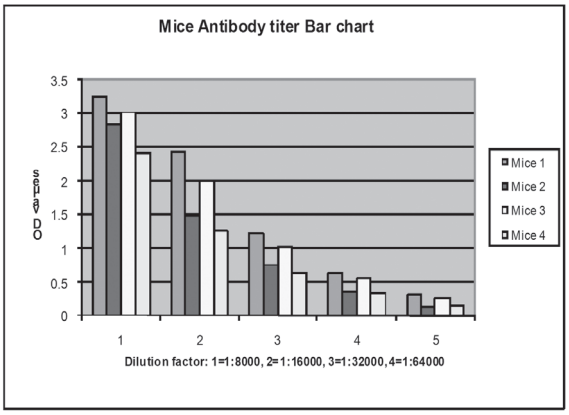
Table:1 Titer values of 4 different mice bleeds checked by ELISA method

S.No	Description	Mice 1	Mice 2	Mice 3	Mice 4
1	Mice test bleed at 1:4000	3.157	3.186	3.123	3.076
2	Mice test bleed at 1:8000	> 3.0	2.922	3.021	2.621
3	Mice test bleed at 1:16000	> 3.0	2.672	2.984	1.461
4	Mice test bleed at 1:32000	2.816	2.452	2.201	1.118
5	Mice test bleed at 1:64000	2.035	1.776	1.229	0.534
6	Mice test bleed at 1:128000	1.173	0.944	0.659	0.271
7	Mice test bleed at 1:256000	0.584	0.452	0.326	0.121
8	Mice test bleed at 1:512000	0.293	0.215	0.163	0.062



Graph.1. Graphical representation of antibody titer values of mice tail bleed.

and rapid doubling time, 13 clones were selected and cultured in T-24 well plate. After 2 days of incubation, supernatant from T-24 well plate was tested against Cry1Ac/Ab protein then transferred and cultured in T-25 flask. Irrespective of single and multiple clone all 13 clones were sub cloned by limiting dilution to ensure monoclonality of the hybridoma. All 13 clones were protected by preserving them in LN2 for further use. After Cryo preservation tissue culture supernatant of 13 different clones were subjected to indirect ELSIA against Cry1Ac/Ab antigens to conform the presence of monocolonal antibodies specific for Cry1Ac/Ab antigen.

Table.2. List of final clones and OD values represents that all 13 clones develops monoclonal antibodies against Cry1Ac/Ab protein

S.no	Clone ID	OD
1	**1F10F5**	3.217
2	**1G8D3**	2.647
3	**2F2C12**	2.321
4	**3AfE1**	1.924
5	**3B9C3**	2.974
6	**3D2B8**	3.471
7	**3E11B11**	2.047
8	**5C7F1**	3.147
9	**5F8F5**	3.262
10	**6H7E4**	2.547
11	**7D2E8H6**	2.742
12	**9G4H8**	2.682
13	**10A8F6**	3.014

Isotyping : The immunoglobulin Isotype refers to the slight phenotypic variations within the immunoglobulin gene family that encode for variant immunoglobulin heavy and light chains. Isotyping involves determining the class and subclass of monoclonal antibody production as it is mandate for selection of appropriate suitable purification method since the antibody obtained form the hybridoma clone is IgM, it can not be purified using protein G or protein A columns. Isotyping requires the use of specific anti immunoglobulin antibodies that are specific to for detecting the different

classes and sub classes of monoclonal antibodies encountered in antibody production. Isotyping of monoclonal antibodies were executed using commercial kit (Envirologix) as per the manufacturer instructions. The kit is used to determine the antibody classes and sub classes (IgG1, Ig2a, IgG2b, and Ig3, IgA or IgM and light chain identity {kappa or lambda}).

Table.3. Isotyping for Cry1Ac/Ab clones.

S.no	Clone ID	Isotype
1	1F10F5	IgG2b k
2	1G8D3	IgG2b k
3	2F2C12	IgG1 k
4	3AfE1	IgG1 k
5	3B9C3	IgG2b k
6	3D2B8	IgG1 k
7	3E11B11	IgG1 k
8	5C7F1	IgG1 k
9	5F8F5	IgG2b k
10	6H7E4	IgG2b k
11	7D2E8H6	IgG2a k
12	9G4H8	IgG2b k
13	10A8F6	IgG2b k

Production of monoclonal antibodies in large quantities by developing Ascites : Ascites fluid is an intraperitoneal fluid collected from the mice peritoneal cavity due to development of peritoneal tumour. Antibodies are produced in large amount from ascites. The tumour is induced by injecting hybridoma cells into the peritoneum which acts as a growth chamber for the cells. The hybridoma cells grow to high densities and continue to secrete the antibody.

Adult male mice of the similar genetic background (Balb/c) were injected with 0.2ml of Pristane (2, 6, 10, 14 tetra methyl deconoic acid) into the peritoneum, as described by Liebermom et al, (1960). These solutions will act as irritants and initiate the mice to secrete nutrients, recirculating monocytes and lymphoid cells into the peritonial area that creates a supporting environment for the growth of the hybridoma cells.

After 7 days of pristine inoculation, the mice are ready to inject hybridomas for ascetic fluid production. The hybridoma cells to be injected in mice for ascites production were grown in tissue culture flask (T-75) to attain the highest cell density (1-1.2 × 10^6/ml). Total number of cells was counted, centrifuge at 1400 rpm for 5 minutes and the supernatant was discarded. The pellet was resuspended in PBS. 2.3×10^6 to 2.8×10^6 hybridoma cells were injected in to each mice intra peritoneally. After 2 weeks following the date of injection of the hybridoma cells, the mice developed a large belly. 5 to 6 ml of fluid per mice was collected using 18-gauge needle attached to 5 ml syringe. After 4 days again 2-3 ml was collected/mice which were considered as second tapping. Ascites fluid was centrifuged at 5000 rpm for 10 minutes to remove cell debris and store at -20ºC.

Purification of monoclonal antibodies through protein G column : Hi-trap protein 'G' HP 5ml column (GE-Health care) was used for antibody purification. Protein G column are made up of polypropylene which is bio compatible and non interactive with biomolecules. The top and bottom parts are made with porous polyethylene. Protein G Sepharose high performance is designed for purification and isolation of monoclonal and polyclonal IgG from ascites, serum and cell culture supernatants. Protein G a cell surface protein G of group G *Streptococci* is type III Fc receptor that binds to the Fc region of IgG by a non immune mechanism. 2 ml of ascites were loaded in 5ml of protein G column and 20 ml of albumin fractions were collected by applying wash buffer (20mM Sodium Phosphate, pH: 7.0) and subsequently IgG were eluted by adding elution buffer (0.1M Glycine-HCl pH 2.7) to the column.

SDS-PAGE was run on 5% ~10% gel, followed by Coomassie blue staining. sample description as follows. **Lane 1**: Cry1Ac Mab clone ID:1F10-F5, **Lane 2**: Cry1Ac Mab clone ID 1G8-D3, **Lane 3**: Cry1Ac Mab clone ID 2F2-C12, **Lane 4**: Cry1Ac Mab clone ID 3A5-E1, **Lane 5**: Cry1Ac

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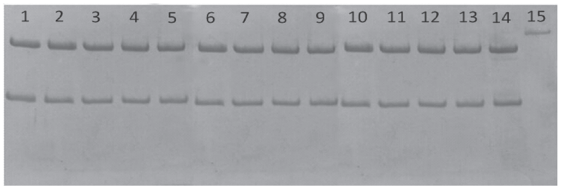

Fig.4. Checking monoclonalantibody purity by SDS-PAGE

Mab clone ID 3B9-C3, **Lane 6**: Cry1Ac Mab clone ID 3D2-B8, **Lane 7**: Cry1AcMab clone ID 3E11-B11, **Lane 8**: Cry1Ac Mab clone ID 5C7-F6, **Lane 9**: Cry1Ac Mab clone ID 5F8-F5, **Lane 10**: Cry1Ac Mab clone ID 6H7-E4, **Lane 11**: Cry1Ac Mab clone ID 7D2-B8, **Lane 12**: Cry1Ac Mab clone ID: 9G4-H8, **Lane 13**: Cry1Ac Mab clone ID: 10A8-F6, **Lane 14** : IgG control, **Lane 15**: Bovine serum albumin (BSA) control.

Sandwich-ELISA for Cry1Ac/Ab Protein: By crosswise testing of the 13 antibodies in a sandwich-type ELISA, clone 1F10F5 was selected as solid phase antibody and 3D2B8 as conjugate antibody.

To determine the limit of detection for Cry1Ab protein, an assay was performed by diluting Cry1Ac/Ab lyophilized protein at different concentration range from 1, 2.5, 5 and 10ng in PBS. PBS was used as a 0nglml control. The results are shown in Figure 5. From these results, the limit of detection was determined as 0.51ngml–1 with a linear range from approximately 1 to 10ngml–1 protein (the limit of detection was calculated as average of the zero values ± three standard deviations of the zero values)

Cross reaction with other Bt Cry proteins : ELISA kit developed was checked for cross reactivity with other Cry protein such as Cry2A, CP4EPSPS (RUR),and Cry1F. These three proteins provided in lyophilized form were initially reconstituted in phosphate buffered saline and then diluted to get concentration range from 100 ng/ml(nanogram) to 2 ng/ml. Cry1Ac/Ab recombinant antigen was used as positive control. OD values at 450nm were recorded. The OD values for Cry1Ac/Ab protein were observed at more than 2.0 and the OD values with other Cry proteins were observed below 0.1 which is similar to PBS blank. Therefore the monoclonal antibodies were Cry1Ac/Ab specific and there is no cross reactivity with other Cry proteins such as Cry2A,CP4EPSPS and Cry1F.

Limit of detection (LOD) was determined by plotting the O.D using standard curve.

Standard value in ng/ml	Absorbance
0	0.009
1.0	0.339
2.5	0.835
5.0	1.646
10.0	2.965

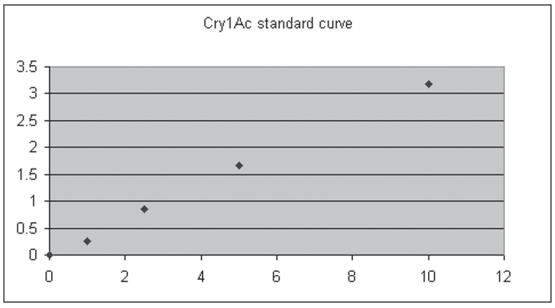

Fig. 5. Calculation of LOD

Discussion:

The objective of this study was to develop mAbs against the insecticidal protein Cry1Ac/Ab from B. thuringiensis and to design sandwich ELISA for the detection of Cry1AC/Ab proteinin in cotton seed and leaf samples.

Although many Bt proteins have been isolated and described (15), the protein which was selected for this study was of special interest in agriculture as biopesticides, particularly due to their use in transgenic plants (EPA, 2001). Only a limited number of insects are targeted whereas soil organisms (16, 17) and beneficial arthropods such as predators (18) or parasitoids (19) remain mostly unaffected. The Purity of the Cry1Ac/Ab

protein was checked by SDS-PAGE. SDS results showed that there is only one protein band visible indicating that the purity of the protein was more than 95%. It was considered as high purity and was used as immunogen to immunize the mice. Total of 4 mice were immunized using Cry1Ac/Ab antigen. The number of booster injections should be limited for the interests of the animal. Usually, a maximum of two or three booster injections are recommended (20). FCA should be used only once because repeated injection of FCA *(Mycobacteria* proteins) may lead to severe tissue reactions. FIA should be used for booster injections Mice with high titre value were selected for fusion.

The titer of anti Cry1Ac/Ab specific IgG antibodies in sera of the immunized mice were monitored throughout the immunization process to verify successful immunization and determine the titer values that would be useful for development of monoclonal antibodies. Titers were performed on the collected bleed from each animal using an indirect non competitive ELISA by coating recombinant antigen on solid phase. The intensity of the yellow coloured product was measured at 450-620nm directly corresponded to the amount of specific antibodies in the serum of each animal. Titration curve was generated using dilution factor on X axis and O.D values on Y axis. Mice with high titer values was selected for fusion. The hybridization or fusion process involves the fusion of mice splenic B cells with histocompatible myeloma cells, such as Sp2/0. Once these hybrid cells are formed and plated into tissue culture wells, the un fused myeloma cells are removed by using a selective medium containing hypoxanthine, aminopterin, and thymidine, known as “HAT. Some hybridomas are unstable and regress. Hence, careful attention should be given to the visual examination of hybridomas using an inverted microscope (21,22). Once Hybridomas are stabilized colony will grow unlimitedly in culture medium (such as RPMI- 1640 with antibiotics and fetal bovine serum) and produce antibody. After 15 days of fusion hybridomas can be propagated in “HT” medium (hypoxanthine and thymidine only) because aminopterin is no longer required (21,22).

Choosing the screening method for selecting the positive clones is very important and critical otherwise numerous unwanted hybridomas will compete for time and nutrients in terms of culture plates and medium (21,22). In this study all fusion plates were individually checked against recombinant antigen. After initial screening against recombinant antigen total 40 positive clones were noted which gave OD of more than 2.5 in ELISA. Those 40 clones were again checked against Cry protein. Based on O.D values observed in ELISA ,13 clones were selected and were expanded to T-24 folled by T-25 and T75 flasks. All clones were cloned twice to get pure single clone.

Isotype determination serves not only to define the immunoglobulin class or subclass but also helps identify the presence of a single isotype for example, IgG1 or a mixture, such as IgM and IgG2b (21).If an antibody is determined to be IgM, in isotyping it can not be purified through protein G or protein A coloumns. Isotyping requires the use of specific anti immunoglobulin antibodies that are capable of detecting the different classes and sub classes of monoclonal antibodies encountered in antibody production.

The up-scaling of MAb production is accomplished by injections of MAb-producing hybridoma cells in the abdominall cavity of mice and then by collecting the ascites that develops after the next 7 to 14 days. The abdominal cavity is serves as an optimal growth chamber for the hybridoma cells because it guarantees a constant temperature, an optimal nutrient and oxygen supply, and the optimal removal of CO2 and metabolic waste products (23). Ascites fluid is an intraperitoneal fluid collected from the mice peritoneal cavity due to development of peritoneal tumour. The hybridoma cells grow to high densities and continue to secrete the antibody. Mab’s were purified from ascites through protein G column and purity was checked by SDS-PAGE. Results indicates that the purity of all mAb’s were more than 95%.

In order to select appropriate mAb for solid phase and to make conjugates all were coated

on solid phase and labeled with HRP. It was conformed that a sandwich ELISA method could be more readily developed for detection of transgenic Bt cotton plants. ELISA developed is Simple qualitative ELISA where presence or absence of Cry1Ac protein can be detected in cotton seed and leaf samples. Results can obtained in 60 minutes. There was no issue of false positive and false negative with the results. To prove that certified known Cry1Ac positive and negative samples were tested by simultaneous comparison with commercially available kit.

The linear range of the assay is approximately 1 to 10ngml–1 protein concentration and the detection limit for purified Cry1Ac/Ab protein was determined as 0.51ngml–1. Since the limits of detection of other published immunoassays for Bt toxins are given in other units, for example ng toxin per microgram soluble protein (Vazquez et al., 1996) or ng of toxin perg dry weight soil (24) they are difficult to compare. However, the detection limit of the assay is in the range which can be expected for an sandwich-type enzyme-linked immunoassay. Based on the determination of three different Cry proteins,

Cross reactivity of all mAB's were checked against other Cry proteins like Cry2A and CP4EPSPS. No cross reactivity was observed.

Summary

In this study 13 different monoclonal antibodies against Cry1Ac protein were developed. When tested in ELISA all antibodies bound to recombinant Cry1Ac protein from *Bacillus thuringeneis* and natural Cry1Ac protein present in cotton leaf & seed. Isotyping of each antibody was done by using commercially available lateral flow strips from Envirologix and all were observed to be IgGk and IgG2bk.

In conclusion,monoclonal antibody based an ELISA for the detection of the B. thuringiensis toxin Cry1AC/Ab was developed. Furthermore, it was demonstrated that this assay can be used for the determination of the Cry1Ac/Ab protein in Bt cotton. No cross reactivity was observed with other Cry proteins such as Cry2A, CP4EPSPS and Cry1F.

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Molecular Characterization and optimization of Bioactive Compounds Production of three Actinomycetes spp Isolated from Waste Dump Soil from Western Uganda

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Abstract

The study was designed to investigate the effect of growth culture conditions, namely: Media composition, incubation period, temperature and pH on the production of antimicrobial compounds from three selected actinomycetes isolated from waste dump soil in Western Uganda. Molecular characterizations of these selected isolates were also carried out. Optimization processes were assessed using shake-flask cultures on eight different media, Portion of filtered fermentation broths were assayed using agar well diffusion method. The remaining portions were extracted using water, ethanol, ethyl acetate and methanol solvents. The extracts were dried and re-dissolved in 2.5% dimethyl sulphoxide to concentration of 2.5mg/ml and tested for antimicrobial activity using agar well diffusion method. Three selected isolates were characterized using conventional PCR and sequenced using Sanger methods. The results showed that, Modified Nutrient broth supplemented with carbon sources (soluble starch and Glycerol at half each) at 12-18g/l, 08-16g/l and 10-18g/l for BRWDc (SP), KBMWDSb6 (M6) and KBRWDSa3 (RF) respectively produced optimum concentration of bioactive compounds. NaCl at concentration of 17-19g/l was found to be suitable for optimum bioactive compound production. The best optimized results were found when cultures were grown under the following conditions: temperature (30-35^{o}C), pH (7.0-7.5) and incubation period (168h). Aqueous and ethanol extracts gave optimum bioactive activity for all the three organisms. The identification of 16SrDNA gene showed that, the three isolates belong to phylum actinobacteria into the genus *Streptomycetes*. This study showed that media compositions, cultural conditions and solvents for extraction play role in bioactive compound production in these *actinomycetes* isolates.

Keywords: Optimization, Actinomycetes spp, Bioactive Compound, Molecular characterization, Waste dump soil, Western Uganda.

Introduction

Over the years, there has been increasing reports of infections with resistant micro-organisms and opportunistic pathogenic infections. This increase has been stated to be among immunocom promised patients with diseases such as HIV, organ transplant, cancer and other conditions (1, 2). In African countries for example in Uganda, there is growing concern on the increasing burden of infectious diseases

among immunocompromised patients and drug resistant pathogens. Literature have shown that, among 35 million populations of Ugandans, 1.14% had HIV and an estimated 9.2% (101,000) have a CD4 count <200 cells/iL (3). Infections caused by bacteria were reported to be 20% and over 1 million cases of fungal infections per year (2, 3). These necessitated the need for search for new antimicrobial compounds to face these current global challenges.

Actinomycete has been shown to be a source of bioactive compounds with pharmaceutical, Agricultural and food industrial importance (4). The ability of this bacteria to produce these important compounds was linked to Non Ribosomal Polyketide Synthase (NRPK), Polyketide Synthase Pathways (PKS) and presence of Larger Genome (>55mol %) with adequate transcription factors which regulates the gene expression which acts in tune of specific environmental requirements (5). Literatures have shown that productions of these compounds depends on genus, species, strains of actinobacteria and could be decreased or increased by using different nutritional, growth conditions and time (6). Extraction solvents could also affect the amount of bioactive compounds (7). Taxonomy of antibiotics producing actinomycetes plays an important role in discovering new antimicrobial compounds (8). Isolation and identifications of actinomycetes using phenotypic methods remain the gold standard but in recent years the process seems to be more accurate using molecular methods (8). The later methods contributed greatly in characterizing novel actinomycetes species and strains from both terrestrial and aquatic environments (9).

This research studied actinomycetes isolated from waste dump soil from Western Uganda and found three isolates ((KBMWDSb6 (M6), BRWDSc (SP) and KBRWDSa3 (RF)) with ability to produce bioactive compounds. The three isolates were tested on bacteria and fungi including drug resistant clinical bacterial isolates (10, 11). This study optimized nutritional requirements, fermentation conditions; extracted fermented broths using different solvents and characterised the selected *actinomycetes* isolates using molecular methods.

MATERIALS AND METHODS

Isolation of Actinomycetes : The three actinomycetes spp were isolated using two different media: Starch casein nitrate agar (12) and yeast extract starch casein agar (YSCA) (13). The pure cultures were maintained on starch casein nitrate agar slants at 4°C for short storage and 30% glycerol at -80°C for long storage (14, 15).

Phenotypic identification of Actinomycetes : The active actinomycetes spp were screened using the API 20A kit (Biomerieux, France) (16) as described in manufacturer's guide and incubated for 48 hours at 37°C in an anaerobic jar after which the results were recorded. The identification was done using the apiweb™ software (V4.0). Other methods used for identification were macroscopic morphology of the colonies, microscopic and conventional biochemical methods (17, 18).

Molecular characterization of actinomycetes

DNA extraction : DNA was extracted from seven (7) day old actinomycetes cultures by suspending the isolates into 3ml of 1XPBS. The suspension was boiled at 90°C for 40 mins and then centrifuged for 5mins at 13000rpm. Five hundred microliter (500µl) of the centrifuged suspension was transferred in to a sterile eppendorf after which 5 µl of RNAase enzyme was added and incubated at room temperature for 10 mins. One millilitre (1mL) of absolute ice cold ethanol was added to the solution and incubated at -80°C for 30 mins. The solution was then centrifuged at 13000 rpm for 10 mins and washed by adding 1ml of 70% ethanol and centrifuged again at 13000 rpm for 10 mins. The solution was then inverted on tissue to dry and after drying, it was eluted in 50 µl of elution buffer.

Amplification of extracted DNA and sequencing : The 16S rDNA gene of three selected actinomycetes was amplified according to the method (19). The following primers 27F

(5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'ACGGCTACC TTGTTACGACTT 3') were used to amplify the extracted DNA. All PCR reactions were performed in a Perkin Elmer DNA thermal cycler (Perkin Elmer 480).

PCR products were washed by precipitation before sequencing and quantified using NanoDrop ND-1000 (Wilmington, DE, USA).Sequencing reactions were carried out using final volume of 7 µl solution containing 5.5µl of nuclease-free water, 0.5µl of (40 ng/µl) primer and 1µl of (100 ng/µl) of DNA template, BD terminator (ABI). The reaction products were analysed using ABI 3500XL Genetic Analyser, POP7™, BigDye® 3.1. Amplification and sequencing reactions were done at Inqab Biotechnological (Africa's Genomic Company) South Africa. The 16S rDNA sequence gene data of the potent actinomycetes were compared to the nucleotide sequences in Gene bank data base in National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov) using Basic Local Alignment Search Tool (BLAST).

Phylogenetic analysis : The Phylogenetic and molecular evolutionary analyses were conducted using MEGA software (version 6) (20).

Optimization for antimicrobial production by three selected actinomycetes

Effect of different media and fermentation period on antimicrobial production : Fermentation was carried out using eight different media in shake-flask culture method (6) in Erlenmeyer flask (500ml). The media used included: **1.** Modified Nutrient broth (MNB) Thermo scientific oxoid (g/l: Lab-lemoco powder 1, Yeast extract 2, peptone 5, Soluble starch 8, Glycerol 3, K_2HPO_2 0.5, NaCl 5, $CaCO_3$ 0.75, $MgSo_4.7H_2O$ 0.5 and pH 7.0±4), **2.** Yeast extract starch broth (YESB) (Composition in media g/l: yeast extract 3, peptone 3, casein 3, starch 8, Glycerol 3, K_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.5, NaCl 12 $CaCO_3$0.75, and pH 7.0±4), **3.** Modified Potato Dextrose broth (MNB) HiMedia-MO96-500G (g/l Potatoes infusion 200, Dextrose 20, Glycerol 3, $CaCO_3$ 0.75, NaCl 4.8, $MgSO_4.7H_2O$ 0.5, K_2HPO_4 0.5 and pH adjusted 7.0± 2**), 4.** Starch casein nitrate broth (SCNB) (Composition in media g/l: Starch 10, Casein 0.3, KNO_3 2, NaCl 2, K_2HPO_4 2, $MgSO_4.7H_2O$ 0.5, $CaCO_3$ 0.02, $FeSO_4.7H_2O$ 0.01 and pH adjusted 7.0 ± 2), **5.** Starch Casein broth (SCB) (the composition in g/l: Soluble starch 10.0; casein 0.3; $NaNO_3$ 2.5; K_2HP0_4 1.0; KH_2PO_4 1.0; $MgSo_4.7H_2O$, 0.5; KCl, 0.5; trace salt solution 1.0 ml : $CuSO_45H_2O$ 0.64; $FeSO_4.7H_2O$, 0.11; $MnCl_2.4H_2O$ 0.79, $ZnSO_4.7H_2O$ 0.15 and pH adjusted 7.0±2), **6.** Starch nitrate broth (SNB) (Composition in media g/l: Starch 20, KNO_3 1, NaCl 0.5, K_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.5, $FeSO_4.7H_2O$ 0.1 and pH adjusted 7.0± 2), **7.** Glycerol Casein broth (GCB) (the composition in g/l: Glycerol 10.0; casein 0.3; $NaNO_3$ 2.5; K_2HP0_4 1.0; KH_2PO4 1.0; $MgSo_4.7H_2O$, 0.5; KCl, 0.5; trace salt solution 1.0 ml : $CuSO_45H_2O$ 0.64; $FeSO_4.7H_2O$, 0.11; $MnCl_2.4H_2O$ 0.79, $ZnSO_4.7H_2O$ 0.15 and pH adjusted 7.0 ±4) and **8.** Glycerol arginine broth (GAB) (the composition in g/l: Glycerol 12; arginine 1; NaCl 1 K_2HP0_4 1.0, $MgSo_4.7H_2O$, 0.5; $CuSO_45H_2O$ 0.001; $FeSO_4.7H_2O$, 0.001; $MnCl_2.4H_2O$ 0.001, $ZnSO_4.7H_2O$ 0.001 and pH adjusted 7.0 ±2). Seven days old culture of actinomycete sp was inoculated and incubated in a digital gas bath thermostats oscillator (THZ-82B) at 28°C and 200 ± 5 rpm. Aliquots of broth were obtained and checked for the antimicrobial activity at intervals: 24, 48, 72, 96, 120, 144 and 168h of incubation to study the effects of incubation period on antimicrobial production.

Effect of pH and temperature on antimicrobial production : At the end of the optimization studies, one media was selected to study the effect of pH and temperature on the production of bioactive compound(s). Modified Nutrient broth medium was selected for this study. The varying pH and temperature used were as shown pH (5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9) and incubation temperature (15°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C and 50°C). Seven days old culture of actinomycete sp was inoculated and incubated in a digital gas bath thermostats oscillator (THZ-

82B) at 200 ± 5 rpm for 7 days after which the broth was centrifuged at 3000rpm for 20 minutes and filtered using filter paper (Whatman No. 1) and tested for antimicrobial activity using agar well diffusion method (21).

Effect of different carbon source on antimicrobial production : Effects of different carbon source were studied on Modified Nutrient broth according to the method (21). The different carbon sources used were: (10g/l): Soluble Starch, Glucose, Glycerol, Lactose, and Sucrose. A second trial was carried out by combining carbon sources as: (5g/l each): Starch and Glucose, Starch and Glycerol, Lactose and Sucrose were added to Nutrient broth medium while all other parameters were kept constant. The tests medium were inoculated with seven days old culture of actinomycete sp and incubated at 28°C in a digital gas bath thermostats oscillator (THZ-82B) at 200 ± 5 rpm for 7 days. After incubation the broth was centrifuged at 3000rpm for 20 minutes and filtered using filter paper (Whatman No. 1). The filtrate was tested for antimicrobial activity using agar well diffusion method.

Effect of different concentration of starch and glycerol combination on antimicrobial production : The effect of glycerol on media containing combine carbon were tested at different concentrations: soluble starch and glycerol at half concentration of each (g/l: 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20) were added to Modified Nutrient broth, other parameters remained constant. The broth was inoculated with seven days old culture of actinomycete sp and incubated at 28°C, 200 ± 5 rpm for 7days. After incubation the broth culture was centrifuged at 3000rpm for 20 minutes and filtered. The filtrate was tested for antimicrobial activity using agar well diffusion method (21).

Effect of sodium chloride and nitrogen source concentration on antimicrobial production : Effects of different concentrations of NaCl on production of bioactive compound were studied according to the method (22). The different concentration of the NaCl and Nitrogen source (Peptone) used were: (g/l: 5, 7, 9, 11, 13, 15, 17, 19, 21 and 23) on Modified Nutrient broth. All other parameters were kept constant. The broth media was inoculated with seven days old culture of actinomycete sp and incubated at 28°C, 200 ± 5 rpm for seven day. The broth cultures were centrifuged at 3000rpm for 20 minutes and filtered. The filtrate was tested for antimicrobial activity using agar well diffusion method (21).

Antimicrobial activity of fermented broths

Test organisms : The following test organisms were used to determine the antimicrobial activity of all fermented broths and different solvents extracts: drug (s) resistant clinical bacterial isolates (*Escherichia coli* 2966, *Pseudomonas aeruginosa* 2929, *and Staphylococcus aureus* 2876) and Standard drug sensitive fungus *Candida albicans* ATCC1023), (obtained from Department of Medical Microbiology Makerere University, Kampala).

Antibacterial and antifungal assay : Cell concentration of test organisms was adjusted to 0.5 McFarland turbidity standards. Bacterial cultures were inoculated on Mueller Hinton agar (HIMEDIA M173-500G), fungal cultures on potato dextrose agar plate. Wells were bored using sterile 1000 µl micro pipette tip (17). The wells were filled with 100 µl of supernatant and the plates were incubated at 37°C for 24 h and 72h at 28°C for bacteria and fungus respectively. Freshly prepared broth media was used as negative control. All experiments were performed in triplicates.

Extraction of bioactive compounds using different solvents : Extraction of bioactive compounds was carried out according to the method earlier described by (8, 17). The remaining centrifuged fermented filtered broth of each isolate was divided into four. Three parts were extracted using three solvents by adding equal volume (1:1) of ethanol (95%), ethyl acetate (95%) and Methanol (95%). The solution was shaken vigorously on a rotatory shaker. The solvent phase was collected and evaporated in hot air oven at 40°C. The remaining one part of the filtered broth was concentrated to get aqueous

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extract. The filtrates were dried and stored at 4°C for further studies.

Antimicrobial activity of different solvent crude extracts : The dried crude extracts (0.43g/200ml, 0.38g/200ml and 0.40g/200ml) for BRWDc (SP), KBMWDSb6 (M6) and KBRWDSa3 (RF) respectively, were re-dissolved in 2.5% dimethyl sulphoxide (DMSO) (THOMAS BAKER) (23, 24) to a concentration of 2.5mg/ml of each extract which was used for antibacterial and antifungal activity (17). Cell concentration of test bacteria and *C. albicans* was adjusted to 0.5 McFarland turbidity standards and inoculated on Mueller Hinton agar (HIMEDIA M173-500G) for bacteria and potato dextrose agar plate for fungus. The test organisms were then spread on to respective media plates. Wells were bored using sterile 1000 µl micro pipette tip and filled with 100µl of 2.5mg/ml ethanol crude extract, ethyl acetate crude extract, Methanol crude extract and aqueous crude extract and 2.5% DMSO was used as negative control. Plates were incubated at 37°C for 24 h and 72 h at 28°C for bacteria and fungus respectively. At the end of incubation, the zones of inhibition were measured. All experiments were performed in triplicates.

Statistical analysis : The data was analysed using GraphPad software (Version 5.04). Results of mean zone of inhibition on the effects of different media used, carbon source, growth temperature, incubation time, pH, nitrogen source, NaCl concentration and different solvents crude extracts were analysed using one way ANAVO using multiple comparison and $p \leq 0.05$ was considered to be significant.

Results and discussion

The results of Analytical profile index using API 20A showed that only actinomycete isolate KBRWDSa3 (RF) can ferment the sugar anaerobically (Table 1). The analysis of this isolates using APIwab™ showed that it's belonging to actinomycetes genus. This also showed that isolate KBRWDSa3 (RF) is a facultative organism. The inability of the isolates BRWDSc (SP) and KBMWDSb6 (M6) to ferment the sugars anaerobically showed that they are either obligate aerobic organisms or other genus of actinobacteria. However, this could also be as result of kit used for the identification which is selective to only actinomycetes genus.

The molecular characterization of the three broad spectrum active actinomycetes isolates were determined and PCR amplification of genomic extracted DNA using actinomycetes forward and reverse primers produced 1600bp for all the three isolates (Fig. 1). The amplified PCR products were also sequenced and produced 885bp, 881bp and 860 bp, of sequences for KBMWDSb6 (M6), BRWDSc (SP) and KBRWDSa3 (RF) respectively. The resultant sequences were BLAST using MEGA software with nucleotide BLAST search in NCBI. The sequences that produced significant alignment

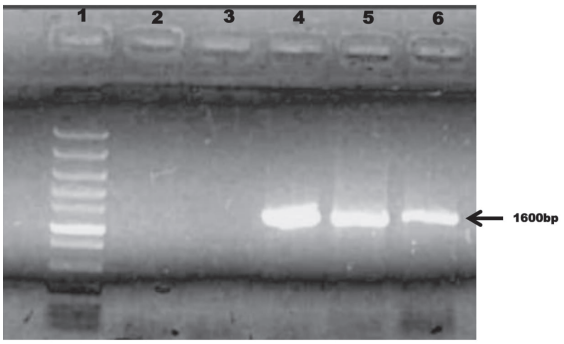

Fig. 1 : Agarose gel showing PCR amplification of 16S rDNA of the actinomycetes isolates: 1: DNA molecular weight ladder, 2: Negative control, 3: MBJ, 4: FR: KBRWDSa3 (RF), 5:KBMWDSb6 M6), 6: BRWDSc (SP).

results showed that actinomycetes isolates KBMWDSb6 (M6) and BRWDSc (SP) were 98% and 97% respectively similar to *Streptomyces* sp AQB.SKKU 20 with accession number JN836957.1. However, these were also found be to be 97% similar to *Streptomyces* sp. MSU 2261 with accession number AY232829.1. Isolate KBRWDSa3 (RF) was found to be 99% similar to *Actinomycete* TGsR-01-04 with accession number AB775551.1 and 33 *Streptomycetes* spp including

Uncultured *Streptomyces* sp. clone 2516 FJ429559.1. The sequences were deposited to Genbank with accession numbers: MG594793, MG594794 and MG594795 for KBMWDSb6 (M6), KBRWDSa3 (RF) and BRWDSc (SP) respectively. The phylogenetic tree analysis based on 16S rDNA was constructed using the Maximum Likelihood method (Fig. 2). The tree result showed that the two isolates (KBMWDSb6 (M6), and BRWDSc (SP)) formed cluster with *Streptomyces* sp. MSU 2261 while KBRWDSa3 (RF) isolates occupied new position in the tree but belong to genus *Streptomycetes*. Base on the phenotypic and molecular data, the isolate KBRWDSa3 (RF) was suggested to be new *Streptomycetes* MG594794 sp

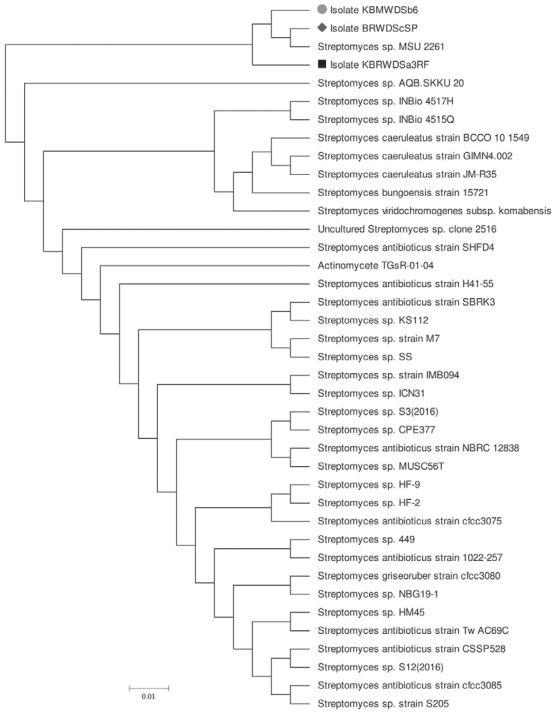

Fig. 2: A phylogenetic tree was constructed using Maximum Likelihood method bootstrap test (100 replicates). The tree showed the position of isolates KBMWDSb6 (M6), BRWDSc (SP) and KBRWDSa3 (RF) with closely related *Streptomycetes* species.

The ability of actinomycetes spp to grow and produce antimicrobial compounds depends on the media compositions and incubation conditions (6, 21). This research has also shown that, selection of media and incubation conditions for production of antimicrobial compounds is necessary.

Out of the eight different fermentation media : Modified Nutrient broth(MNB), Yeast extract starch broth (YESB), Modified Potato Dextrose broth (MPDB), Starch casein nitrate broth (SCNB), Starch casein broth (SCB), Starch casein arginine broth (SCAB), Glycerol casein nitrate broth (GCNB), Glycerol arginine broth (GAB), three actinomycetes spp (BRWDc (SP), KBMWDSb6 (M6) and KBRWDSa3 (RF) isolates grown on two media MNB and YESB were found to support the production of antimicrobial compound (Fig.3). Actinomycete sp KBMWDSb6 (M6) fermented in all the media tested but did not show activity to resistant clinical isolates *S. aureus* 2876 while actinomycete spp (BRWDc (SP) and KBRWDSa3 (RF) fermented in all media showed no activity against two clinical resistant isolate *P. aeruginosa* 2929 and *E. coli* 2966. It was also observed that, all the three actinomycetes fermented broths showed activity to standard test fungus *Candida*

Table 1: Analytical profile index of active actinomycetes isolates (Using API20A kit)

Active Isolates	IND	URE	GLU	MAN	LAC	SAC	MAL	SAL	XYL	ARA	GEL	ESC	GLY	CEL	MNE	MLZ	RAF	SOR	RHA	TRE	CAT
BRWDSc (SP)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
KBMWDSb6 (M6)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
KBRWDSa3 (RF)	-	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	-	+	+	+

KEY: IND: Indole, URE: Urease, GLU: Glucose, MAN: Mannitol, LAC: Lactose, SAC: Saccharose, MAL: Maltose, SAL: Salicin, XYL: Xylose, ARA: Arabinose, GEL: Gelatin, ESC: Esculin, GLY: Glycerol, CEL: Cellobiose, MNE: Mannose, MLZ: Melezitose, RAF: Raffinose, SOR: Sorbitol, RHA: Rhamnose, TRE: Trehalose, CAT: Catalase.

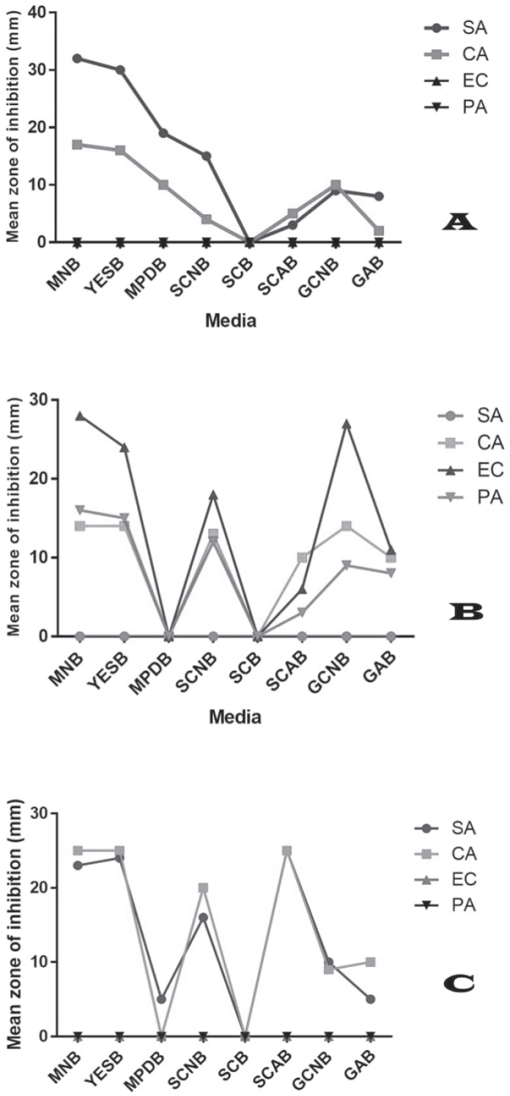

Fig. 3: Media selection for antimicrobial productions. MNB: Modified Nutrient Broth, YESB: Yeast extract starch broth, MPDB: Modified Potato Dextrose broth, SCNB: Starch casein nitrate broth, SCB: Starch casein broth, SCAB: Starch casein arginine, GCNB: Glycerol casein nitrate broth and GAB: Glycerol arginine broth. A: BRWDSc(SP), B:KBMWDSb6 (M6), C: KBRWDSa (RF), EC: *E. coli* 2966, PA: *P. aerugenosa* 2929, CA: *C. albicans* ATCC1023, SA: *S. aureus* 2876. The results of multiple comparisons showed statistical significance at p value $\leq$ 0.05 using one way ANOVA.

*albicans*ATCC1023. The zone of inhibition of the three actinomycetes spp from all test media ranged from 02 to 32mm. The results showed that the three selected isolates produced low amount of antimicrobial compound in 71.42% of the media used. The p values (0.0051, 0.0002, and 0.0003 for KBMWDSb6 (M6), KBRWDSa3 (RF) and BRWDc (SP) respectively) of multiple comparison between the different media used for extraction of antimicrobial compound showed statistical significance at $p \leq 0.05$. This confirmed the reports of (6, 21) who also found out that, different actinomycetes isolates from different environments do not produce bioactive compounds in all test media.

The results on different incubation periods showed that antimicrobial production of the three isolates started after 72h of incubation but maximum production was obtained after 168h (Fig. 4). This could be as a result of the depletion the nutrients from medium after 168h of incubation which enhance the production of antimicrobial compounds by actinomycetes (25). This was contrary to the findings of (6) who reported that, maximum productions of antimicrobial compounds by *Streptomyces* strain ERI-1, ERI-2 and ERI-3 were obtained after 96h of incubation. The p values (0.0133, 0.0012, and 0.0006 for KBMWDSb6 (M6), KBRWDSa3 (RF) and BRWDc (SP) respectively) of multiple comparisons on the influence of incubation time on antimicrobial production showed statistical significance at $p \leq 0.05$. This showed that different actinomycetes isolates require different growth conditions for optimum production of bioactive compounds.

Modified Nutrient broth medium was selected for study on the effect of different carbon source on the production of antimicrobial compounds. The combined carbon source (Starch and Glycerol) gave a higher production of antimicrobial compounds when compared to the other carbon sources used (Fig.5). The p values (0.1026, for KBMWDSb6 (M6) and 0.0001 for KBRWDSa3 (RF) and BRWDc (SP)) of multiple comparisons on the effects of different carbon source on antimicrobial production showed statistical

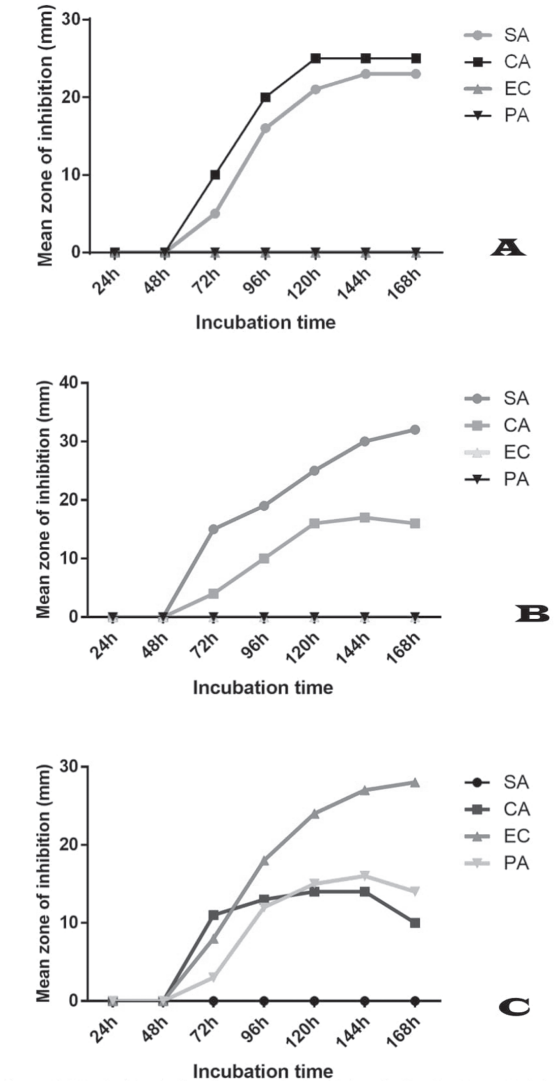

Fig. 4: Effect of incubation time on antimicrobial production by three selected actinomycetes spp. on modified nutrient broth medium, A: BRWDSc (SP), B: KBMWDSb6 (M6); C: KBRWDSa (RF). EC: *E. coli* 2966, PA: *P. aerugenosa* 2929, CA: *C. albicans* ATCC1023, SA: *S. aureus* 2876.The results of multiple comparisons showed statistical significance at p value ≤ 0.05 using one way ANOVA.

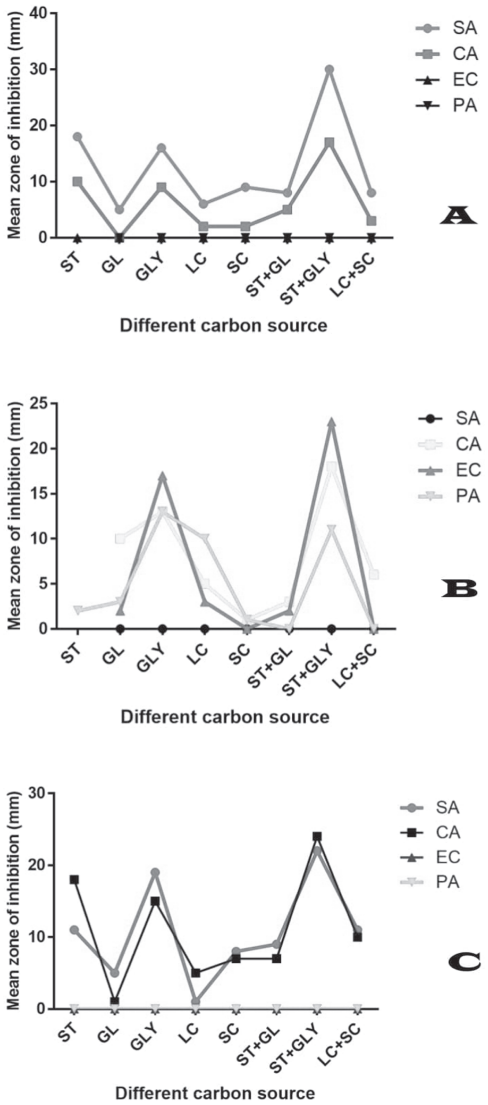

Fig. 5: Effect of different carbon source on production of antimicrobial compound in modified nutrient broth medium. A: BRWDSc (SP), B : KBMWDSb6(M6); C : KBRWDSa (RF). EC: *E. coli* 2966, PA: *P. aerugenosa* 2929, CA: *C. albicans* ATCC1023, SA: *S. aureus* 2876, ST: starch, GL: Glucose, GLY: Glycerol, LC: Lactose, SC: Sucrose, ST+GL: Starch and Glucose, ST+GLY: Starch and Glycerol, LC+SC: Lactose and Sucrose. The results of multiple comparisons showed statistical significance at p value ≤ 0.05 using one way ANOVA.

significance at $p \leq 0.05$ for KBRWDSa3 (RF) and BRWDc (SP) while statistical insignificance was observed on the isolate KBMWDSb6 (M6). This result was similar to those of (26, 27, 28) though they used the two carbons separately. Arasu*et al.* (6) reported the maximum antimicrobial production by *Streptomyces* spp from medium with glucose and fructose combinations. However, studies by (29, 30) reported that media containing single carbon such as Glucose, Sucrose, and Lactose supported higher production of bioactive compounds.

Different concentrations of selected carbon source (Soluble starch and Glycerol) were also studied for their influence on the production of antimicrobial compounds. The results are shown on (Fig. 6). The influence of combine Starch and Glycerol concentrations on the production of antimicrobial compounds ranged from 12-18g/l, 08-16g/l and 10-18g/l for BRWDc (SP), KBMWDSb6 (M6) and KBRWDSa3 (RF) respectively. These combinations gave better results than those of single carbon sources. The p values (0.0081, 0.0057, and 0.0011for KBMWDSb6 (M6), KBRWDSa3 (RF) and BRWDc (SP) respectively) of multiple comparisons on the effects of different concentration of combine carbon source (Starch and Glycerol) on antimicrobial production showed statistical significance at $p \leq 0.05$. Similar finding were reported by Sengupta *et al.*(27). He used, yeast extract medium combined with two carbon source (starch 8g/l and Glycerol 3g/l) though different concentration on antimicrobial production was not established.

The effects of temperatures on the production of antimicrobial compounds are shown on (Fig. 7). The minimum temperature for the antimicrobial production was found to be 25°C while the maximum temperature was 35°C. It was observed that, higher antimicrobial activity was when cultures were grown at 30-35°C. The ability to produce antimicrobial compounds below room temperature (20°C) could be due to their adaptations to the environments where they were isolated. The p values (0.0945, 0.0400, and

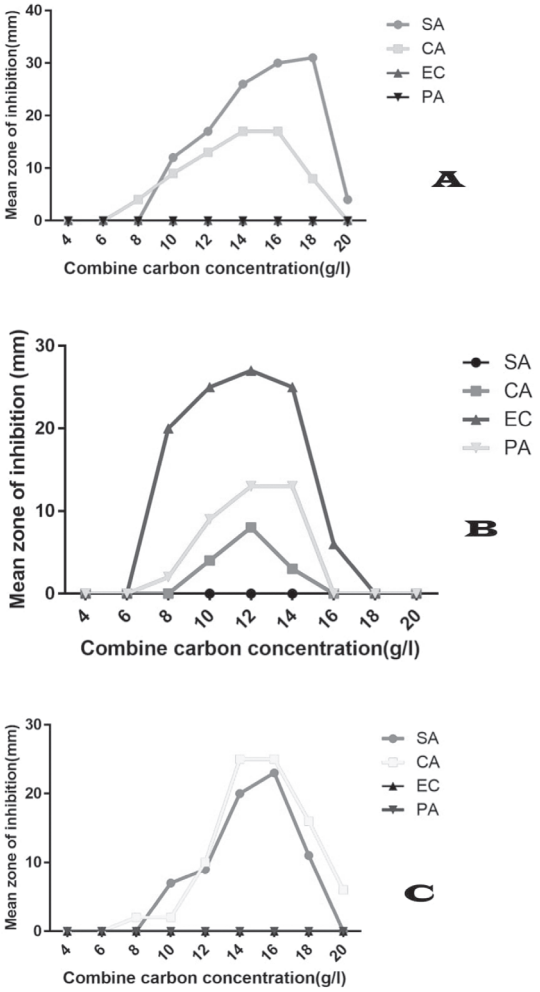

Fig. 6: Effects of different combine carbon source(Starch and Glycerol) on antimicrobial production from three selected actinomycetes spp. A: BRWDSc (SP), B : KBMWDSb6(M6); C : KBRWDSa (RF). EC: *E. coli* 2966, PA: *P. aerugenosa* 2929, CA: *C. albicans* ATCC1023, SA: *S. aureus* 2876.The results of multiple comparisons showed statistical significance at p value ≤ 0.05 using one way ANOVA.

0.0403for KBMWDSb6 (M6), KBRWDSa3 (RF) and BRWDc (SP) respectively) of multiple comparisons on the effects of growth temperature on antimicrobial production showed statistical significance at $p \leq 0.05$ for KBRWDSa3 (RF) and BRWDc (SP), but no statistical difference was observed from isolate KBMWDSb6 (M6). Other

studies have reported high production of antimicrobial compounds by most actinomycetes spp when grown at temperature 30 - 35°C (6, 22, 28, 30; 31).

Furthermore, this study showed that the production of antimicrobial compounds were high when the three isolates were grown under pH 6.7 to 8.5. Higher zone of inhibitions were observed from organisms grown at pH 7.0-pH 7.5 (Fig. 8).

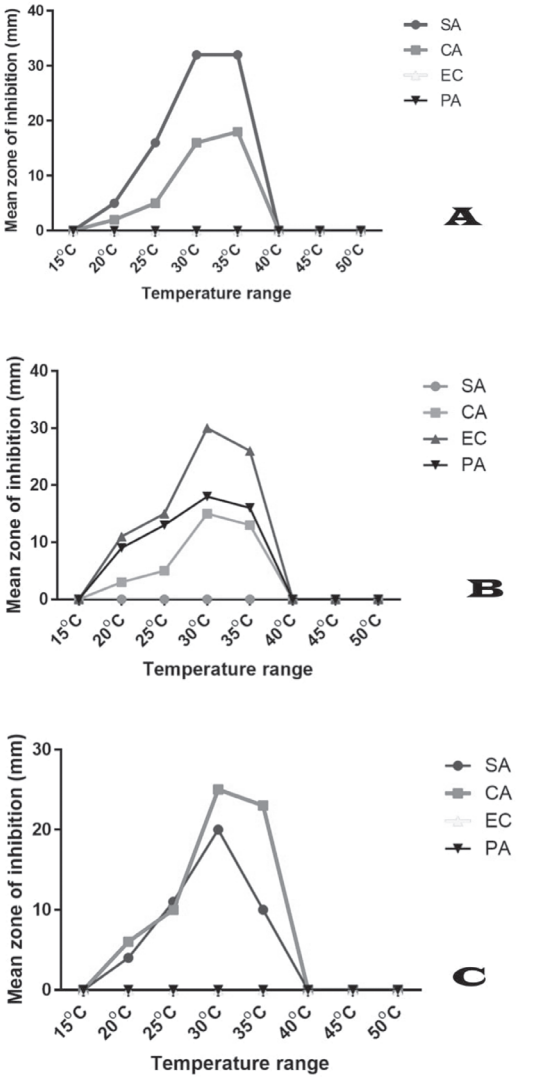

Fig. 7: Effect of temperature on the production of antimicrobial compound from three selected actinomycetes ssp. **A**: BRWDSc (SP), **B**: KBMWDSb6 (M6); **C**: KBRWDSa (RF). **EC**: *E. coli* 2966, **PA**: *P. aerugenosa* 2929, **CA**: *C. albicans* ATCC1023, **SA**: *S. aureus* 2876. The results of multiple comparisons showed statistical significance at p value ≤ 0.05 using one way ANOVA.

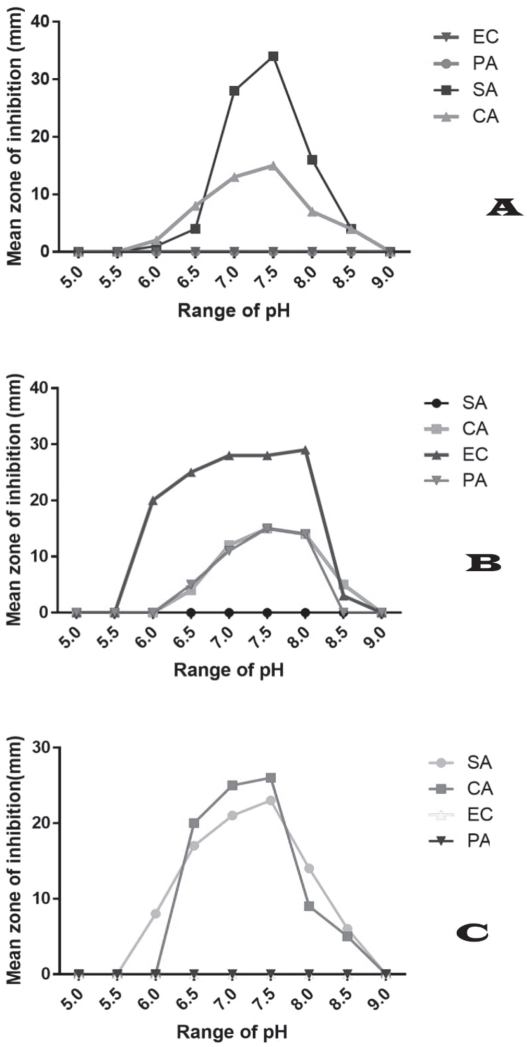

Fig. 8: Effect of pH on the production of antimicrobial compounds by three selected actinomycetes isolates. **A**: BRWDSc(SP), **B**: KBMWDSb6(M6); **C**: KBRWDSa(RF). **EC**: *E. coli* 2966, **PA**: *P. aerugenosa* 2929, **CA**: *C. albicans* ATCC1023, **SA**: *S. aureus* 2876. The results of multiple comparisons showed statistical significance at p value ≤ 0.05 using one way ANOVA.

The p values (0.0054, 0.0042, and 0.0186 for KBMWDSb6 (M6), KBRWDSa3 (RF) and BRWDc (SP) respectively) of multiple comparisons on the effects of pH on antimicrobial production showed statistical significance at $p \leq 0.05$. Our finding was in line with previous results reported in similar studies (21, 22, and 28). However, other studies reported high zone of inhibition at neutral to slightly acidic pH (30, 31).

Figure 9 below showed the results of the effect of different NaCl concentrations on the production of antimicrobial compounds. The suitable concentration of NaCl for the production of antimicrobial compounds ranges 15-21g/l for the selected actinomycetes spp but highest antimicrobial activity was observed at 17 and 19g/l. The p value (0.0001 each for KBMWDSb6 (M6), KBRWDSa3 (RF) and BRWDc (SP)) of multiple comparisons on the effects of different NaCl concentration on antimicrobial production showed statistical significance at $p \leq 0.05$. Different actinomycetes spp produced antimicrobial compounds at different concentration of NaCl (22). Other studies have shown optimum antimicrobial production by actinomycetes spp with NaCl concentration of: (20g/l) Singh *et al*. (32) and (10g/l): El-Refai*et al.* (33). Mangamuri*et al.* (22) findings showed that the optimum NaCl concentration for the production of bioactive compounds from *Streptomyces tritolerans* DAS 165T was equivalent to 50g/l.

Figure 10 showed the effect of different concentration of nitrogen source (peptone) on antimicrobial production by three actinomycetes spp. The suitable nitrogen source (peptone) for the production of antimicrobial compounds ranged from 9-21g/l and 11-21g/l for BRWDc (SP) and KBRWDSa3 (RF), andKBMWDSb6 (M6) respectively. The optimum activity was observed at 11-21g/l for all three isolates. The p value (0.0001 each for KBMWDSb6 (M6), KBRWDSa3 (RF) and BRWDc (SP)) of multiple comparisons on the effects of different nitrogen source (peptone) on antimicrobial production showed statistical significance at $p \leq 0.05$. The concentration obtained from this study was higher than that of

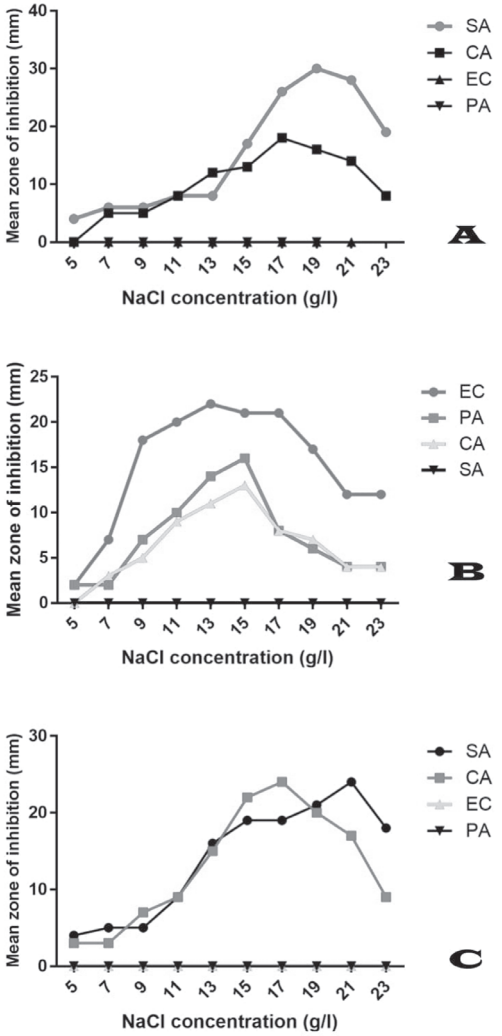

Fig. 9: Effect of NaCl concentration on the production of antimicrobial compounds by three selected actinomycetes isolates. **A**: BRWDSc (SP), **B**: KBMWDSb6 (M6); **C**: KBRWDSa (RF).). **EC**: *E. coli* 2966, **PA**: *P. aerugenosa* 2929, **CA**: *C. albicans* ATCC1023, **SA**: *S. aureus* 2876. The results of multiple comparisons showed statistical significance at p value ≤ 0.05 using one way ANOVA.

Al-Ghazali and Omran (34) who reported 0.05g/100ml as the required concentration for maximum antimicrobial production by *Streptomyces* sp. LH9.

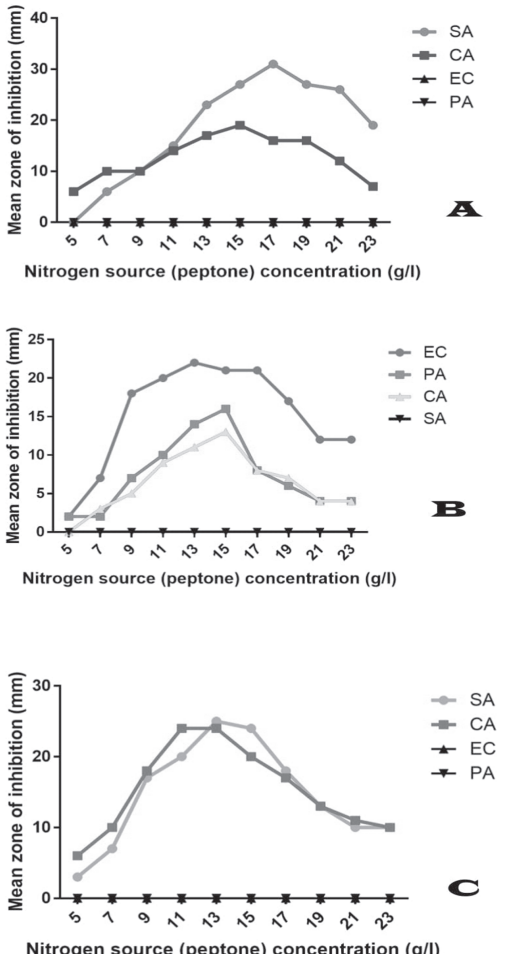

Fig. 10: Effect of nitrogen source (Peptone) on the production of antimicrobial compounds by three selected actinomycetes spp. **A**: BRWDSc (SP), **B**: KBMWDSb6 (M6); **C**: KBRWDSa (RF).). **EC**: *E. coli* 2966, **PA**: *P. aerugenosa* 2929, **CA**: *C. albicans* ATCC1023, **SA**: *S. aureus* 2876. The results of multiple comparisons showed statistical significance at p value ≤ 0.05 using one way ANOVA.

The results on antimicrobial activity from different solvents crude extracts showed that aqueous and ethanol crude extracts had the highest antimicrobial activity (Figure 11). The mean zones of inhibition from all crude extracts ranged from 8.33 - 31.67, 3.66 - 22.33 and 9.67-24.16±0.17 for BRWDc (SP), KBMWDSb6 (M6) and KBRWDSa3 (RF) respectively. Aqueous crude extract of actinomycetes isolate KBMWDb6 (M6) did not show activity against *S. aureus* 2876, but had activity when ethanol and methanol were used for extraction. The p values (0.0001 each for KBMWDSb6 (M6), BRWDc (SP) and 0.0019

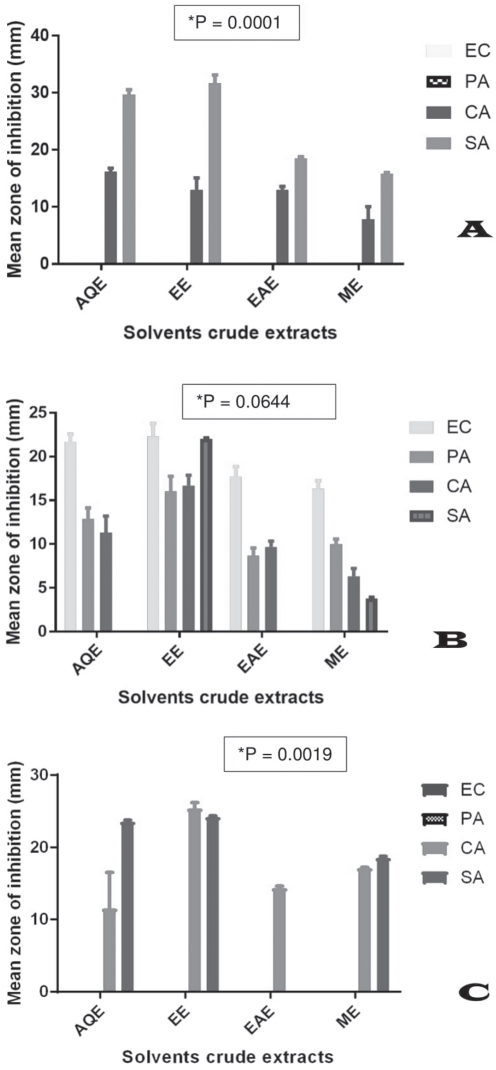

Fig. 11: Mean and standard error zone of inhibition of different solvents crude extracts from three actinomycetes spp. A: BRWDSc (SP), B: KBMWDSb6 (M6); C : KBRWDSa (RF). EC: *E. coli* 2966, PA: *P. aerugenosa* 2929, CA: *C. albicans* ATCC1023, SA: *S. aureus* 2876. AQE Aqueous extract, EE: Ethanol extract, EAE: Ethyl acetate extracts, ME: Methanol extract, * P value of multiple comparisons using one way ANOVA.

forKBRWDSa3 (RF)) of multiple comparisons between the crude extracts showed statistical significance at ($P \leq 0.05$). The ability of ethanol crude extract to produce higher activity could be as result of universal polarity of the solvent which make it to extract both polar and weak polar compounds (35).

CONCLUSION

Production of antimicrobial agents by actinomycetes species is dependants on media compositions, growth conditions and extraction methods. This study revealed that suitable medium, optimum concentration of combine carbon sources, growth conditions, and solvent are important for optimum production of antimicrobial compounds by actinomycetes spp. The molecular identification of 16S rDNA gene showed that all the three isolates belong to phylum Actinobacteria into the genus *Streptomycetes*.

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Identification of Thermophilic *Flavobacterium* and *Anoxybacillus* in Unexplored Tatapani Hot spring of Kishtwar District of Jammu and Kashmir: A North Western Himalayan State

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ABSTRACT

Two industrially important thermophilic bacteria were isolated from Tatapani hot spring located in Kishtwar district of Jammu and Kashmir, India and were named as KSI and KSII. Water sample from Tatapani hot spring was collected for screening of industrially important thermophillic bacteria. Only two thermophilic bacteria (KSI and KSII) were isolated from Tatapani hot spring water sample. The strict thermophilic nature of both the bacterial isolates (KSI and KSII) was tested by growing them at different temperatures ranging from 40 °C-70 °C. KSI and KSII bacterial isolates showed their optimum growth at temperature 60 °C and at pH 9.0. Morphological and biochemical analysis of both the bacterial isolates was carried out. Results revealed that KSI bacterial isolate was non-motile, Gram negative, large rods and whitish in color. While, KSII bacterial isolate was non-motile, Gram positive, medium rods and creamish in color. Molecular identification of both the bacterial isolates (KSI and KSII) was done by 16s rDNA sequencing. Phylogenetic analysis of 16s rDNA sequences of KSI and KSII bacterial isolates obtained after sequencing revealed that KS1 (GenBank accession no KU248487) had closest homology (99%) with *Flavobacterium thermophilum* G-21 (GenBank accession no NR 104891.1) and bacterial isolate KSII (GenBank accession no KU248486) showed 99% homology with *Anoxybacillus sp.* DR01 (GenBank accession no EU621359.1). Thermophillic bacteria (KSI and KSII) were tested for production of industrially important thermophilic enzymes. Both KSI and KSII bacterial isolates could produce catalase enzyme (catalase positive), nitrate reductase enzyme (nitrate positive) and could produce acetoin (Voges Proskaue positive). KSI was efficient in producing enzymes such as amylase and cellulase, while KSII was efficient in producing glutaminase. The bacteria (KSI and KSII) isolated in the present study are thermophilic in nature and could produce industrially important thermophilic enzymes. Thus, these microorganisms can be used commercially for large scale production of industrially important thermostable catalase, nitrate reductase, amylase, cellulase and glutaminase enzymes.

Keyword (s): Kishtwar, Himalayas, *Flavobacterium thermophilum, Anoxybacillus*

INTRODUCTION

Thermophiles show optimum growth between 60-70°C and a very little growth below 45 °C (1). Thermophilic bacteria are being explored extensively for production of novel thermostable

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enzymes as compared to the plant and animal sources. Thermostable enzymes can withstand high temperature during their large scale production and processing in industries as compared to the mesophilc enzymes. Variety of thermophilic enzymes including proteases, amylase, isomerase and lipase are being utilized in beverages, food, and detergent industries. Whereas, ribonuclease, malate dehydrogenase, Taq DNA polymerase, T4 DNA ligase, and lysozyme are useful in research laboratories (2). Thus, these thermophilic microbial sources fulfill the industrial demands of detergents, pharmaceuticals, food, textiles, and research and development industries. Thermostable enzymes are stable and active under harsh conditions of high temperature thus provide new opportunities for biocatalysis and biotransformation (3).

Geothermally heated regions of the Earth like hydrothermal vents, hot springs, geysers and compost are the main sources of thermophiles. According to the geological survey of India, there are more than over 350 hot springs in India and are classified on the basis of their geo-tectonic setup (4, 5). Most of the Indian hot springs are being explored for microbial diversity. Cellulase and amylase producing thermophilic bacteria named as *Geobacillus sp*. have been isolated from Tattapani hot spring of Himachal Pradesh India (6, 7); Panamik (Ladakh, Jammu and Kashmir). There is also a report on cellulase producing *Thermophilic bacilli* from Tattapani Hot Spring sediment in North West Himalayas (8); two alkaline Indian hot springs, Yumthang (Sikkim) and Jakrem (Meghalaya) are enriched with bacterial and archaeal diversity e.g., *Firmicutes, Chloroflexi and Thermi* dominant in Jakrem and *Proteobacteria* in Yumthang (9).

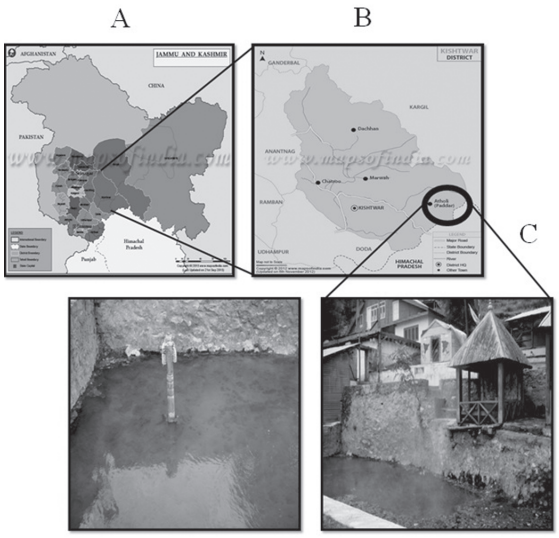

Fig. 1: Geographical location of Tatapani hotspring. (A) Map showing Jammu and Kashmir. (B) Enlarged view of Kishtwar district showing location of Tatapani hotspring circled (black) and (C) Actual view of Tatapani hotspring.

The Himalayan regions of Jammu & Kashmir State (A North Western Himalayan state) posseses more than 20 hot spring sites. In this study, a hot spring located in Himalayan sub range of Kishtwar district of Jammu and Kashmir, India (Fig. 1A-C) was explored for isolation of industrially important thermophilic bacteria. This hot spring is not even in the list of thermal hot springs of India and has not been explored for microbial diversity.

Two thermophilic bacteria were isolated from Tatapani hot spring and were named as KSI and KSII. Both the thermophilic bacterial isolates were analyzed morphologically and by biochemical/ molecular characterization. After molecular characterization, KSI and KSII were identified as thermophilic bacteria of genus *Flavobacterium* and *Anoxybacillus,* respectively. Temperature and pH optimization for KSI and KSII bacterial isolates was carried out. Thermophilic isolates were further analyzed for thermophilic hydrolytic enzyme production. In this study, authors have also compared the characteristics of KSI and KSII thermophilic bacterial isolates with that of PW4, isolated from Tattapani hot spring Himachal Pradesh, India. Identification of PW4 was also done by 16s rDNA sequencing in the present study.

MATERIALS AND METHODS

Isolation and screening of thermophilic bacteria : Water sample was collected aseptically

in sterile falcon tube (50 ml) from Tatapani hot spring located in Kishtwar district of Jammu and Kashmir, India (33° 19' 0" North, 75° 46' 0") East. For isolation of thermophilic bacteria, Tatapani hot spring water sample was 10 fold serially diluted and plated on nutrient broth medium containing 2 % agar and the plates were incubated for 24 h at 60 °C. After incubation, morphologically distinct colonies were selected and re-streaked on a nutrient broth medium containing 2 % agar to get pure colonies. Purified colonies were preserved in 50 % glycerol at -80 °C.

Microscopic analysis of thermophilic bacterial isolates : Morphology of the isolated bacteria was studied by Gram's staining (10) and the bacteria were visualized under light microscope.

Effect of temperature and pH on the growth of thermophilic bacterial isolates : The optimum temperature and pH for the growth of thermophilic bacterial isolates were studied by streaking and by quantitative measurements either in nutrient broth medium containing 2 % agar or in only nutrient broth medium, respectively. The cultures were grown at different temperature in the range of 40-70 °C. The pH of medium was adjusted to different pH ranges (5-12). Glacial acetic acid was used to adjust the acidic pH 5 and 6; whereas, alkaline pH of 8.0-12.0 was adjusted by 5N NaOH. Cultures were incubated at 60 °C for 24 hours. After incubation, plates were observed for growth of the bacterial isolates and growth was also observed by quantitative estimation of the culture medium by monitoring the optical density (OD) of liquid culture at A_{600} nm on a double beam UV/ VIS spectrophotometer.

Biochemical characterization : In order to test the production of industrially important enzymes by thermophilic bacteria isolated from Tatapani hot spring water sample, various biochemical tests were performed as described earlier (11, 12). For catalase test, a small amount of 24 h old grown culture of thermophilic bacterial isolates was placed aseptically on the clean surface of glass slide. A drop of H_2O_2 (3 %) was placed over bacterial culture on the slide. The slide was observed for release of oxygen bubbles. In glutaminase test, thermophilic bacterial isolates were inoculated in nutrient broth medium (10 ml) supplemented with 1 % glutamine and a drop of phenol red. Culture medium was incubated for 72 h at 60 °C and was observed for change in color from yellow to pink.

Methyl red test was performed by inoculating fresh thermophilic bacterial cultures into Methyl Red Voges Proskauer (VP) broth (10 ml) followed by incubation at 60 °C for 72 h. After incubation, 3-4 drops of methyl red indicator solution was added to the test tubes containing bacterial isolates. The test tubes were observed for change in color of the culture medium from yellow to red. Voges Proskauer test was carried out by inoculating fresh thermophilic bacterial cultures into Methyl Red Voges Proskauer broth (10 ml) followed by incubation at 60 °C for 72 h. After incubation, 1 ml of 5% alpha naphthol and 0.5 ml of 40% KOH were added to the culture medium followed by gentle shaking. The test tubes were observed for change in color of the culture medium from yellow to red.

Indole test was done by inoculating the tryptophan broth (10 ml) with fresh bacterial culture followed by incubation at 60 °C for 24 h. After

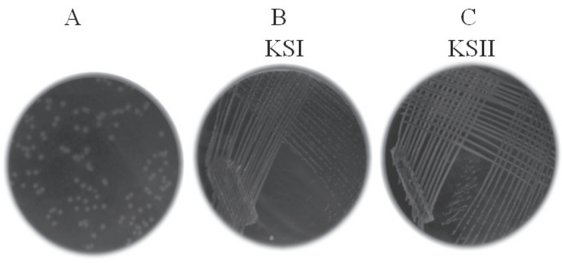

Fig. 2: Growth of bacterial isolates from the water sample of Tatapani. Growth of bacterial colonies from water sample of Tatapani hot spring on LB agar (2 %) medium. (A) 10^{-4} dilution was plated on LB agar medium. Two bacterial isolates (B) KSI and (C) KSII were streaked on LB agar (2 %) medium. Petriplates were incubated at 60 °C for 12 h and observed for growth.

incubation, 0.5 ml of Kovac's reagent was added to the culture medium. The test tubes were observed for formation of pink colored ring. In nitrate reductase test, nitrate broth (10 ml) was inoculated with fresh bacterial culture followed by incubation at 60 °C for 24 h. After incubation, one drop of sulfanilic acid and one drop of α-naphthylamine was added to the culture medium. The test tubes were observed for change in color of the culture medium from yellow to red.

The amylase and cellulase activity of the thermophilic bacterial isolates was tested by spotting equal number of cells on nutrient broth medium containing 2 % agar and starch (1 %) or caboxy methyl cellulose (CMC) (1 %) plates respectively. Plates were incubated at 60 °C for 24 h. After incubation plates were flooded with Gram's iodine and the plates were observed for zone of clearance around bacterial colonies.

Thermophilic bacterial isolates were also screened for protease activity based on the hydrolysis of casein protein at 60 °C. For this test, equal number of the bacterial cells were spotted on nutrient broth medium containing 2 % agar and casein (1 %). Plates were incubated at 60 °C for 24 h. After incubation, plates were observed for zone of clearance around the bacterial colonies. For lipase activity equal number of cells of thermophilic bacterial isolates were spotted on nutrient broth medium containing 2 % agar and tributyrin (1 %) and observed for the zone of clearance around bacterial colonies after incubation at 60 °C for 24 h. Thermophilic bacteria named as PW1, PW4 PW12, PW10 and PS3 isolated previously from Tattapani hot spring, Himachal Pradesh, India (6, 7) were used as either positive or negative controls in the present study.

Genomic DNA preparation and PCR amplification of 16s rDNA : For the molecular identification of thermophilic bacterial isolates, genomic DNA extraction and purification from each sample (KSI, KSII and PW4) was done as described (13). Each genomic DNA sample (100 ng) was used as template for 16s rDNA gene amplification using universal primer 17F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492R (5´-TACCTTGTTACGACTT-3´). The amplification was done by initial 2 min denaturing at 94 °C , while 30 sec at every cycle of denaturation at 94 °C , annealing at 40 °C for 30 sec, extension at 72 °C for 2 min and final extension at 72 °C for 10 min. The PCR products were resolved on 1% agarose gel and visualized under UV gel documentation system (Alpha Innotech, USA). PCR products were purified by using a gel extraction kit (Thermoscientific). Gel purified PCR products of 16s rDNA of all bacterial isolates (KSI, KSII and PW4) were sequenced on both the strands using 27 F and 1492 R primers at Eurofins, Bangalore, India (https://www.eurofins.com).

The complete 16s rDNA sequence for each bacterial isolate was generated manually by removing overlapping sequences. The 16s rDNA gene sequence of each bacterial strain was compared against other bacterial 16s rDNA sequences available in the Gene bank data base by using BLAST (blastn) search (14). The nucleotide sequences were aligned using Clustal W 1.74 (15). Phylogenetic tree was constructed by neighbor joining using MEGA4 (http://www.megasoftware.net) (16) and bootstrapping was used to estimate the reliability of the phylogenetic reconstructions (1,000 replicates). The nucleotide sequences were submitted in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

RESULTS

Water sample of Tatapani showed the presence of thermophilic bacteria : The Tatapani hot spring water sample was plated on nutrient broth medium containing 2 % agar. Whitish and creamish colored colonies were observed after incubation for 24 h at 60 °C. To estimate the total bacterial count, bacterial colonies were counted using colony counter and the colony forming unit (CFU) was determined. CFU count of water sample was 3×10^4 ml^{-1} (Fig. 2A). Both the bacterial isolates showed good visible growth at 60 °C and pH 9.

Microscopic and biochemical characterization of KSI and KSII : Two different thermophilic bacterial isolates were observed on the basis of colony color and were named as KSI and KSII. Both the bacterial isolates (KSI and KSII) were then selected for their biochemical characterization and for their thermophilic enzyme producing abilities, (Fig. 2B-C). Bacterial isolate KSI showed whitish colony and was observed as Gram negative. On the other hand, KSII isolate showed creamish coloration and was Gram positive in nature (Fig. 3A-B). KSI bacteria were large rod shaped as compared to KSII. Moreover, both the bacterial isolates were non-motile in nature (Table 1).

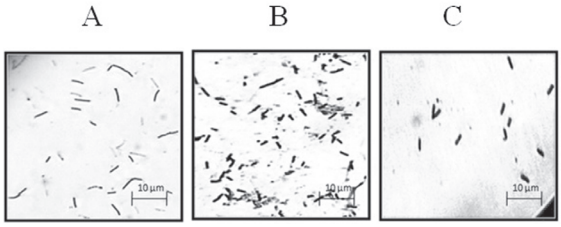

Fig. 3: Light micrograph (100X) of the isolated thermophilic bacteria. (A) KSI, (B) KSII, and (C) *Bacillus sp.* as known control.

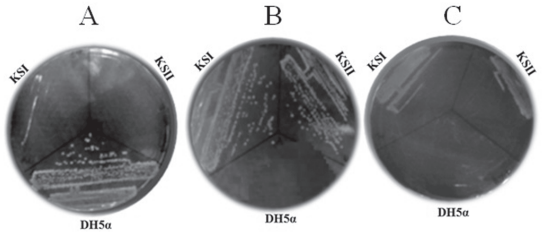

Fig. 4: Growth of thermophilic bacterial isolates at different temperature. Thermophilic bacterial isolates were streaked on LB agar (2 %) medium and incubated for 24 h at different temperatures ranging from 40 °C to 70 °C. (A) Only *E. coli* showed observable growth 40 °C (B) Both KSI and KSII were able to grow at 60 °C and (C) Little growth of KSI and KSII was observed at 65 °C. No growth of KSI and KSII was observed at 50 °C, 55 °C, 70 °C (Data not shown).

Strict thermophilic bacterial isolates were identified from Tatapani hot spring of Kishtwar : To study the effect of temperature on growth, thermophilic bacterial isolates were streaked on nutrient broth medium containing 2 % agar or were grown in only nutrient broth medium parallely. Both the culture medium were incubated at different temperatures (40 °C, 50 °C, 55 °C, 60 °C, 65 °C and 70 °C) for 24 h. Both the bacterial isolates showed detectable growth between 60-65 °C when grown either in nutrient broth medium containing 2 % agar or in only nutrient broth medium. Whereas, none of the bacterial isolates could grow at 40 °C, 50 °C, 55 °C, 70 °C (data not shown), even when incubated for 72 h. In contrast, a mesophile *E. coli* strain DH5α showed detectable growth at 40 °C and no growth at high temperature (Fig. 4A-C and Fig. 5). This indicates the strict thermophilic nature of the bacteria (KSI and KSII) isolated from Tatapani hot spring water sample.

Further, the growth of thermophilic bacterial isolates (KSI and KSII) was tested by streaking of the bacterial isolates on nutrient broth medium containing 2 % agar or by growing the bacterial isolates in only nutrient broth medium parallely. Both the media, either nutrient broth medium containing 2 % agar or only nutrient broth medium

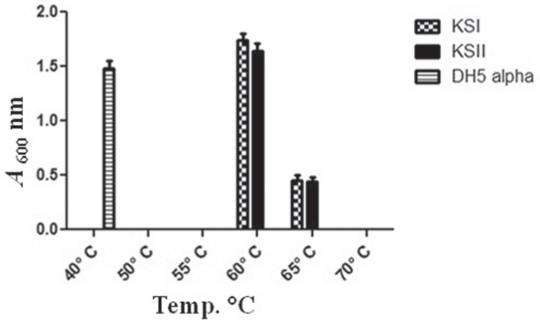

Fig. 5: Effect of temperature on the growth of thermophilic bacterial isolates. Equal number of cells were inoculated in LB medium and incubated at different temperature (40-70 °C) as indicated for 24 h. Cell density was measured by measuring absorbance at A_{600} nm and plotted against the incubation temperature. Data of three independent experiments was plotted with standard deviation.

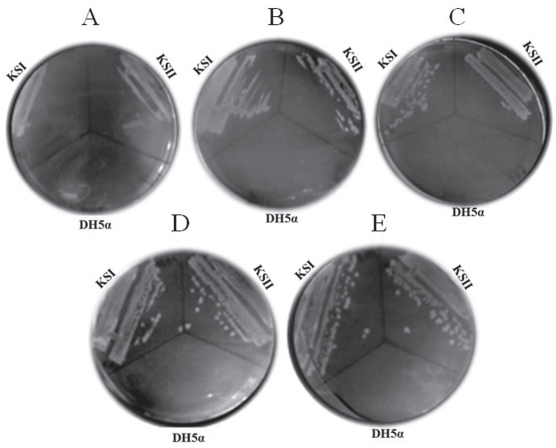

Fig. 6: Growth of thermophilic bacterial isolates at different pH. Thermophilic bacterial isolates were streaked on LB agar medium adjusted at (A) pH 5.0 (B) pH 6.0 (C) pH 7.0 (D) pH 8.0 (E) pH 9.0 (F) pH 10.0 (G) pH 11.0 (H) pH 12.0. Petri plates were incubated at 60 °C for 24 h. Mesophilic bacterial strain DH5α was used as control.

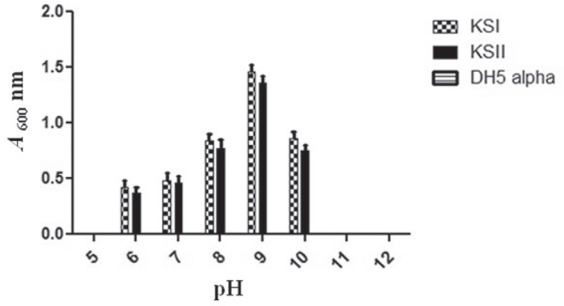

Fig. 7: Effect of pH on the growth of thermophilic bacterial isolates. Equal numbers of cells were inoculated in LB medium adjusted to pH ranging from 5-12. Cultures were incubated at 60 °C for 24 h. Cell density was measured at A_{600} nm and plotted against the different pH. Data of three independent experiments was plotted with standard deviation.

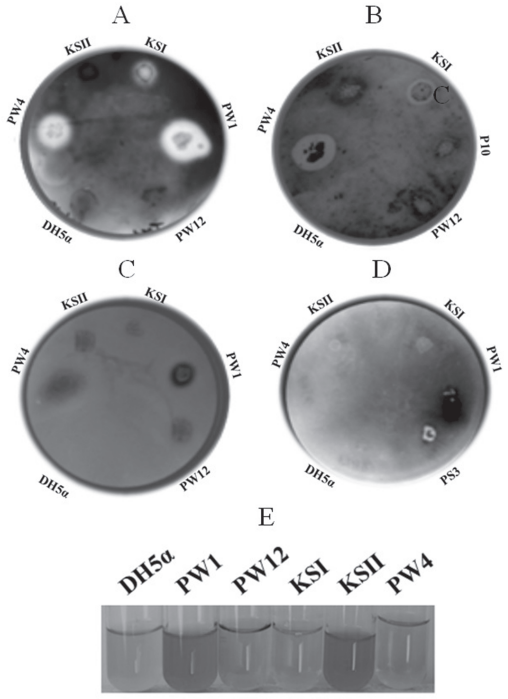

Fig. 8: Qualitative screening of thermophilic bacterial isolates for amylase, cellulase, protease, lipase and glutaminase activity. Equal number of cells of thermophilic bacterial isolates, positive control, negative control and DH5α were spotted on their respective LB agar medium supplemented with (A) 1% starch, (B) 1% CMC (C) 1% skim milk (D) 1% trybutyrin for the detection of amylase, cellulase, protease and lipase respectively. All the cultures were incubated for 24 hr of incubation at 60 °C. After 24 hr of incubation, CMC and starch containing plates were flooded with Gram's iodine and observed for the zone of clearance. Equal number of cells of all thermophilic bacterial isolates, positive control, negative control and DH5α were inoculated in LB medium supplemented with 1% L-glutamine and phenol red. (E) The cultures were incubated at 60 °C for 24 h and visualized for change in color from yellow to pink .

were adjusted to different pH ranging from 5-12 (Fig. 6A-E) and (Fig. 7). Though, the optimum pH for growth of both the bacterial isolates (KSI and KSII) was observed to be pH 9.0, but both the isolates showed detectable growth even at pH 6.0, 7.0, 8.0 and 10.0. No detectable growth was observed at pH 5.0, 11.0 and 12.0 (data not shown).

Production of industrially important thermophilic enzymes by thermophilic bacterial isolates : KSI and KSII bacterial isolates were studied for the production of various industrially important thermophilic enzymes. PW4 a thermophilic bacterial isolate from Tattapani hot spring of Himachal Pradesh, India, was used for

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Table 1. Biochemical analysis of thermophilic bacterial isolates (KSI and KSII)

S. No.	Bacterial strains	Methyl red test	Voges Proskaue test	Indole test	Nitrate reductase test	Catalase test
1.	KSI	-	+++	-	+++	+++
2.	KSII	-	+++	-	+++	+++

comparison of enzyme production. Both the bacterial isolates (KSI and KSII) showed catalase, Voges-Proskauer and nitrate reductase positive reaction. Bacterial isolate PW4 was catalase and Voges-Proskauer positive (Table 1).

To check the amylase and cellulase activity of the thermophilic bacterial isolates, starch and caboxy methyl cellulose (CMC) plates with good bacterial growth were flooded with Gram's iodine. After flooding starch agar plates with Gram's iodine, a varied size clear zone was observed around the bacterial isolate KSI, PW1 (positive control) and PW4. Clear zone of 8 mm size was observed for KSI (Fig. 8A). No zone of clearance was observed around the bacterial isolate KSII, and PW12 (negative control).

Cellulase activity was checked by flooding CMC agar plates with Gram's iodine (17). KSI, PW4 and, PW12 showed zone of clearance of about 8 mm, 16 mm and 8 mm respectively, which indicates production of cellulase (Fig. 8B). Thermophilic bacterial isolates KSII and PW10 (negative control) showed no zone of clearance in CMC agar plates.

Thermophilic bacterial isolates were screened for protease production based on the hydrolysis of casein protein at 60 °C (Fig. 8C). The appearance of zone of clearance due to digestion of casein by the action of extracellular protease indicated the presence of protease activity. Only thermophilic bacterial isolates PW4 and PW1 (positive control) showed protease activity, whereas protease activity was not detected in KSI, KSII and PW12 (negative control) bacterial isolates.

Equal number of cells of thermophilic bacterial isolates were spotted on LB agar medium containing 1 % tributyrin (18) and observed for the zone of clearance as an indication of lipase activity (Fig. 8D). Only the bacterial isolate PW1 (Positive control), showed zone of clearance of 7 mm, while KSI, KSII, PW4 and PS3 (negative control) did not show the zone of hydrolysis.

For glutaminase activity, thermophilic bacterial isolates were inoculated on nutrient broth medium supplemented with 1 % glutamine and a drop of phenol red and observed for change in colour from yellow to pink (19). The bacterial isolates KSII and PW1 (positive control) showed change in color of the medium from yellow to pink (Fig. 8E). No glutaminase activity was detected in KSI, PW4, and PW12 (negative control) isolates.

16s rDNA sequencing identified KSI and KSII as Flavobacterium and Anoxybacillus thermophilic bacteria : For identification of the bacterial isolates, genomic DNA of bacterial isolates was extracted (Fig. 9A). Total genomic DNA of three isolates (KSI, KSII and PW4) which were not identical were subjected to PCR amplification of 16s rDNA using 16s rRNA gene specific primers. 16s rDNA amplified at ~1.5 kbp fragment in all the three isolates as shown in (Fig. 9B). Gel purified DNA fragments were sequenced on both the strands. Overlapped nucleotide sequences obtained by the two primers were removed manually.

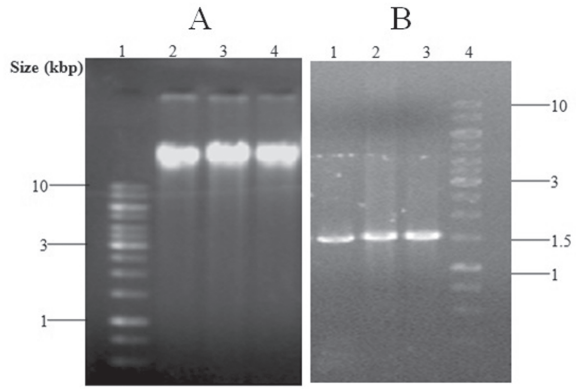

Fig. 9: Genomic DNA isolation and PCR amplification of 16S rDNA gene of KSI, KSII and PW4 bacterial isolates. Total genomic DNA analysis of the thermophilic bacterial isolates as indicated was electrophoresed on 1% agarose gel. (A) Lane 1 indicates 1kb DNA marker, genomic DNA of KSI, KSII and PW4 in lane 2, 3 and 4 respectively. Genomic DNA of the three isolates as indicated was subjected to PCR amplification for 16S rDNA by using primer 1492R and 17F. PCR amplified DNA was electrophoresed on 1 % agarose gel. (B) Lane 1, 2 and 3 represents PCR products of KSI, KSII and PW4 respectively. Lane 4 indicates the molecular size marker (kb).

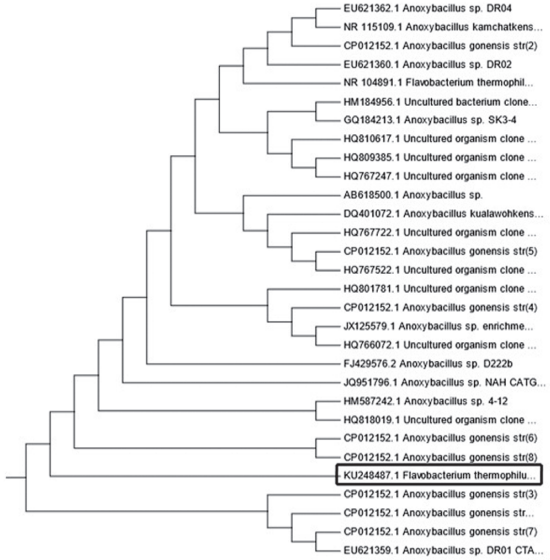

Fig. 10: 16S rDNA based dendrogram showing phylogenetic relationship of newly isolated thermophilie KSI. 16S rDNA sequence of KSI was subjected to BLAST search and hits showing >95% similarity were selected and aligned by using CLUSTAL W. Phylogenetic tree was constructed using MEGA 4 version 3.22. *Flavobacterium thermophilum* KSI KU248487 is indicated in the box.

The complete assembled sequence of 1487, 1474 and 1458 bps were obtained for KSI, KSII and PW4, respectively. Similar nucleotide sequences were identified by BLAST (blastn) search. The 16s rDNA nucleotide sequences of all three bacterial isolates were submitted to the NCBI GenBank database under the accession numbers- KU248487 (*Flavobacterium thermophilum* KSI), KU248486 (A*noxybacillus* sp. KSII) and KU248488 (*Bacillus* sp. PW4). The highest level of nucleotide sequence similarity of isolate KSI (GenBank accession no KU248487) was (99%) with *Flavobacterium thermophilum* G-21 (GenBank accession no NR 104891.1) (Fig. 10) whereas KSII (GenBank accession no KU248486) showed 99% similarity with *Anoxybacillus sp.* DR01 (GenBank accession no EU621359.1) (Fig. 11) while PW4 (GenBank accession no KU248488) showed 99% similarity with *Bacillus sp.* SP22 (GenBank accession no JQ808133.1) (Fig. 12).

Discussion

Till date, a number of microorganisms have been isolated from extreme environments like high/ low pH, temperature, salt, pressure for large scale production of industrially important thermophilic enzymes. Among all these, thermophilic bacteria are getting more importance (20). Thermophilic bacteria produce thermozymes, which are highly stable at high temperatures. Hot springs are the main source for thermophilic bacteria. Keeping this into mind, in the present study we initiated the survey of microbiological organisms of Tatapani, an unexplored hot spring of Jammu and Kashmir, India.

In the present study, two industrially important thermophilic bacterial isolates KSI and

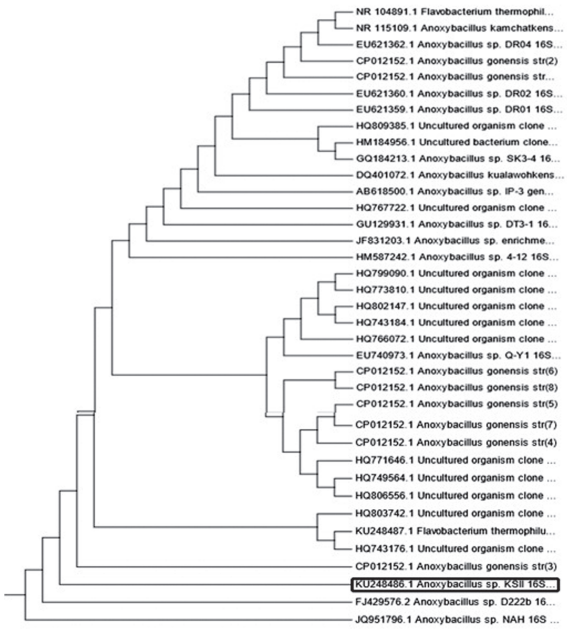

Fig. 11: 16S rDNA based dendrogram showing phylogenetic relationship of newly isolated thermophilie KSII. 16S rDNA sequence of KSII was subjected to BLAST search and hits showing >95% similarity were selected and aligned by using CLUSTAL W. Phylogenetic tree was constructed using MEGA 4 version 3.22. *Anoxybacillus sp.* KSII KU248486 is indicated in the box.

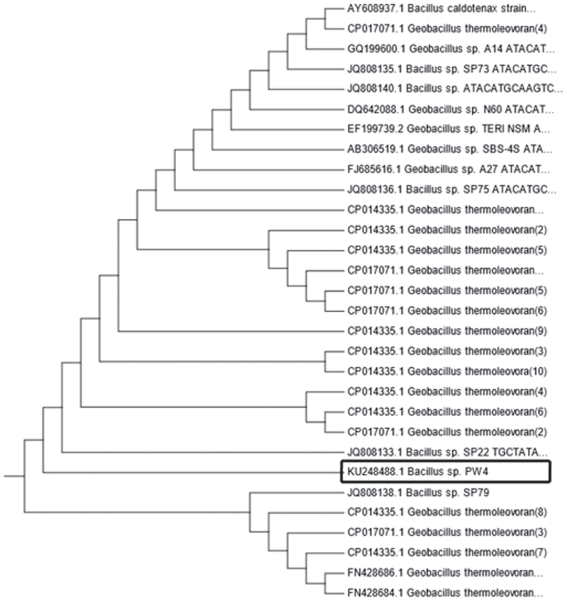

Fig. 12: 16S rDNA based dendrogram showing phylogenetic relationship of newly isolated thermophilie PW4. 16S rDNA sequence of PW4 was subjected to BLAST search and hits showing >95% similarity were selected and aligned by using CLUSTAL W. Phylogenetic tree was constructed using MEGA 4 version 3.22. *Bacillus sp*. PW4 KU248488 is indicated in box.

KSII were isolated from Tatapani hot spring of district Kishtwar, Jammu and Kashmir, India. By 16s rDNA sequencing, it was revealed that the bacterial isolates KSI and KSII were related to genera *Flavobacterium* and *Anoxybacillus* respectively. Previously isolated and characterized thermophilic bacteria PW4 from Tattapani hot spring, Himachal Pradesh, India, was also identified and showed similarity with *Bacillus sp*.

As expected, these thermophilic bacterial isolates (KSI and KSII) produce industrially important thermozymes. *Anoxybacillus* genus was first introduced in 2000 and a number of species are being isolated till now (21). A Novel thermophilic α-Amylase producing *Anoxybacillus flavithermus* SO-13 has been isolated from hot spring mud sample in Afyonkarahisar (Omer) (22). There is also a report on hydrocarbon degrading *Anoxybacillus sp.* isolated from a deep petroleum reservoir (23). KSII showed glutaminase activity and is a glutaminae producing thermophilic bacteria.

Although *Flavobacteroium* is a genus with diverse species, very few reports are available in literature pertaining to the selective isolation and screening of this rare thermophile. *Flavobacterium thermophilum* KSI showed cellulase and amylase activity. KSI is a rare isolate and to our best knowledge, this is the first report on isolation of cellulase producing *Flavobacterium thermophilum* from an Indian hot spring. Amylase producing *Flavobacterium thermophilum* was previously isolated from thermally polluted river in Belgium (24). No such cellulase producing *Flavobacterium*

thermophilum has been reported till now. Oshima and Yamakawa isolated and characterized a novel glycolipid from *Flavobacterium thermophilum,* which was further studied for fatty acid composition (25, 26). Recently, *Flavobacterium arcticum* sp. nov., has been isolated from Arctic seawater (27).

Bacillus sp. survives under wide range of physiological abilities. *Bacillus sp.* PW4 with various enzyme producing abilities was previously isolated from Tatapani hot spring Himachal Pradesh, India. A thermophilic *Bacillus sp*. with protease activity has been isolated from hot spring of Tarabalo, Odisha, India (27). A thermophilic *Bacillus sp*. with extracellular enzymatic activities has recently been isolated from hot spring of Ganeshpuri, Maharashtra, India (28).

Both the bacterial isolates (KSI and KSII) were VP positive, which indicates the production of a compound known as acetoin. Acetoin is an industrially important compound used in food industry as a flavor enhancer and it also gives buttery taste (29). Acetoin is currently produced, commercially by chemical synthesis, which is not safe and not human friendly as it is used mostly in food and cosmetic industry. Thus, production of acetoin by microbial fermentation using KSI and KSII bacterial isolates can replace the chemical synthesis process for natural acetoin production. Moreover, KSI and KSII could produce thermostable acetoin which can withstand high temperature during industrial food processing.

Both the bacterial isolates were also catalase positive, thus can be used for large scale production of thermophilic catalase, which can withstand high temperature treatments in food and textile industries. Nitrate reductase enzyme has important industrial application, as it is mostly used in waste water treatment. In the current study, both the bacterial strains were able to produce thermostable nitrate reductase enzyme and can be used for large scale production of thermostable nitrate reductase enzyme, which can be used further in waste water treatment. Moreover, *Flavobacterium thermophilum* KSI of the present study is a very rare thermophile that can be further explored for the production of new biomolecules of industrial importance.

Conclusion

Tatapani hot spring located in Kishtwar district of Jammu and Kashmir, India has never been explored previously for industrially important microbes. In the present study, two industrailly important thermophilic bacteria namely, *Flavobacterium thermophilum* (KSI) and *Anoxybacillus* (KSII) were isolated from the water sample of Tatapani hot spring. This is the first report of isolation of any bacteria from Tatapani hot spring located in Kishtwar district of Jammu and Kashmir, India. Both the bacterial isolates (KSI and KSII) are industrially very important, as they can produce industrially important thermostable enzymes viz. catalase, nitrate reductase, cellulose, amylase and glutaminase. In addition, both the bacterial isolates (KSI and KSII) can also be used for large scale production of an industrially important thermostable compound known as acetoin. Moreover, from the present study it was reported that Tatapani hot spring contains a rare and primitive thermophile namely, *Flavobacterium thermophilum*.

Acknowledgements

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Fingerprint Profile of an important therapeutic plant of Astavarga *Crepidium acuminatum* (D. Don) Szlach by HPTLC

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Abstract

Nature is crucial source of drugs. Plants have played important role in identifying important drugs and are basis of various modern pharmaceuticals. Therefore ethnomedicinal plants must be exploited to identify lead compounds. *C. acuminatum* is an important plant of Astavarga (combination of 8 drugs, most commonly used in Ayurveda) *C. acuminatum* is used in breathing disorders, burning sensation, cough, decrease in bone tissue, bleeding disorders, blood disorders, tuberculosis, insect bites and rheumatism. It is refrigerant, febrifuge and aphrodisiac. It is utilized as tonic and in general debility. HPTLC is a valuable analytical tool for the investigation of herbal products and drugs. Hence, HPTLC Fingerprint profile has been developed for this plant.

Objective: The present study was aimed to develop the fingerprint profile by HPTLC of *Crepidium acuminatum* (D. Don) Szlach.

Materials and Methods: HPTLC System (CAMAG, Switzerland), equipped with linomat 5 applicator, development chamber, scanner, derivatizer, vision Cats software, was used. The plate was scanned at 580 nm using Tungsten lamp and images were captured at visible light, UV 254 nm and UV 366 nm.

Results: HPTLC method for separation of phytoconstituents using different solvent system has been developed for *C. acuminatum.* The study revealed the presence of 2 saponins, 3 bitter principles, 2 steroids, 2 Sterols, 2 essential oils, 1 anthraquinones, 2 coumarin and 5 flavonoids in methanol extract of pseudobulbs of this plant.

Conclusion: The HPTLC fingerprint profile developed for methanol extract of *C. acuminatum* can be used for routine quality control of herbal formulations comprising of this plant and serve as a base for qualitative, quantitative analysis and standardization of the drug 'Jeevak'. It will also help in identification and quantification of active/ marker compounds. By isolating and identifying marker compounds, new drugs can be formulated to treat various diseases.

Keywords: HPTLC, Fingerprint profile, *C. acuminatum* (Jeevak), *Malaxis acuminata* Astavarga, Phytochemical analysis

Introduction

Traditional plant medicine is becoming an area of ever-increasing importance in the health care systems. Since times immemorial, plants form the basis of various traditional therapeutic systems like, Ayurveda, Unanai, Sidha. Uses of plant based remedies in healthcare preparations have been reported in Vedas and the Bible. Plants produce a diverse group of bioactive molecules, making them a rich source of different types of medicines (1). These days medicinal and neutraceutical herbs are receiving immense scientific attention for their holistic effects (2). Thus, natural products with pharmacological or

biological activities are playing a very important role in medicine (3). World Health Organization (WHO) has confirmed that herbal medicines are serving the health needs of about 80 percent of the world's population especially in rural areas of developing countries. Attention has also been paid due to the side effects of most modern drugs. It has been estimated that in the mid-1990s over 200 companies and research organizations worldwide were screening plant and animal compounds for medicinal properties (4, 5). Important drugs like vinblastine, vincristine, topotecan, taxol, teniposide, etopside, irinotecan etc. have come from plant sources. Curiosity is escalating in the overall fitness & wellness of man due to nutraceutical plants.

Crepidium acuminatum (D.Don) Szlach (Syn. *Malaxis acuminata*) is having immense ethnomedicinal potential. The dried pseudobulbs known as 'jeevak' are important ingredients of various formulations and a polyherbal immune-booster nutraceutical 'Chyavanprash'. This drug has been stated in various ayurvedic formulations like Astavarga churna, chyavanprash Rasayan, Ghrita, Taila, Gutika, Agada etc. (6). *C. acuminatum* is used in breathing disorders, burning sensation, Cough, decrease in bone tissue, bleeding disorders, blood disorders, tuberculosis, insect bites, rheumatism. It is reported to be refrigerant therefore used to reduce fever (Febrifuge). It has been described as aphrodisiac and used in emaciation, seminal weakness. It is utilized as tonic and in general debility (7, 8, 9) Although its antimicrobial (10), antioxidant (11) essential oil analysis (12), and anti-inflammatory activities (13) have been reported but work has not been described on fingerprint profile of this plant by HPTLC.

High Performance Thin Layer Chromatography (HPTLC) is a sophisticated analytical technique pedestal on the full potential of thin layer chromatography. Automation, scanning, full optimization, selective detection principle, minimum sample preparation, hyphenation enable it to be a powerful tool in modern research for qualitative and quantitative analysis of complex mixtures of bio molecules (14). It has gained popularity to become a leading type of analysis in fingerprinting of herbal drugs. Although thin layer chromatography (TLC) is commonly used for the analysis of herbal drugs since long time back. Various pharmacopoeias such as American Herbal Pharmacopoeia (AHP), Chinese Drug Monographs and Analysis, Ayurvedic pharmacopeia of India (API) Pharmacopoeia of People's Republic of China, etc. still use TLC to provide first characteristic fingerprints of herbal drugs. Fingerprint analysis approach using chromatography is becoming the most powerful tool for identification, authentication and quality control of herbal products. For quality control, the concept of phyto-equivalence is utilized. Chromatographic fingerprint can demonstrate 'sameness' and 'differences' between various formulations and the authentication and identification of herbal medicines can be accurately carried out even if the number and concentration of chemically characteristic components are not very similar in different samples of herbal formulations (16). As per World Health Organization (WHO), the quality, quantity, safety and efficacy data on traditional medicine are not sufficient and there is still a lack of adequate/accepted research methodology for evaluating traditional medicine till date. In this situation, HPTLC is playing pivot role f for the fingerprinting of medicinal plants. It is a realistic alternative to meet the need for effectual and powerful assessment of herbal products. HPTLC is mainly used for expressing various patterns which are preserved as 'databases' known as 'fingerprints' for future studies. In such a situation, there is dire need that chromatographic fingerprint, for a herbal product should be constructed. Hence, this technique was adopted for deriving the fingerprint patterns of the crude drug of *Crepidium acuminatum.*

Materials and Methods

Pseudobulbs of the plant were screened for profiling. Pseudobulbs were shade dried in air at room temperature, powdered and stored in air

tight container. It was authenticated and identified as *Crepidium acuminatum* (D. Don) Szlach from National Institute of Science Communication and Information Resources (NISCAIR), New Delhi and deposited in herbarium of Panjab University, Chandigarh with PAN 21262.

For analysis 500mg of powder was dissolved in 10 ml of methanol with occasional shaking for duration of 2 hrs., then filtration was done by using membrane filter. Sample of 1, 2, 4 ,6 ìl aliquot were loaded as 8 mm band length on a 5 X10 silica gel 60 F_{254} TLC plate using LINOMAT 5 auto sampler instrument (CAMAG, Muttenz, Switzerland). The samples-loaded plates were kept in TLC twin trough developing chamber after saturation, (saturation time 5 min) with solvent vapour using respective mobile phases (as shown in table 1) and the plates were developed in the respective mobile phase up to 70 mm. The developed plates were dried by hot air to evaporate solvents from the plate. The plates were kept in a photo-documentation chamber (CAMAG) and the images were captured. Software Visioncats-serv, version 2.4.17207.2 was used for data analysis.

Results

The present study, which was performed to develop fingerprint of the drug by using HPTLC technique showed valuable results. 2 saponins, 3 bitter principles, 2 steroids, 2 sterols, 2 essential

Table 2. Showing various class of compounds, mobile phase and derivatization reagent used

S. No.	Class of Compounds studied	Mobile Phase/composition	Derivatization Reagent	Result & Interpretation
1.	Saponins	Chloroform: acetic acid: methanol: water/ 6.4:3.2:1.2:0.8	Anisaldehyde sulphuric acid reagent	Band at Rf 0.80 and 0.50 , violet color shows presence of saponins.
2.	Bitter Principle	ETHYL acetate: methanol; water/ 7.7:1.5:0.8	No	Band at Rf 0.37 ,0.60 and 0.90, bluish violet color indicates presence of bitter principles.
3.	Essential oils	Toluene: ethyl acetate/ 9.3:0.7	Vanillin Sulphuric acid	Band at Rf 0.29 and 0.75, violet color indicates presence of essential oil (terpinoids).
4.	Anthraquinones	Ethyl acetate: Methanol; Water/ 16:4:02	Alcoholic KOH	Band at of Rf 0.65 in UV 366nm yellow florescence is observed.
5.	Sterols	Chloroform: ethyl acetate/ 4:6	Anisaldehyde Sulphuric acid	Band at Rf 0.10 and 0.79 in visible(Reddish–violet color) indicates presence of sterols.
6.	Coumarin	Chloroform	Vanillin Sulphuric acid reagent	Bands at Rf 0.71, 0.05 ,0.18 yellow fluorescence confirms presence of coumarins.
7.	Flavonoids	Ethyl acetate: Formic acid: acetic acid: water/ 10:0.5:0.5:1.3	Natural product Reagent	Fluorescence at Rf 0.10, 0.25, 0.75, 0.80, 0.97 indicates presence of flavonoids.
8.	Steroids	n-butanol: methanol: water/ 3:1:1	Anisaldehyde sulphuric acid reagent	Band at Rf 0.60 and 0.67 red-violet color indicates presence of steroids.

oils, 1 anthraquinones, 2 coumarin and 5 flavonoids were observed in methanol extract.

Methanol extract was subjected to different composition of the mobile phase (Table 1) to separate different secondary metabolites. After derivatization with appropriate reagents, the colour development was noted. Based on colour development and fluorescence (15), the secondary metabolite were differentiated and Rf values were calculated and presented in figure Best solvent system for phytcohemical are presented in table 1.

HPTLC fingerprinting studies of methanol extract showed distinct band pattern before and after spraying with derivatizing reagent. Rf values under different wavelengths before and after derivatization are taken and presented in figure 1-8 and Table1. HPTLC, now a days is applied not only to obtain "Fingerprint" patterns of herbal formulations, quantification of active ingredients but also for the detection of adulterant.

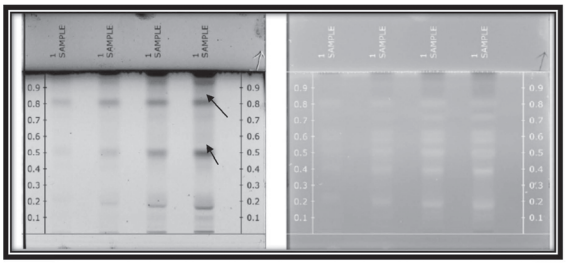
Discussion

The preliminary phytochemical screening of crude drug indicated the presence of saponins, essential oils, anthraquinones, Sterols, coumarins, flavonoids, steroids, tannins and glycosides by HPTLC.

Saponins are a large family of phytochemicals which are structurally related compounds. 'Sapo' is a Latin word for soap. They possess soap like properties and form lather in aqueous solution. They possess surfactant properties and are used as natural detergent. Chemically they are glycosides of steroids (C27) and triterpenes (C30). Saponin = glycone + aglycone. Glycone is sugar moiety, which is polar in nature and consists of hexoses/pentoses/uronic acid, whereas aglycone part, is known as sapogenin, which is steroidal/triterpene. They have received industrial, commercial, pharmaceutical attention. They are used as food additives, as ingredients in photographic emulsions, in fire extinguishers etc. (17) Biological effects of saponins are in the membrane-permeabilising, immunostimulant, hypocholesterolaemic and anticarcinogenic properties. They have also been found to affect significantly reproduction in animals. These structurally diverse compounds have also been observed to kill protozoans and molluscs, to have an effect on cold blooded animals, also to have the analgesic, antinociceptive, antioxidant activity, to impair the digestion of protein, to cause hypoglycemia and to act as antifungal and antiviral agents (18). Investigations are going on towards the development of new natural medicines and prove the efficacy of traditional herbal medicine. The plant under investigation showed two types of saponins at Rf 0.80 and 0.50 which can be isolated and characterized for further studies.

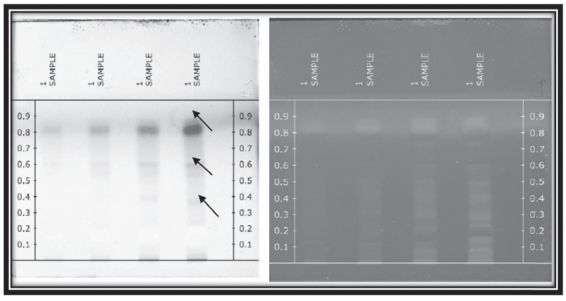
The term 'essential oil' derives its name from the drug *Quinta essentia*, named by Paracelsus von Hohenheim of Switzerland in sixteen century. Essential oils are 'essences' which are responsible for different scents that plants emit. They are odours, flammable, volatile products which have tendency to evaporate on exposure to air even at ambient conditions and therefore also referred to as volatile oils or ethereal oils. Essential oils are indispensable in food, cosmetic and human health field. They are extensively used in perfumery and aromatherapy (therapeutic technique including massage, inhalations, or baths using these volatile oils). They serve as chemical signals allowing the plant to control or regulate its environment (ecological role), attraction of pollinating insects, repellent to predators, inhibition of seed germination, and communication between plants. They have antibacterial, antioxidant, anti-inflammatory, cancer chemoprotective activity, antifungal or insecticide and deterrent activities etc. (19, 20). Essential oils reported in this plant are

Anthraquinones, another class of phytochemicals, are aromatic compounds having anthracene ring with 2 keto groups (9, 10-dioxoanthracene). These are derivatives of phenolic and glycosidic compounds but in living plants they are generally found as glycosides. They are derived from anthracene and giving



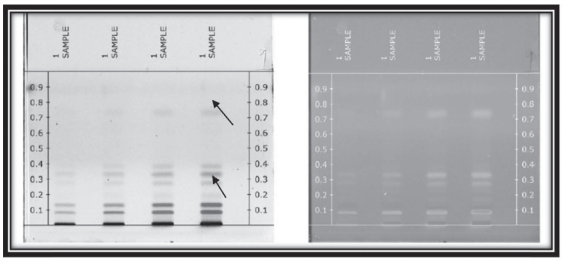
1 (a) 1 (b)

Fig. 1: represents results for saponins captured after derivatization 1 (a) under white light ; arrows at band Rf 0.80 and Rf 0.50 observed under white light indicates presence of saponins (b) same plate under UV 366 nm



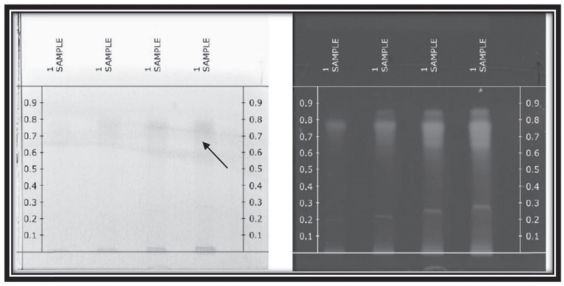
2(a) 2(b)

Fig. 2: shows image representing results for bitter principles , visualized under 29a) white light and 2 (b) UV 366nm arrows at bands in 2 (a) corresponding to Rf 0.37 , 0.60 and 0.90 of bluish violet color indicated presence of bitter principles.



3 (a) 3 (b)

Fig. 3: Image obtained after derivatizing with vanillin and Sulphuric acid and observed under 3(a) white light and 3 (b) under UV 366 nm. Arrows pointing at bands corresponding to Rf 0.29 and 0.75 of violet color in 3 (a) indicated presence of different essential oils (terpenoids).



4(a) 4(b)

Fig. 4. Image acquired after derivatizing with alcoholic KOH and taken under 4(a) white light and 49b) UV 366 nm. Arrow at band Rf 0.65 in 4 (a) showing yellow florescence is pointing towards anthraquinone

variable groups based on the degree of oxidation e.g. anthrones, anthranols, chrysophanol, poramide, luteolin, emodin etc.(21). They are most commomnly utilized as laxatives and possess antiviral and antifungal properties. These compounds impart color to plants and have been extensively employed as natural dyes (22). One anthraquinone has been observed in methanol extract of this plant in current study.

Sterols are a class of phyto-compounds, derived from hydroxylated polycyclic isopentenoids which is having a 1,2-cyclopentanophenthrene structure. These compounds contain a total of 27-30 carbon atoms in which a side chain with carbon atoms is attached at the carbon 17 position. The number and position of double bonds in the polycyclic and side chain systems of sterols can be different. Generally, the sterols can be categorized into three subclasses: (I) 4, 4 desmethylsterols (II) 4a-methylsterols and (III) 4, 4-dimethylsterols. Phytosterols are important products for health and nutrition industries. They have hypocholesterolemic activities, and are used as cholesterol-lowering agents contributing towards cardiac health benefits. They are useful emulsifiers for cosmetic manufacturers and used as precursors for the production of hormones. They are known to inhibit oxidative deterioration of oils, therefore serving as potential antipolymerization agents for frying oils and used as markers for the

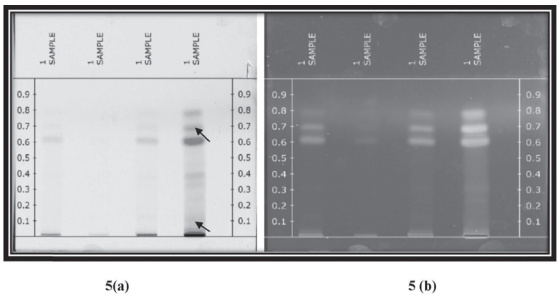


Fig. 5. Image taken after derivatizing the plates with Anisaldehyde Sulphuric acid and observed under 5 (a) white light and (b) UV 366 nm . Arrows at Rf 0.10 and 0.79 in 5(a) of reddish–violet color band confirmed the presence of sterols.

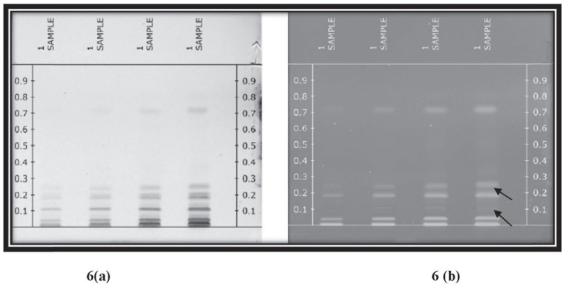


Fig. 6. Image taken after derivatization and observed under (a) white light and (b) UV 366 nm . Arrows pointing towards bands at Rf 0.05 & 0.71 showing fluorescence of pale yellow colour indicated presence of coumarins.

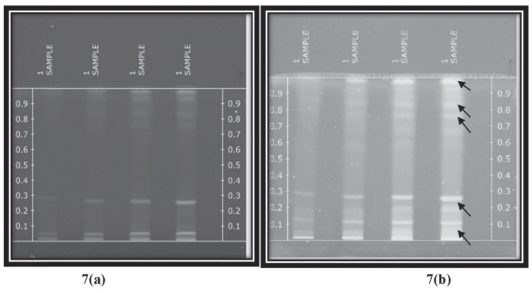


Fig. 7. Image captured under UV 366 nm 7(a) before derivatization and 7(b) after derivatization. Arrows pointing towards fluorescent bands at Rf 0.10, 0.25,0.75,0.80 , 0.97 indicated presence of flavonoids.

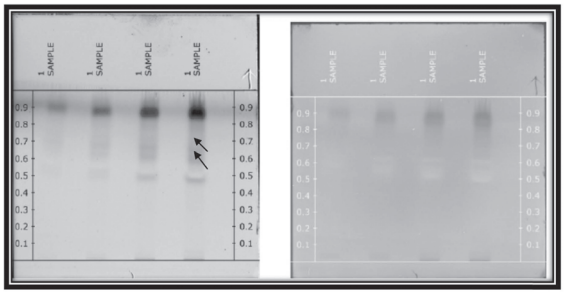


Fig. 8. Image scanned under white light and UV 366 nm after derivatization. Arrows at Rf 0.60 and 0.67 bands depicting red-violet color proved the presence of steroids.

assessment of adulterated oils. Sterols are present in the nonsaponifiable fraction of plant oils. Since these are of plant origin and not synthesized in humans therefore are poorly absorbed and are excreted faster than cholesterol. Therefore they have lipid lowering efficacy. Main phytosterols which are used in the human diet are sitosterol, stigmasterol, and campesterol. (23, 24). Two types of sterols observed in the present study at Rf 0.10 and 0.79 of reddish–violet color band confirmed the presence of sterols.

'Coumarins' word is derived from 'Coumarou', the vernacular name of the tonka bean (*Dipteryx odorata*), from which coumarin, was first time isolated in 1820. There are four classes of coumarin; simple coumarins; furanocoumarins; pyranocoumarins; and pyrone-substituted coumarins. The coumarins have bacteriostatic and anti-tumor activity and these compounds are being screened as novel therapeutic agents (25). Two types of coumarins are first time reported in existing study at Rf 0.05 & 0.71 showing fluorescence of pale yellow colour indicated presence of coumarins.

Flavonoids are polyphenolic compounds, ubiquitous in nature and are more than 4,000 commonly occur in vegetables, fruits and beverages like tea, coffee and fruit drinks. They occur as aglycones, glucosides and methylated derivatives. They are utilised as food and pharmaceutical supplements. The flavonoids appear to have played a major role in successful medical treatments (26). Flavonoids are linked to their potential cytotoxicity and their capacity to

interact with enzymes through protein complexation (27). They have antioxidative activity, free-radical scavenging capacity, anticancer activity and have role in coronary heart disease prevention and anti-human immunodeficiency virus functions They are reported to be hepatoprotective, anti-inflammatory, and antiviral also (28). Five types of flavonoids are reported in present investigation carried out.

Steroids have the fundamental structure of four carbon rings called the steroid nucleus. The addition of different chemical groups at different positions on backbone leads to the formation of many different types of steroidal compounds. Plant steroids are synthesized by cyclisation of 2,3-epoxysqualene into cycloartenol, which are further metabolized to produce biologically active steroids. Plant steroids classified in different classes based on their chemical structure, pharmacological activities and source from which they have been isolated. Plant steroids possess many interesting medicinal, pharmaceutical and agrochemical activities like anti-tumor, immunosuppressive, hepatoprotective, antibacterial, plant growth hormone regulator, sex hormone, antihelminthic, cytotoxic and cardiotonic activity (29).

Chemically, it is difficult to define tannins as they include diverse number of oligomers and polymers. Tannins are a heterogeneous group of high molecular weight polyphenolic compounds which form reversible and irreversible complexes with proteins, polysaccharides (cellulose, hemicellulose, pectin etc.), alkaloids, nucleic acids and minerals etc. Structurally, tannins can be categorised into four groups: Gallotannins, ellagitannins, complex tannins, and condensed tannins. Gallotannins are all those tannins in which galloyl units or their derivatives are joined to polyol-, catechin-, or triterpenoid units. Ellagitannins are those tannins in which at least two galloyl units are C–C coupled to each other, and do not contain a glycosidically linked catechin unit. Complex tannins are tannins in which a catechin unit is bound glycosidically to a gallotannin or an ellagitannin unit. Condensed tannins are all oligomeric and polymeric proanthocyanidins. Tannin-containing plant extracts are used as astringents, anti-inflammatory, as antiseptic, as diuretics, antioxidant and haemostatic. They are also used against diarrhoea and various types of tumours. Tannins are used in the dye stuff industry and also in the production of inks. In the food industry tannins are used to clarify wine, beer, and fruit juices. Other industrial uses of tannins include textile dyes, as antioxidants in the fruit juice, beer and wine industries, and as coagulants in rubber production. Recently the tannins have attracted scientific interest for treatment of AIDS and various cancers (25, 30)

Glycoside is a generic term used for phytochemicals that are bound to a sugar. They are the compounds that yield one or more sugars upon hydrolysis, Hence the glycoside consists of two parts: the sugar and the aglycone part. The aglycon may be a terpene, flavonoid, coumarine etc. Among the sugars found in natural glycosides, D-glucose, L- rhamnose and L-fructose, L-arabinose are commonly found. The sugar part can be disaccharide also. The classification of glycosides is a difficult matter. They are usually mixed acetals. The sugar moiety of a glycoside is joined to the aglycone, according to the chemical group of the aglycone involved into the acetal union, they are O-glycoside (OH group); S-glycoside (SH group), N-glycoside (NH group), C-glycoside (C group). The systematic names are formed by replacing the 'ose' suffix of the parent sugar with "oside". Classification can be based on the sugar group for e.g., lucosides and rhamnosides, classification can be based on aglycone group, Examples are lignan glycosides, alkaloidal glycosides etc. Glycosides which show soap like properties are called saponin. Glycosides that release hydrocyanic acid on hydrolysis are known as cyanogenic glycosides. Based on functional group they can be Phenolic glycosides, Aldehyde glycosides, Anthraquinone glycosides etc. (31)

The compounds which were reported earlier in *C. acuminatum* are alkaloid, carbohydrates, flavonoids, resin, saponin, steroids, tannins,

whereas triterpenoids have been reported negative qualitatively (32), whereas in same report proteins are also documented as negative. Essential oils such as Limonene, Eugenol, citronellal,1-8-cineole,Piperitone and p-cymene were reported by TLC (33),where as in another study beta – sitosterol, cetyl alcohol, glucose, rhamnose are reported. (34) Volatile oils are estimated as 0.54 ± 0.28(%v/w) in Tarikhet sample (32). In one recent studies (35) dietary fatty acids, alpha – hydroxy acids, phenolic acids, sterols, amino acids, sugars and glycoside are reported in this plant by GC-MS. Another group of scientists (36) analysed quantitatively metal content and volatile constituents of the plant by Atomic absorption spectrophotometer and GC-MS. Alpha tocopherol and gamma tocopherol along with terpenoids are also reported by(36), whereas presence of Acidic polysaccharides, Anthocyanins, Lignin, Phenolic substances, cutin, suberin, lignin and starch have been documented (37). Scientists (38) also confirmed the presence of polyphenols, flavonoids, while synthesising gold nanoparticles of the extracts of pseudobulbs. Whereas comprehensive report and fingerprint profilile has been presented in this paper.These secondary metabolites are accountable for the therapeutic activity of plants (39). Hence, great potential of *Crepidium acuminatum* as a nutraceutical and herbal drug is confirmed in the present study.

Conclusion:

C. acuminatum is an important plant of Astavarga. It has a lot of therapeutic potential. The present study revealed the presence of saponins, bitter principles, steroids, Sterols, essential oils, anthraquinones, coumarin and flavonoids in methanol extract. The HPTLC fingerprint profile developed for methanol extract of *C. acuminatum* can be used for routine quality control of the drug and serve as a base for qualitative & quantitative analysis and standardization of the drug. It will also help in identification and quantification of active/marker compounds. By isolating and identifying marker compounds, new drugs can be formulated to treat various diseases.

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Optimization of a simple methodology for Extraction, purification of SDG from *Linumusitatissimum*and its characterization

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Abstract:

This research was conducted to optimize a simple methodology to extract and purify a lignan present in *Linumusitatissimum(*Flax seed also called as line seed). Flax seeds are the richest plant source of lignans and omega-3fatty acid. SDG - Secoisolariciresinol Diglucoside is the principallignan present in flax seeds.The extraction and purification methods vary from simple to complex depending on separation, fractionation, identification and detection of the analytes. To isolate SDG, flaxseeds were defatted by four volumes of n-hexane with continuous stirring and the wet cake was collected. Then the wet cake was heated at 70°C and added 80% (v/v) aqueous methanol, brought to alkaline pH by adding sodium hydroxide with continuous stirring for about 3 h to extract maximum yield (of SDG). Neutralization was made by adding a few drops of conc. H_2SO_4 (pH 5-6), distilled under vacuum in a rotary evaporator. The isolated lignan was purified by column chromatography using ethanol, ethyl acetate as elution system. Desired lignan fractions were collected, combined and the solvent was evaporated in a lyophilized to get the colorless solid which was identified by TLC and has been characterized by NMR, IR and LC MS. "Despite the abundant research conducted on the isolation of SDG this article presents a simple, inexpensive and ecofriendly procedure for extraction."The isolation and purificationwas done by using ecofriendly solvents and provided complete characterizationinformation.

Key words: flaxseed; lignan; secoisolariciresinoldiglucoside; TLC; IR; LCMS; NMR

Introduction:

*Linumusitatissimum*also known as common flaxseed or line seed. *Linumusitatissimum*isa member of the genus *Linum* of the family *Linaceae*. It is a food and fiber crop cultivated in cooler regions of the world. Flax is grown for its oil [1],used as a nutritional supplement, an ingredient in many wood-finishing products, used to produce linen and also cultivated as an ornamental plant.

Flaxseed is found in all kinds of today's foods from crackers to frozen waffles to oatmeal. The Flax Council estimates close to 300 new flax-based products were launched in the U.S. and Canada in 2010 alone. Not only has consumer demand for flaxseed grown, agricultural use has also increased. Flaxseed is what's used to feed all those chickens that are laying eggs with higher levels of omega-3 fatty acids [2].

SDG is an antioxidantphytoestrogen present in flax, sunflower, sesame, and pumpkin seeds, among these seeds, flaxseed is, by far the nature's richest source of plant lignans [3]. In food, it can be

found in commercial breads containing flaxseed [4, 5]. It is a precursor of mammalian lignans[6] which are produced in the colon from chemicals in foods. SDG is the major lignan found in flaxseed, which is known to have antioxidant [7, 8], anti-hyperglycemic[9,10] and anticancer properties [11]. It is believed to play a crucial role in reducing the incidence of several diseases such as hypertension, cardio vascular diseases, cancers and inflammatory diseases [11-16]. Studies have proved that lignan rich diets help reduce the risk of various cancers and heart diseases [7, 8, 11-16].

Althoughample research been conducted on flaxseeds and isolation of SDG[17, 18], there's lot of scope for research because of its vast dietary assets and therapeutic values and antioxidant properties [7-12, 16, 17]. This articleprovided a simple and inexpensive procedure for extraction in less time and purification by using ecofriendly solvents[19] and provided complete characterization information.

Materials and methods

Materials:

Flax seedswere collected from local market. The solvents and reagents n- hexane, methanol, ethyl acetate, ethanol, sulfuric acid, sodium hydroxide andsilica gelused in the extraction methodology were obtained from Fisher, Ranbaxy and Merck. SDG standard used was from Chengdu Bio purify Phytochemicals Ltd. Wenjiang Zone, Chengdu, Sichuan, 611130 China

Flax seeds (100 g) were collected from a local market and powder using a mechanical grinder.Infrared (IR) spectroscopy was performed in an Agilent carry 630 FTIR apparatus. ESI-MS were recorded on a Thermo Finnigan LCQ ion trap mass spectrometer equipped with electron spray ionization. An NMR spectrum was obtained on a Bruker 300 MHz spectrometer using D2O as solvent. The purity was checked by a Waters Alliance HPLC connected to an UV detector by using reverse phase column chemically bonded to Octadecylsilane, Phenomenex Luna C8 (250*4.6mm) 5µ.

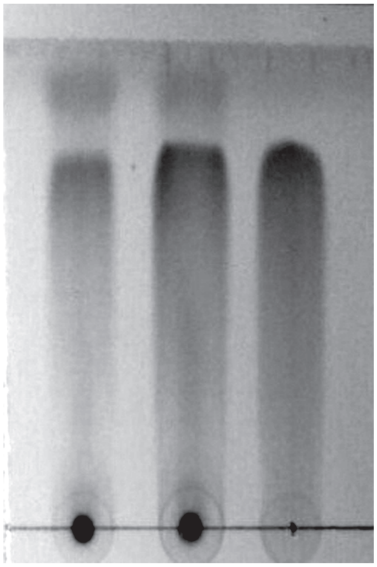
Infrared (IR) spectroscopy was used for the detection of the isolated SDG compound.The experiment performed in Agilent carry 630 FTIR.ESI-MS were recorded on ThermoFinnigan LCQ ion trap mass spectrometer equipped with electron spray ionization. High resolution mass spectra were obtained by using ESI-QTOF mass spectrometer. The experiment performed on a Bruker 300 MHz spectrometer in appropriate solvent using D_2O. The purity was checked by Waters Alliance 2695, High performance liquid chromatography (HPLC) connected with UV detector by using reverse phase column chemically bonded to Octadecylsilane, Phenomenex Luna C8 (250*4.6mm) 5µ.

Defatting and Extraction:

Defatting process was done by adding four volumes (1000mL) of n-hexanewith continuous stirring for 30mins.The resulting mixture was filtered through a Buchner funnel using a Whatman filter paper No.1 the filtrate was discarded and the wet cake collected for the isolation of SDG. Four volumes of 80% (v/v) aqueous methanol [26] and 12g of NaOH was added to the wet cake to get alkaline pH ($\geq$ pH 10). The reaction mass was then heated at 70°C with continuous stirring for about 3 h to extract maximum yield of SDG. After 3 h the reaction mass was cooled to room temperature and neutralized (to pH 5-6) by adding a few drops of conc.H_2SO_4. The aqueous methanol extracted the lignin and then the solvent was removed by rotary evaporator. The result was then evaluated by TLC.

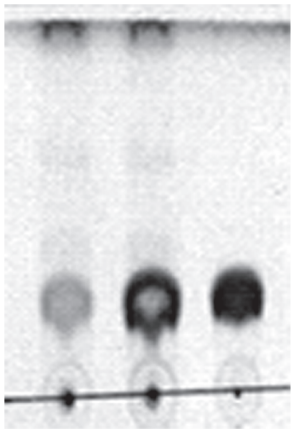
Evaluation by TLC:

Taken a part of the resulting residue, dissolved in ethanol and compared with standard by TLC-Thin layer chromatography method [27] by using solvent system /mobile phase as ethanol: ethyl acetate (90:10) ratio.The resulting TLC pattern was in band manneras shown in Figure A.



1 2 3
1.Sample 2.Co Spot 3.Standard
Figure A

Further TLC wasperformed by using solvent system /mobile phase as ethyl acetate: methanol: ethanol: aqueous (81:11:4:8) ratio to get the spots as desired. The resulting TLC was not in band manner and observed spots for fraction/sample, Co-spot and Standardas shown in Figure B.



1 2 3
1.Sample 2.Co Spot 3.Standard
Figure B

The extract was tested using TLC. The plant extracts were spotted using a capillary tube on TLC plates. The plates developed usingethanol: ethyl acetate (90:10) and ethyl acetate: methanol: ethanol: aqueous (81:11:4:8) ratioswere then viewed under UV fluorescence light at wavelength 254 nm and also exposed to iodine vapors in a chamber.

Purification by Column chromatography technique: A glass column with diameter 20mm, length of 90cmswas clamped upright and packed with silica gel of GF 254 ofMerck, mixed with theappropriate mobile phase and poured into the column as a compact even suspension.This constituted the stationary phase. The extract was then mixed with a small amountof the mobile phase and loaded as a thin band to the silica gel. Once the extract was introduced onto the silica gel, the mobile phase was added at a constant flow rate. Gradientelution of increasing polarity was initiated consisting of successive elution of severalfractions using different solvent mixtures composed of n-hexane, ethyl acetate andethanol.

The ethanol fraction was subjected to column chromatography on silica gel andeluted with stepwise gradient polarity using n-hexane:ethyl acetate (100:0, 80:20, 60:40, 30:70; 20:80; 0:100; each 50 ml fraction collected) as solvent system to given the 16fractions (F1-F16). The system was further eluted with ethyl acetate: ethanol (100:0,95:5, 90:10, 80:20, 70:30, 50:50, 30:70; 20:80; 0:100 each 50 ml fractions collected) togive 32 fractions (F17-F48). These fractions were further subjected toTLC studies andcompared with authentic sample found that Fraction number (F18-F28) contained themajor compound.These fractions were combined together and the solvent wasevaporated to get the colourless solid by using SP Scientific lyophilizer.

Results & Discussion

The resulting compound was a colorless solid, very hygroscopic in nature. The compound was confirmed via different characterization

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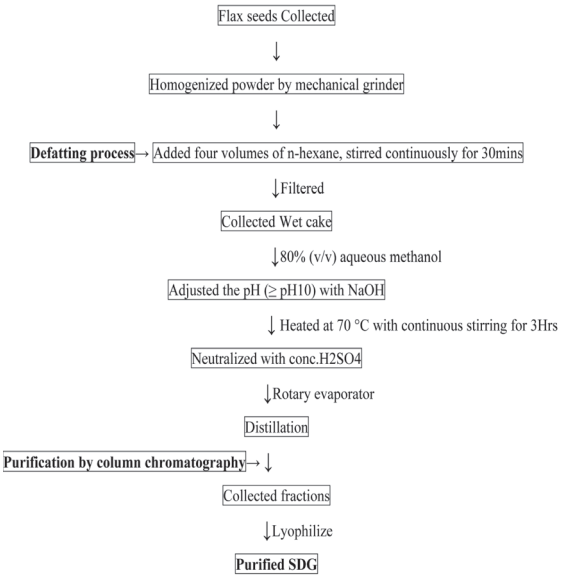

techniques likeFTIR, LCMS, NMR and purity by High Performance Liquid Chromatography.

Isolation and Purification of SDG by reversed phaseHPLC, preparative HPLC[23, 25,31]methods were prominent, but also expensive when compared to column chromatography. The occurrence of probable safety issues in some of the column chromatography methodsdue to the presenceof toxic solvents [17].

However this article indicating the simple and inexpensive procedure for extraction in less time and purification by using ecofriendly solvents [19].The Purified compound showed single spot in TLC and it showed similar Rf value (0.24) with authentic compound.

Fourier Transform Infra-Red (FTIR) for pure SDG : FTIRspectrophotometers were used for recording spectra in the region 4000 cm^{-1} to 670 cm^{-1} (2.5ìm to 15 ìm) or in some cases down to 200 cm^{-1} (50 ìm).Lignan has many functional groups.Phenolic– OH group stretching at wave number 3675.2, Aromatic C=C at wave number 1654.9.49 and Aliphatic C– O at wave number 1054.8.

As shown inFigureC shows the infrared spectra for the extracted pure lignan and matches with the previous IR spectra,I.R Frequencies of extracted SDG were in the range of standard group $cm^{-1}$of the functional groups phenolic-OH group (3650-2500), Aromatic C=C(1680-1620) and Aliphatic C-O(1300-1000)[17].

LCMS: SDG sodium adduct formed *m*/*z* 710.30 $(M+Na)^+$and 688.35$(M+H)^+$ detected in the positive ESI mode. The resulting MS spectrum was shown in Figure D.The formation of sodium and ammonium adducts in LCMS is apparent depending on the source, mobile phase and usage

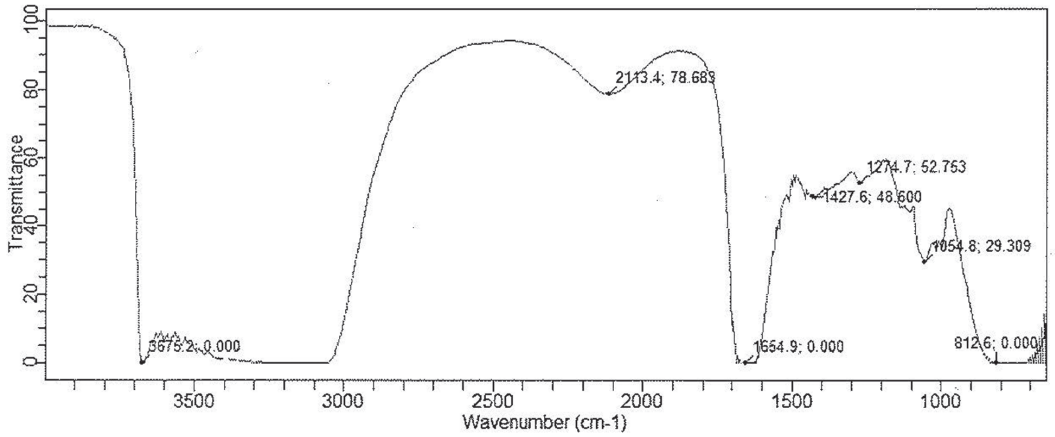

Figure C

of reagents in the process. The *m*/*z* 710.30 $(M+Na)^+$in accordance with previous spectra [28, 29].

Proton magnetic resonance spectroscopy (1H NMR) : Molecular formula of SDG is $C_{32}H_{46}O_{16}$ solvent signal 4.80; sugar resonances obscured by NMR (300MHz): 6.67 (7 & 7', d, 2H); 6.62 (4 & 4', d, 2H:) 6.51 (8 & 8', s, 2H); 4.37 (13 & 13', d, 2H); 3.75 (11 & 11', m, 4H); 3.67 (10 & 10', s, 6H); 3.31 (19 & 19', m, 4H); 3.01 (16, 16', 17 & 17', m, 4H); 2.86 (2&2', m, 4H); 2.23 (18& 18', m, 2H), 2.08-1.99 (15 & 15',m, 2H); 1.99 (1 & 1',

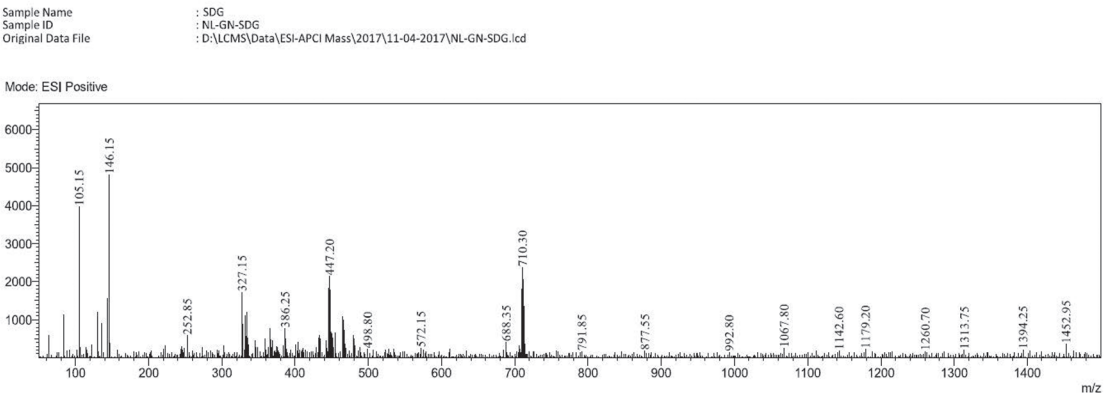

Figure D

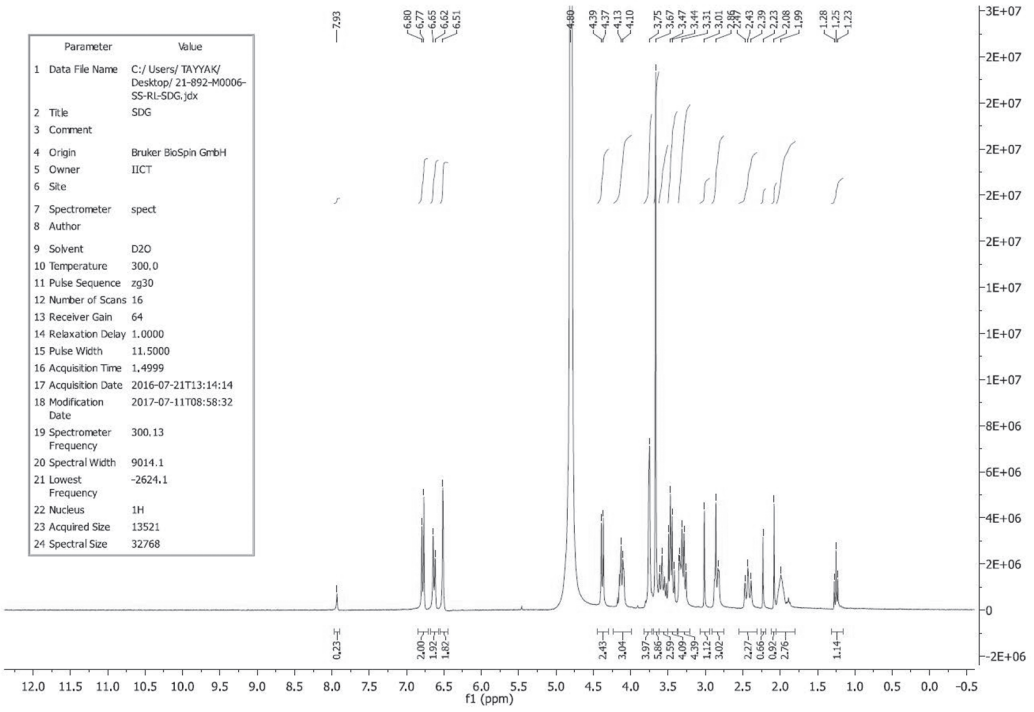

Figure E

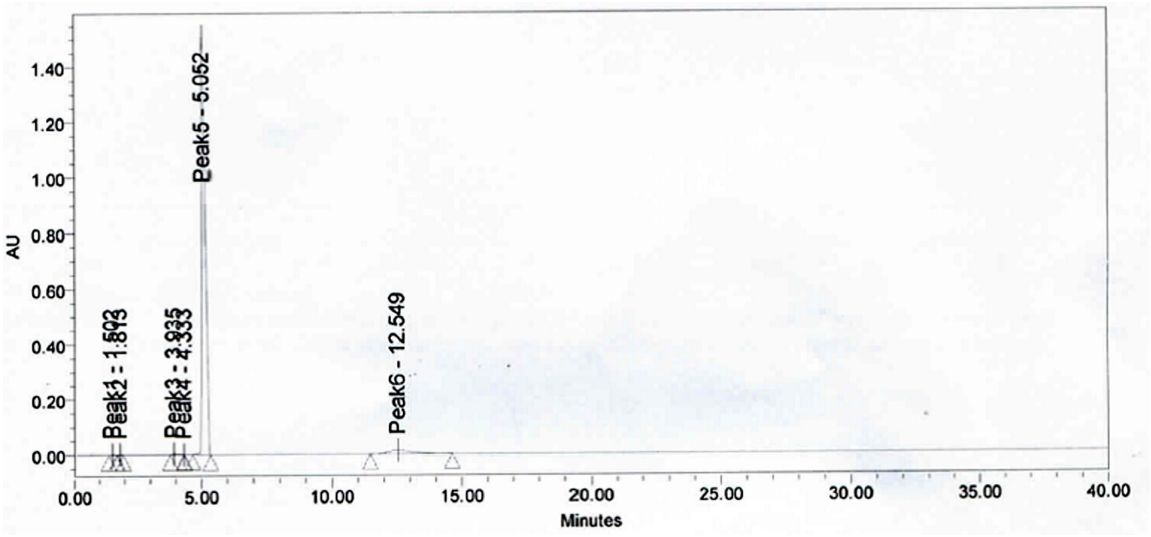

Figure F

m, 2H)spectra shown in FigureE.The NMR spectrum wasin agreement with previousNMR spectra [25, 28, 30].

Purity by High Performance Liquid Chromatography : HPLC connected with UV detector by using reverse phase columnchemically bonded to Octadecylsilane,Phenomenex Luna C8 (250*4.6mm) 5µ. Isocratic system with 1.0mL/min flow rate, mobile phase used was 85:15 ratio of 0.1 % acetic acid: 100% acetonitrile, detected at UV 280 nm. Column compartment temperature was 30 °C as and runtime 40min.Chromatogram shown in FigureF.

Chromatograph obtained major peak with the purity 94.3%.Various methods reported previously for detection purity and quantification [28, 31-33].

Conclusion

In this article we endow the simple and inexpensive procedure for extraction and purification, provided complete characterization information by IR, LC-MS, and NMR. The method above which was optimized for the extraction was taking less time in accordance with many of the existing methods.We obtained about 65mg of pure SDG from 100g of flaxseeds.The purification method was done by column chromatography, very economical and obtained much pure compound(94.3%),which was determined by HPLC.Animmense amount of purified SDG can be obtained by this method to evaluate its bioactivity.

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Characterization and Evaluation of Antibacterial, Antioxidant and Cytotoxicity of synthesised silver nanoparticles (AgNps) using chloroform crude callus extracts of *Wrightia tinctoria* (Roxb.)

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ABSTRACT:

The present work established that the chloroform callus extract taken from the leaf midrib of *in vitro* grown seedling of *Wrightia tinctoria (Roxb.)* is very efficient for the synthesis of AgNPs. The produced silver nanoparticles were preliminarily conformed by UV Visible spectroscopy, SEM, TEM, FTIR spectroscopy and Energy Dispersion Spectrum. UV-Vis spectroscopy analysis revealed the peak at 425nm which corresponds to the surface plasmon resonance of AgNPs. The uniform spherical shape and size was detected by SEM and TEM. The size and stability of AgNps were detected using Dynamic light scattering (DLS), Zeta potential. The synthesised silver nano particles were evaluated for its antibacterial by agar well diffusion, antioxidant activities by DPPH assay and cytotoxicity on MCF-7 and HEK 29 were tested by MTT assay. The result indicated decrease in cell viability and cell growth inhibition. The present study highlighted the possibility of utilizing nanoparticles synthesised from *in vitro* derived callus of WTR for animal or human applications as it enhanced antibacterial, antioxidant activity and Cytotoxicity.

Key words: *WTR* leaf midrib callus, Silver nanoparticles, Bio-reductant, DPPH inhibition, Antibacterial activity, Antioxidant and Cytotoxicity.

Abbreviations :
AgNp - silver nanoparticles
AgNO3 - silver nitrate
UV-Vis - Ultraviolet Visible
FT-IR - Fourier transform infrared spectroscopy
SEM - Scanning electron microscopy
TEM - Transmission electron microscopy
EDS - Enzyme dispersion spectrum
DLS - Dynamic light scattering

INTRODUCTION

The synthesis of nanoparticles have achieved a significant recognition at present scenario because of their excellent applications in techno commercial products(1).Nanoparticles are smaller, ranges from 1 to 100nm which can exhibit high surface to volume ratio and differ greatly from those atoms and bulk materials (2).The nanoparticles has several advantages in numerous fields which includes health care, environmental health, food, cosmetics, biomedical science, energy science, chemical industries, electronics, nano drug and gene delivery (3,4,5); and are still charming the researchers to explore the nanoparticles in various new dimensions (6,7).The noble metal nanoparticles shows variety of applications in various fields. (8). Nanoparticles based treatment has been accepted clinically for diseases, vaccines and renal diseases (9).

Among various nanoparticles, silver nanoparticles have attracted significant consideration due to their effective antibacterial property which exhibits very less toxicity and in various *in vitro* and *in vivo* applications (10,11).

There are several conventional methods for the production of nanoparticles such as chemical, electrochemical (12) and photochemical (13). However, these methods are more expensive and also toxic which leads to biological risk. Bio-assisted production of silver nanoparticles by using plant crude extracts, enzyme and microorganisms pave the way to overcome such limitations. It is cost effective, economic, eco-friendly which is non toxic and free of chemicals (14) and have benefits that the plants are easily available everywhere. Moreover, the silver nanoparticles synthesized from plant extracts are rich in source of secondary metabolites (15) when compared to other methods. The plant extracts contains the organic compounds like carbohydrate, proteins, phenols, flavanoids, triterpenoids, alkaloids, tannins etc. These active ingredients are capable of donating electron and answerable for reduction of Ag to AgNps (16). In addition also aids for the superior antibacterial activity (18,19).

Wrightia tinctoria (Roxb.) is an deciduous tree which belongs to the family Apocynaceae (native to India and Burma). (17) having medicinal importance in curing human ailments like tuberculosis, psoriasis etc.,

This is the first report on synthesis of silver nanoparticles using callus extract of *Wrightia tinctoria* (Roxb.). Since we used plant based biological materials (callus of *Wrightia*) for the synthesis of nano particles they could be safer for human or animal applications.

MATERIALS AND METHODS:

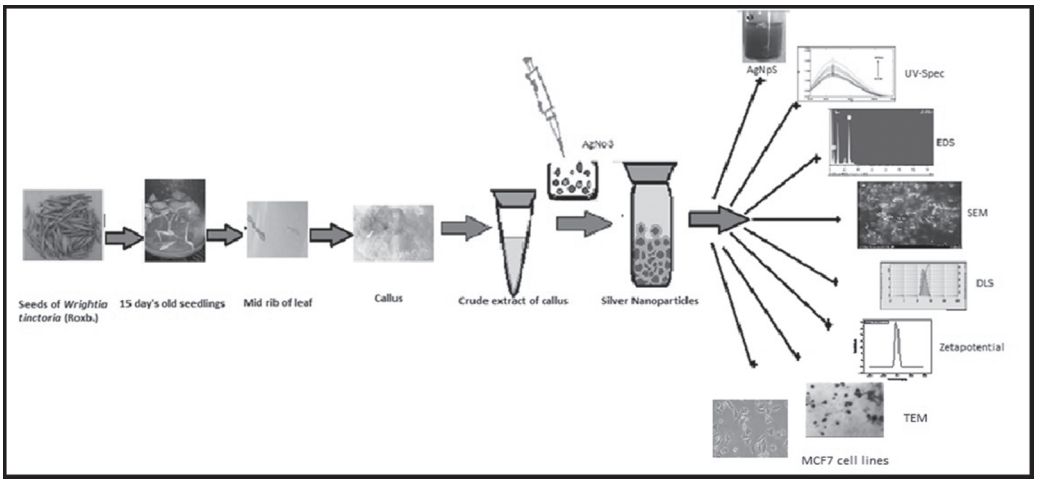
Chemicals and Micro Organisms : The Chemicals required for this study i.e., MS medium, hormones, nutrient medium and DPPH were purchased from Hi-media (Mumbai, India). The reagents were prepared according to standard protocols. pH of the MS media were maintained to 5.8 and 7.4. The strains of bacteria were obtained from the microbial type culture collection (MTCC, Chandigarh, India). The codes of the bacterial culture are as follows: *Pseudomonas aeruginosa* (10636), *Staphylococcus aureus* (6908), *Bacillus subtilis* (1305), *Pseudomonas putida* (1194), *Proteus vulgaris* (744), *Escherichia coli* (9537), *Klebsiella pneumonia* (10309), *Salmonella paratyphi* (ATCC 9150), *Shigilla boydii* (ATCC 9207) and *Enterococcus feacalis* (MTCC 459).

Collection of Plant Materials : The fruits of *WTR* were collected from the Botanical garden of Yogi Vemana University, Kadapa, Andhra Pradesh, India. The gathered fruits were dried at room temperature for 20 days. The seeds were separated from the dried seed pods and stored at room temperature for further use.

Raising of Aseptic Seedlings and Callus Initiation : The dried seeds of *WTR* were first washed gently with tap water for 10 minutes, followed by Tween-20 in distilled water for several times. The seeds were then treated with 1% sodium hypochlorite for 2 min subsequently washed with 70% of alcohol for 60 Sec and rinsed well in distilled water for three times. The sterilized seeds were then dried on a sterile Whatman filter paper and were inoculated on to the MS basal medium pH of 5.8 (20).The leaf midrib of *in-vitro* grown seedlings were excised under aseptic conditions and inoculated on MS medium amended with growth regulators such as KIN, NAA, 2,4-D and BAP in different concentrations. The cultures were incubated at 25°C in dark condition for initial period of one week followed by exposing to photoperiod of 16 h light followed by 8 h dark which resulted in the initiation of callus.

Preparation of Chloroform Callus Extracts : The *in vitro* grown friable calluses were dried at 40°C and then grinded in a blender. The fine powdered callus was stored in a glass container for further use. About 10 g of the powdered crude callus was used for extraction with chloroform in a soxhlet apparatus for 2 days and followed by rotary evaporator to get fine powder extract and named it as callus chloroform extract.

Synthesis of AgNps : In a clean sterile 250 ml Erlenmeyer flask, 10 ml of crude callus extract was assorted with 90 ml of 1mM $AgNO_3$ under dark conditions. The mixture was then incubated at 37°C on shaker incubator under dark conditions until the colour reform from light yellow to brown.



The bio synthesized nanoparticles were then complying by UV Visible spectrophotometer.

Characterization : The biological reduction of $AgNo_3$ to AgNps was observed periodically using UV-Vis spec (UV-1800 240V Shimadzu, made in Japan) and the absorbance values was noted at a range of 200-700nm. AgNps suspension was dried and made into powder. 1mg fine powder was used for the characterization of SEM and EDS using the instruments JEOLJSM 6360A (SEM), and JEOLJSM 1600(EDS). A fine coater for uniform coating of Platinum on the sample. Analysis was carried out on JEOLJED-2300 Analysis Station. The structure of nano size particles was observed by TEM Hitachi h-7500. The nano particles were coated on to the carbon coated copper grid and observed under various magnifications using transmission electron microscope. The size distribution were identified by using Dynamic light scattering, Zeta potential, EDS and FTIR,

Antibacterial Assay : Pathogenic strains from MTTC were used in this assay. Bactericidal activity was done by using Agar well diffusion method. Bacterial cultures were cultured in nutrient broth for 24h at 37°C. These bacterial strains were spread on nutrient agar medium by L-shaped rod and wells were made using cork borer. The samples viz, chloroform callus extract, silver nanoparticles, silver nitrate, +ve control as ampicilin and -ve control as DMSO were loaded to the wells carefully and was incubated for 24h at 37°C. The inhibition zone diameter was measured in centimeter. The experiment was repeated for three times and the graphs was plotted using graphpad prism 5.

Antioxidant Activity : The free redical activity was performed by DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging method. The various concentrations of synthesized silver nanoparticles from callus extract are dissolved with 3 ml of the DPPH and ethanol. The combination was vigorously assorted and kept in dark condition for 30mins. The absorbance of optical density was taken at 517 nm by using UV-Vis spectrophotometer against standard ascorbic acid as +ve control and blank DPPH as -ve control. The free radical scavenger activity was calibrated by a formula given below (21).

$$\text{Inhibition \%} = I_0\text{-}I/I_0 \times 100$$

Where I_0 was the absorbance of control/or blank and I was the absorbance of sample.

Cytotoxicity: Viability of MCF-7 and HEK293 cells was assessed by the MTT Assay with six different concentrations of extracts in triplicates. Cells were trypsinized. The trypan blue assay was performed to know the viable cells in cell suspension. Cells were counted using hemocytometer and seeded

at density of 5.0 X 10 ^{3} cells / well. 100µl media was poured into 96 well plate and incubated at 37^0 C for overnight. After incubation, the old media was replaced with 100µl of fresh media along with different concentrations of test compound in representative wells in 96 plates. After 48 hrs., the drug solution was discarded and the fresh media was added with solution of MTT (0.5 mg / mL^{-1}) to each well and incubated for 3 hrs at 37^{0}C. Precipitates were formed at the end of incubation time. The reduction of MTT salt to chromophore formazan crystals by the cells with metabolically active mitochondria was observed. Compute the solubilised crystals at 570 nm using micro plate reader. The percentage inhibition of growth was quantified using the formula. The concentration of test drug needed to inhibit cell growth by 50% values is produced from the dose-response curves for each cell line using origin software (22).

$$\% \text{ Inhibition} = \frac{100 \text{ (Control - Treatment)}}{\text{Control}}$$

RESULTS and DISCUSSION

Callus Initiation : The tender leaf midrib explants from the *in vitro* grown seedlings were used for the initiation of callus. The callus was grown in almost all the combinations and concentrations, tested. Among different concentrations and combinations of hormones tested for callus induction, the friable callus from MS medium supplemented with NAA (2.5mg/l) + (KIN 1mg/l) (Fig-1) has chosen for the study.

The friable light greenish callus obtained from the 4th week of tissue culture were used for the photochemical analysis. The chloroform extract of *in vitro* grown callus showed the presence of carbohydrates, phenols, phytosterols, saponins, alkaloids, xantho proteins, quinine, tannins and coumarin. There is a rich amount of phytochemicals because of the actively dividing cells present in callus. Various biomolecules present in the callus acts as catalyst by helping in bio reduction and stabilization of synthesized nanoparticles (23).

The friable callus from MS medium supplemented with NAA 2.5mg/l+ KIN 1mg/l was chosen for the synthesis of AgNps.

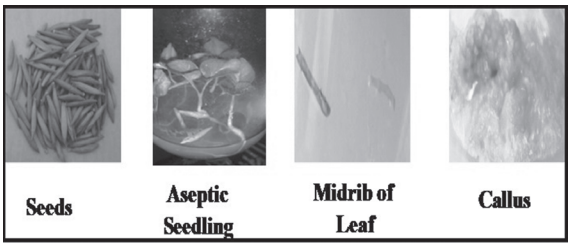


Fig. 1: Different Stages of Callus Initiation

Synthesis of AgNps : The visual observation of colour modification was noted after the treatment with callus extract of *WTR* to $AgNO_3$ solution with continuous shaking at room temperature (Fig. 3). The colour changed from pale green to brown colour. The callus extracts did not show any change in colour in the absence of $AgNO_3$ (Fig.4). The change of colour to light brown is mainly due to diminution of $AgNo_3$ to AgNps and the excitation of plasmon resonance of the AgNps (24). It may also due to the bioactive compounds present in the sample (25).

UV Spectrophotometer : Due to the size of nanoparticles, UV-Vis spectrum revealed an absorption maxima at 425nm. The absorbance (max) also showed blue tendency (distribution of size>35nm) with a peak at 425nm (distribution of

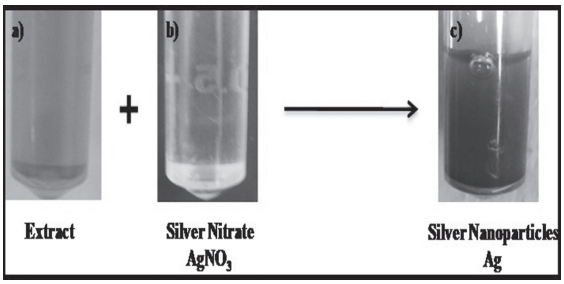


Fig. 3. Synthesis ofSilver Nanoparticles (a) *Wrightia tinctoria* (Roxb.) *Callus Extract* (Yellow colour) *(b)* $AgNO_3$ Solution and (c) Synthesized Silver Nanoparticles (Brown colour).

size =/<35nm) because of the various sized nanoparticles which confirms the formation of bio-reduced silver nanoparticles (26). SNps peaks at 420-480 (27) and 440-460 (28) UV were observed. These noble metal nanoparticles exhibited a strong absorption peak at 425nm in visible range which is named as surface plasmon resonance peak. The bioactive compounds in callus extract aids in the stabilization of synthesized silver nanoparticles. The absorption peaks at 425 nm gets sharper and colour intensity increased with incubation time which results in the formation of larger amount of nanoparticles (29).

SEM and EDS

The SEM analysis showed the formation of the properly distributed (or) dispersed and

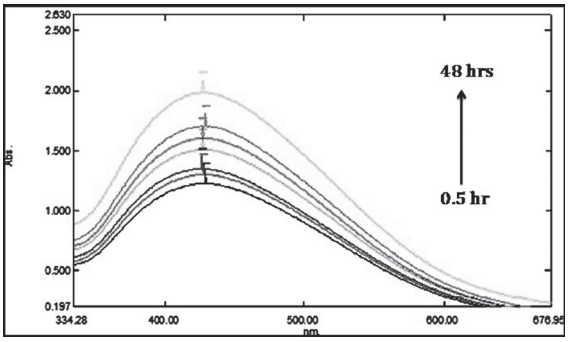


Fig. 4. UV Spectral Analysis of Silver Nanoparticles Synthesised from *In Vitro* Derived Callus of *Wrightia tinctoria* (Roxb.) Mid Leaf Vs Incubation time (0.5 h to 48 hrs).

morphologically stable AgNps obtained from the callus. As shown in Fig. 5, synthesized Ag nanoparticles seemed to be roughly spherical ranging in the size from 35nm to 89nm.The results suggested that, the callus extract of *WTR* act as good bio-reductant for synthesis of AgNps. The surface plasmon resonance (max) observed in the UV-Vis spectroscopy because of the size which resemblance with the SEM. Further, the EDS gives both qualitative and quantitative information about the presence of elemental silver metal (37 %).

TEM analysis : The transmission electron microscopy reveals the presence of well dispersed nano particles obtained from chloroform callus extracts, acts as capping agents and the formed nanoparticles. Most of the particles that are synthesised are in nano range which are round in shape and the diameter size ranges from 25 to 80nm (Fig.6). By increasing the concentration of callus extract, the size of the particle is decreased.(30)

DLS and Zeta potential : The size of the monodispersed silver nano particles is around 25 to 100nm in diameter which was measured by DLS. The histogram bars indicate the percentage of the volume for the AgNps.The wide range distribution of AgNps was quantified from 20-95nm. The calculated mean average particle size distribution of AgNps is 59.9nm (Fig.7) (31,32). The Zeta potential of the synthesised silver nanoparticles was found as a sharp peak at -14.2mV. It is recommended that the medium

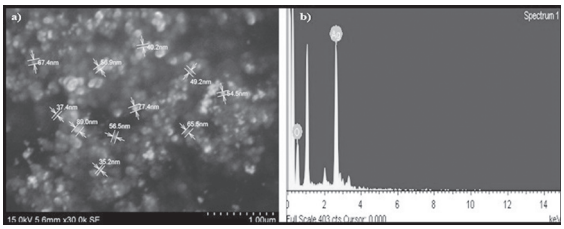


Fig. 5. (a) Scanning Electron Microscopic image (b) Energy Dispersive Spectrum of Ag nanoparticles Synthesized Using Callus Extract of *Wrightia tinctoria* (Roxb.)

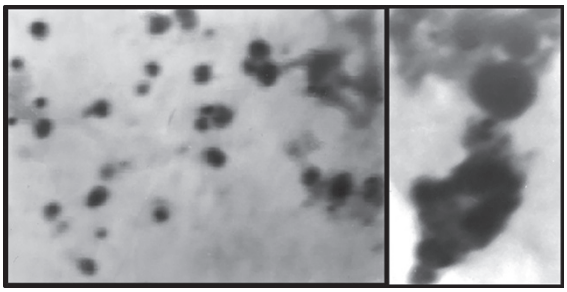


Fig. 6. (a) Transmission Electron Microscopic image of Ag nanoparticles Synthesized Using Callus Extract of *Wrightia tinctoria* (Roxb.)

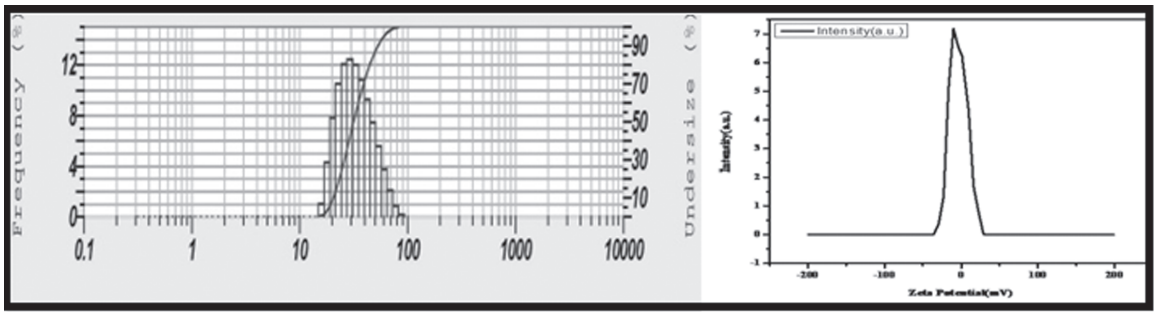


Fig. 7. Dynamic light scattering and Zeta potential of Ag nanoparticles Synthesized Using Callus Extract of *Wrightia tinctoria* (Roxb.)

dispersed silver nanoparticles with negative charge confirms the repulsion among the particles which proves that the AgNps are stable.

Fourier Transform Infrared (FTIR) Spectroscopy : The bioactive compounds act as an bio-reductant and capping agent for the creation of AgNps. The chemical interaction between silver and biological compounds present in the callus extract was investigated by the FTIR spectra. The FTIR spectra of callus extract of *WTR* was presented in Fig 8. Biological compounds present in the callus extract were also investigated by the FTIR spectra. The appearance of peaks at 2850 cm^{-1} and 2341 cm^{-1} was ascertained to aliphatic group and –C-H stretching of alkanes respectively. Similarly, the bands at 1384 cm^{-1} corresponds to nitro N-O bending respectively. Further, the peak at 578 cm-1 correspond to the alkylhalides (C-Cl) and the characteristic OH stretching vibrations of phenol/carboxylic group present in extract was observed at 3471 cm-1. Moreover, the appearance of bands at 1631 cm-1 is assigned to stretching mode of C=O stretching or amide bending. The peak at 1114 cm^{-1}corrosponds to C-O-C stretching of aromatic ring. With this study, it may be inferred that the presence of all these bioactive compounds of *WTR* callus extracts acts as a capping agent and aids in the bio-reduction /stabilization of silver nanoparticles (33,34).

Antibacterial Activity: The synthesized AgNps were tested against ten pathogenic bacteria viz., *Pseudomonas aeruginosa, Staphylococcus aureus, Pseudomonas putida, Bacillus subtilis,*

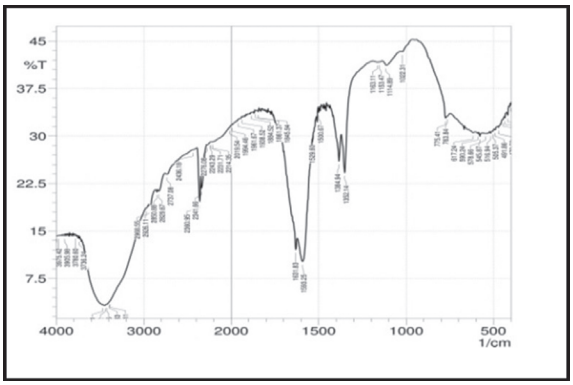


Fig. 8. FTIR Spectra of *Wrightia tinctoria* (Roxb.) Callus Extracts and Synthesized Silver Nanoparticles.

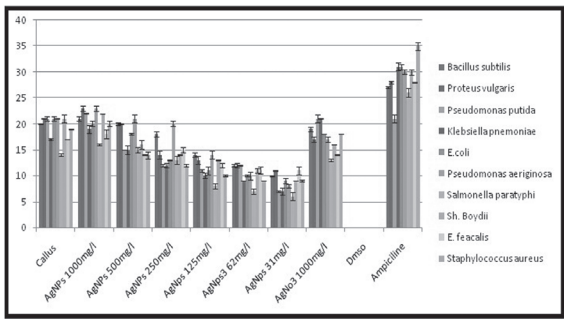


Fig. 9. Zone of inhibition (mm) of Callus, Silver Nanoparticles, Silver, Antibiotic, DMSO *against* ten Different Human Pathogens.

Escherichia coli, Salmonella paratyphi, Klebsiella pneumoniae, Proteus vulgaris, Shigilla boydii and Enterococcus feacalis. Due to the large surface area of silver nanoparticles it is showing efficient antibacterial activity. The synthesized nanoparticles demonstrate a superior antibacterial activity against 10 pathogenic bacteria. The antibacterial activity of AgNps against bacteria is as follows as *Sh. boydii* > *Proteus vulgaris*> *Pseudomonas aeruginosa*> *Pseudomonas putida*> *Bacillus subtilis* > *E-coli*> *Klebsiella pneumonia*> *Staphylococcus aureus* > *Enterococcus feacalis* >*Salmonella typhi*. Moreover, the inhibition zone of synthesized silver nanoparticles, callus, silver and antibiotics was compared against all the 10 bacteria and the values are tabulated in Fig.9. It was found that zone of inhibition of synthesized silver nanoparticles is more than that of callus and silver.The leaf derived silver nanoparticles of *Wrightia tinctoria* (Roxb.) showed effect on *Staphylococcus aureus*, *Klebsiella pneuminiae* (35).

Antioxidant Activity : The antioxidant activity of *WTR* leaf callus assisted silver nanoparticles was estimated by using DPPH assay, The percentage of inhibition was calculated based on the absorbance values taken from UV-Visible spectrophotometer at 517 nm. The synthesized silver nanoparticles showed very good percentage of free radical scavenging activity than that of *in vitro* derived callus extract. The values are tabulated in Fig.10 and Table 1. The valuable secondary metabolites present in the plant extracts showed antioxidant activity (36). These secondary metabolites may involve in the inhibition of the oxidative chain reaction as a result of oxidation of the molecules. Due to redox property of phenols, it may be showing antioxidant activity and in turn plays a role in neutralizing oxygen free radicals, quenching of singlet and triplet oxygen. The anti oxidant activity has been attributed by the capacity of the phyto compounds in donating H- ions (37).

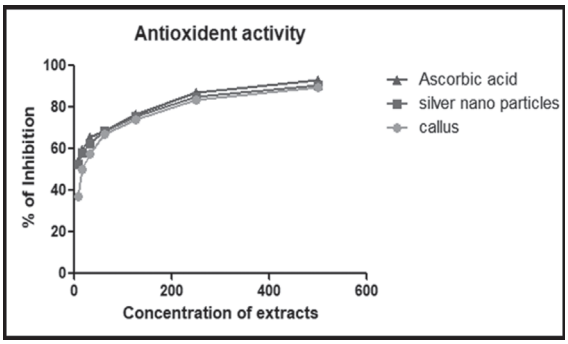


Fig. 10. Percentage of Inhibition of Free Radical Scavenging Activity of *Wrightia tinctoria* (Roxb.) Callus, Silver Nanoparticals and with the Standard.

Table. 1. Percentage of Inhibition of Free Radical Scavenging Activity of *Wrightia tinctoria* (Roxb.) Callus and Silver Nanoparticals Compared with the Standard.

Name of the Extract/ Standard	**Antioxidant activity IC50 (μg/ml)**
Chloroform Extract	17.04±0.5
Silver nanoparticles	13.70 ±0.8
Ascorbic acid	6.499±0.87

Cytotoxicity: The cytotoxicity of the crude and silver nanoparticles of callus extracts of *WTR* on MCF-7 cells from human breast cancer and normal cells HEK 293 cells was investigated by MTT assay. Based on the dose of extract the cell growth as well as the viability of cells were decreased. The IC_{50} values of MCF 7 for crude chloroform extract, silver nanoparticles, commercial nanoparticles and standard cisplatin were 62.21, 104.96, 46.192 and 3.609 where as for HEK 293 no activity on cells was detected. When compared to the crude extract, silver nanoparticles demonstrated more anti oxident, and antiproliferative activity(Fig.11). The bark extract of *WTR* cytotoxic activity on MCF-7 cell lines and HL-60 cell line (38); leaf methanolic extract of *WTR* showed 50% effect on Huh5.2 cell line (39). Petroleum ether and ethyl acetate fractions of *WTR* are more potent on MCF-7 cell lines (40).

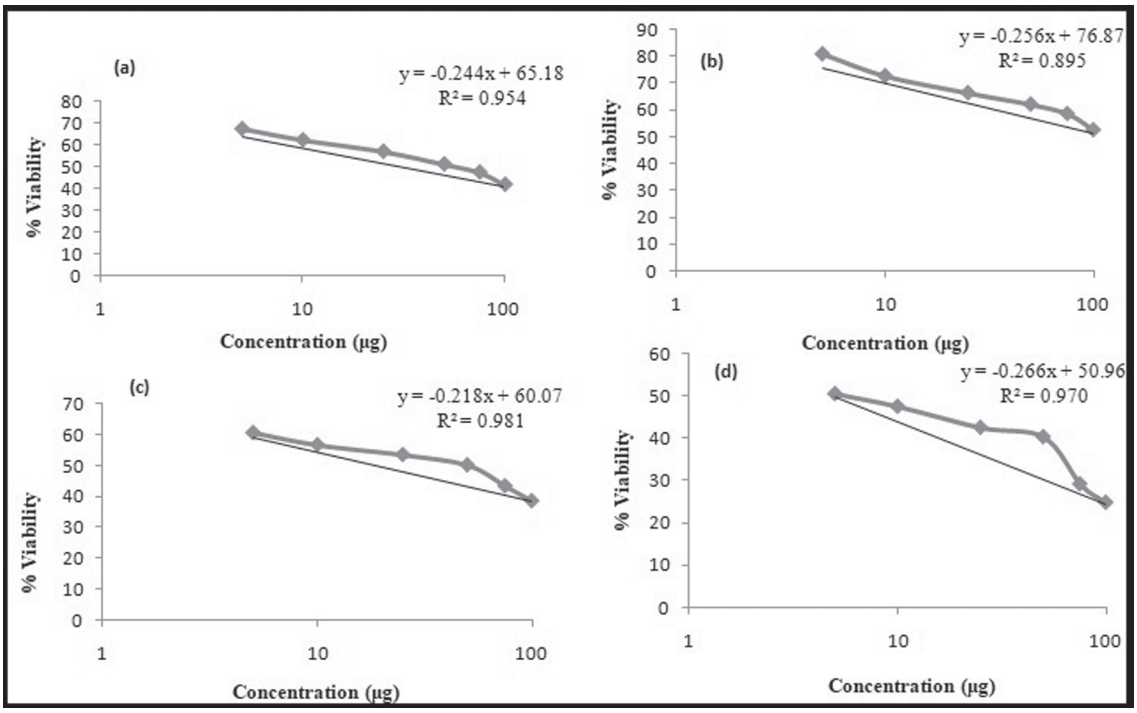


Fig. 11: Cytotoxic Effect of the (a) crude exract, (b) Synthesised Silver nanoparticles (c) Nanoparticles (d) cisplatin on MCF 7 Cell Line

Table. 2. Cytotoxic effect of callus crude extracts of Wrightia tinctoria (Roxb.) and silver nanoparticals compared with the nanoparticles and Cisplatin on MCF 7 and HEK293 Cell Lines

S. No.	Sample Description	IC_{50}(µg) MCF 7	HEK293
1	Crude extract	62.21	ND
2	Nanoparticles	104.96	ND
3	Silver nanoparticles of callus	46.192	ND
4	Cisplatin	3.609	-

CONCLUSION :

The bioreduction of Ag^+ ions by the *in vitro* derived callus of *WTR* leaf midrib leaf has been demonstrated. It is believed that *in vitro* derived callus extract are rich in phyto-chemicals which can act as a bioreducing agents for the production of AgNps with a size ranging from 10-100nm. The resulted AgNps were preliminarily categorized by UV–Vis spectroscopy, FTIR and SEM equipped with EDS. The silver nano particles synthesized from callus extract are eco friendly and efficient. It showed enhanced antibacterial activity against ten bacteria and also efficient antioxidant activity. Antibacterial studies of synthesized plant mediated-silver nanoparticles on human pathogens pave a way to develop nano-medicine against various human and veterinary pathogens.

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Phytochemical analysis and *in vitro* antimicrobial activities of *Terminalia arjuna* leaf, bark and fruit extracts in different solvents

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Abstract

The soxhlet extracts of fruits, bark, and leaf of *Terminalia arjuna* were obtained using different solvent viz. water, methanol, ethanol, acetone, chloroform and petroleum ether and were analyzed for their phytochemical, antibacterial and antifungal activity. The phytochemical activity of leaf, bark and fruit extract of *T. arjuna* were performed using all six solvent. Results clearly indicate the presence of alkaloids, carbohydrates, cardiac glycosides, proteins, phytosterols, flavonoids, tannins, terpenoids, saponins, and phenols/ polyphenols. Moreover, proteins, flavonoids, tannins, and phenols were present in almost all leaf, bark and fruit extracts of *T. arjuna*. Antibacterial activity of the crude extract was studied against two each of gram-positive and gram-negative bacterial strains along with three fungal strains. All microbial strains (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Aspergillus niger, Aspergillus flavus* and *Candida albicans*) were procured from IMTech Chandigarh. Antimicrobial activity was performed using the disc diffusion method. The antibiogram of bark extract in methanol, acetone and petroleum ether showed significant ($p < 0.01$) antimicrobial activity. Similarly, significant antimicrobial activity ($p < 0.01$) was observed within the chloroform and aqueous extract of fruits. Maximum antibacterial and antifungal activity was found to be present in the aqueous extract of fruit indicating its probable significance in the reduction of infectious diseases within the feeding livestock population.

Keywords: *Terminalia arjuna,* Bark, Leaves, Fruit, Phytochemical Screening.

Introduction

Study of medicinal plants as natural products is widespread throughout the world [1]. From pre-historical period, medicinal plants have been used for traditional and conventional medicine formulations. People, in general, prefer these medicinal formulations due to their safe, effective and inexpensive mode. Henceforth, medicinal plants are the indispensable part of human healthcare system [2].

Terminalia arjuna tree has a cosmopolitan distribution and is found throughout the Indian subcontinent. It is present in the form of rows within the dry hill areas of several plants near water bodies - rivers, streams, and ravines. It is also planted for ornamental purposes. It thrives best on loose moist, fertile alluvial loams soil and shallow soil, often overlying more or less impervious rock. *T. arjuna* is an evergreen large deciduous tree reaching up to a height of 60-85 feet, bearing yellow flowers and conically shaped leaves [3]. Fruit is fibrous woody, 2.5-3.5 cm long, having five hard wings, striated with numerous curved veins. It has a buttressed trunk and a vast spreading crown from which the branches bent downwards. Flowering occurs between March to

June and fruiting between September to November [4].

Traditional Indian medicinal herb i.e., *T. arjuna* has many therapeutic applications in Ayurvedic, Unani and Homeopathic [4]. Its barks find applications in various medicinal practices as it is rich in calcium, magnesium salts, and glycosides which are prominently used in Ayurvedic medicines [5]. Juice of its leaves finds applications in the treatment of dysentery and headache [6, 7]. Due to the high antioxidant property of its fruits which is similar to Vitamin E, it helps in maintaining the cholesterol level [8]. It strengthens the heart muscle and also improves cardiovascular output [9]. *T. arjuna* is used to cure coronary artery disease, angina, heart failure, edema, and hypercholesterolemia [9]. Due to its diuretic, prostaglandin enhancing and coronary risk factor modulating properties, it is used in the treatment of asthma.

Active phytochemicals present in medicinal plants along with antimicrobial activity plays a key role in the prevention of various infectious diseases and could be a potential tool in combating antibiotic resistance among pathogenic microbes [10]. It has also been used traditionally as a milk decoction [11]. *T. arjuna* has been mentioned in Vagbhata and Ashtânga Hridayam as a medicine for the treatment of haemorrhage, wounds, and ulcers. Thus the study was conducted in order to investigate the presence of secondary metabolites in the leaf, bark, and stem (fig. 1) of *T. arjuna* plant and it's *in vivo* antimicrobial activity.

Materials and Methods

Collection of Plant Material: *Terminalia arjuna* leaves, bark and fruits were collected from a location situated at the bank of Ajhari kund (Geographical location: 27°362 15.043 N; 77°352 00.863).

Bacterial Culture: *Bacillus subtilis* (MTCC 2057), *Escherichia coli* (MTCC 294), *Staphylococcus aureus* (MTCC 3160) and *Pseudomonas aeruginosa* (MTCC 2581) bacterial strains were employed in the antimicrobial analysis.

Fungal Cultures: *Aspergillus niger* (MTCC 282), *Aspergillus flavus* (MTCC 873) and *Candida albicans* (MTCC 227) fungal strains were used in the antimicrobial analysis.

Preparation of Extracts: 30 gm of each plant part (leaf, bark, and flower) was sequentially extracted with different solvents (200 ml each) on the basis of their decreasing polarity (Water> Methanol> Ethanol> Acetone> Chloroform> Petroleum ether) by using Soxhlet apparatus for 18 hours at a temperature equivalent to the boiling point of the solvent [12]. These extracts obtained were filtered by using Whatman No. 1 filter paper

Fig. 1. Plant parts of *T. arjuna* employed in the study. **(A)** Leaf and flowers. **(B)** Fruit. **(C)** Bark.

and concentrated by evaporating the solution at 40°C in a hot air oven for 24 hrs. Few drops of chloroform were added to the extract to prevent the growth of fungal contaminants and ultimately the dry extract obtained was stored at 4°C in sterilized sample bottles [13]. Percent extractive values of each extract were calculated by the following formula.

$$Percent\ Extract = \frac{Weight\ of\ dried\ extract}{Weight\ of\ dried\ plant\ material} \times 100$$

Phytochemical screening of Extracts: The standard methods of Harbarne [14] were used to test for the presence of phytochemical in the different extracts of *T. arjuna* leaf, bark and fruit extracts.

Alkaloids [Mayer's Test]: 0.5-1 ml of the sample was taken in a test tube and few drops of Mayer's reagent were added to it. The solution was well shaken and allowed to stand for some time. The appearance of cream color ppt. indicates the presence of alkaloids.

Carbohydrates [Benedict's test]: 0.5-1 ml of the sample was taken in a test tube and few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) were added to it. Upon boiling in a water bath, the appearance of a reddish-brown ppt indicates the presence of a reducing sugar.

Cardiac glycosides [Sodium nitroprusside test]: 0.5-1 ml of the sample was taken in a test tube and a pinch of sodium nitroprusside powder was added to it. 2-3 drops of 10% sodium hydroxide solution was added, mixed and allowed to stand for 2-3 min. The appearance of red color indicates the presence of cardiac glycosides.

Proteins [Ninhydrin test]: 0.2 % solution of Ninhydrin was added to 0.5-1 ml of the sample and subsequently heated. The appearance of a purple/violet color indicates the presence of protein.

Detection of phytosterols [Salkowski test]: 0.5-1 ml of sample was taken in a test tube, few drops of chloroform and conc. sulphuric acids were added. The solution was mixed well and kept undisturbed for some time. The appearance of a lower yellow layer indicates the presence of triterpenoids.

Flavonoids [Alkaline reagent test]: 0.5-1ml of sample was taken in a test tube; few drops of 10% sodium hydroxide solution were added. The appearance of an intense yellow color, which turns colorless on the addition of few drops of dil. Hydrochloric acid (HCl), indicates the presence of flavonoids.

Tannins and phenolic compounds [Ferric chloride test]: 0.5 ml of sample was taken in a test tube. Add few drops of ferric chloride. The appearance of blue- green color confirms the presence of tannins and phenols.

Test for Terpenoids: 2 ml of sample was taken in a test tube, 2 ml of chloroform and conc. sulphuric acids were carefully forming a layer of each. The appearance of reddish brown color indicates the presence of terpenoids.

Test for Saponins: 2 ml of sample was dissolved in 2 ml of Benedict's reagent. The appearance of a blue-black color precipitate indicates the presence of saponins.

Phenols/polyphenols: A small amount of sample was dissolved in distilled water and 0.5 ml Folin-ciocalteu reagent was added to it. 2 ml of 20% sodium carbonate was added to the solution. The appearance of a bluish color indicated the presence of phenols.

Screening for in vitro antimicrobial activity: The antimicrobial susceptibility tests were carried out using disc diffusion assay [15]. Sterile filter paper discs (Whatman no. 1, diameter 5 mm) were impregnated with 40 ml of the extract (10 mg/ ml) and left to dry in vacuum so as to remove residual solvent. The bacterial and fungal pathogens were initially grown on Mueller-Hinton broth and Czapekdox broth medium respectively. Bacterial and fungal suspensions were prepared by obtaining the inoculum size 1×10^7 CFU/ml in a sterilized medium [12]. Under aseptic condition

Mueller-Hinton agar media and Czapekdox Agar medium agar medium were poured in sterilized petri dishes for growth of different pathogenic bacterial and fungal strains [16]**.** Using a sterile cotton swab, 500 µl of the suspensions were spread over the Mueller-Hinton and Czapekdox agar plates for obtaining uniform microbial growth on test plates. Different extract discs were then placed in triplet on the Mueller-Hinton and Czapekdox agar plates at concentration of 10 mg/ml. Results were compared with that of the standard Himedia antibiotic disc i.e., Gentamicin (10 mcg/disc) and Ketoconazole (10 mcg/disc) as standard for bacterial and fungal strains [17]. The plates were then incubated at 37°C for bacterial strains (24 hrs) and at 27°C for fungal strains (48 hrs**)** [18]. Each experiment was repeated thrice and the average inhibition zones for leaf bark and fruit extracts were recorded and compared with the standard reference antibiotics [19].

Statistical Analysis: Statistical analysis of the data (Zone of inhibition) obtained was carried out using one way analysis of variance (ANOVA) using SPSS ver. 20.0 software and Duncan's multiple range test (DMRT) at $p < 0.05$ and $p < 0.01$ to determine the significant difference in mean values among the treated and the control. All values were expressed as mean ± S.E.M (standard error of the mean).

Results and Discussion

Phytochemical screening: Phytochemical analysis of methanol, ethanol, chloroform, acetone, petroleum ether and aqueous (water) extracts of leaves, bark, and fruits of *T. arjuna* revealed the presence of alkaloids, carbohydrates, cardiac glycosides, proteins, phytosterols, flavonoids, tannins, terpenoids, saponins and phenols/ polyphenols. Similar results have also been obtained by other researchers globally [20, 21]. The presence/ absence of various phytochemicals in different plant part (leaf, bark, and fruit) extracts are shown in table 1. The plant leaf extract revealed the presence of flavonoids, proteins, phenols/ polyphenols and carbohydrates ("+" in all extract) while the bark extract displayed the presence of all the active phytochemical ingredients except cardiac glycosides and phytosterols [22].

The fruit extract displayed the high content of proteins, flavonoids, tannins and phenol/ polyphenol ("+" in all extracts) indicating its potential to act as a source for fruit protein concentrate (FPC) along with leaf protein concentrate (LPC) for the feeding livestock's [23]. Almost similar results with slight variations have also been reported in other studies [20, 21, 22, 23].

Antimicrobial activity: Antimicrobial activity of the leaf, bark and fruit extracts were tested against selected microorganisms and inhibition zone was recorded (table 2). Plant extracts of leaf, bark, and fruit of *T. arjuna* showed significant ($p < 0.01$) antimicrobial potential against *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *A. niger*, *A. flavus* and *C. albicans* (fig. 2). Among all the extracts, aqueous extract of fruit displayed maximum

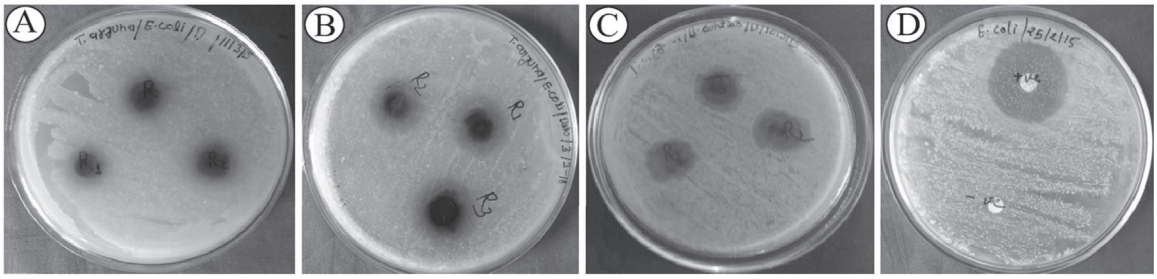

Fig. 2. Antimicrobial activity of *T. arjuna* methanolic extracts. **(A-C)** Antimicrobial activity of leaf **(A)**, bark **(B)** and fruit **(C)** extract against *E. coli* culture. **(D)** Positive control (G: 10 mcg) and negative controls (disk rinsed in methanol) employed during the study.

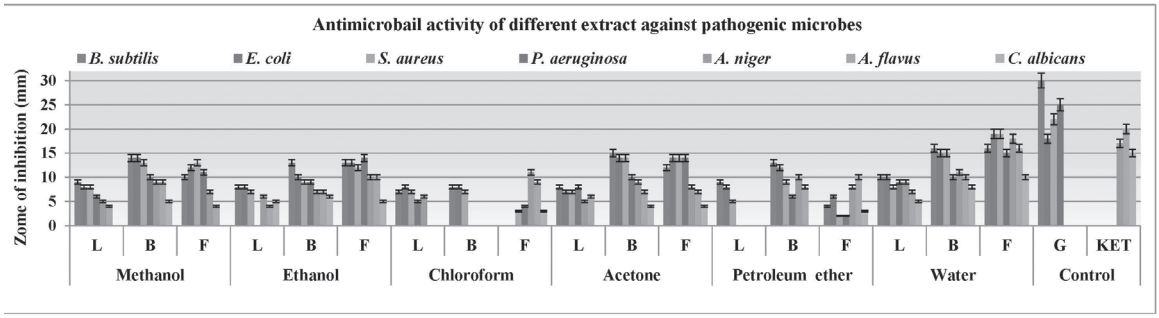

Fig. 3. A comparative antimicrobial activity of different extracts of leaf, bark and fruits of *T. arjuna* in different solvents employed. Considerable high antimicrobial activity was found to be associated with the aqueous extract of the fruits as compared with the activity of other extracts. The antimicrobial activity was compared with that of control.

antimicrobial activity against both bacterial and fungal strains (fig. 3). Most susceptible bacterial pathogens were found to be *E. coli* and *S. aureus* against which, the extracts displayed maximum inhibition zone (7 to 19 mm in leaf, bark and fruit extracts) similar results have also been obtained by other researchers [23, 24]. Among fungal strains genus, *Aspergillus* (*A. niger* and *A. flavus*) displayed maximum inhibition zone (4 to 18 mm in leaf, bark and fruit extract) while other researchers have reported the antifungal activity of leaf and bark extract against *C. albikans* [24]. The antimicrobial activity accessed was compared with that of standard antibiotics (Gentamycin and Ketoconazole) (table 3) (fig. 3).

The values represent the mean SEM of experiments performed in triplet sets. Statistical analysis was performed using one way ANOVA followed by DMRT revealed the results to be significant ($p < 0.01$).

Leaf extract in methanol, ethanol, chloroform, acetone, petroleum ether and water displayed significant ($p < 0.01$) antibacterial activity among all bacterial strains except that of ethanol and petroleum ether against *P. aeruginosa*. Leaf extracts in different solvent exhibited antifungal activity against genus *Aspergillus* except that of petroleum ether (table 2). Similar work on leaf extract carried out by different researchers has produced synonymous findings [24, 25]. Bark extract in almost all (except chloroform extract against *P. aeruginosa* and all fungal strains) displayed significant ($p < 0.01$) antibacterial antifungal activity (fig. 3) which was in accordance with the findings of other research workers [23, 24, 25]. The fruit extract demonstrated maximum antimicrobial among all the plant part extracts. It displayed antimicrobial activity against all pathogenic bacterial and fungal strains except for *B. subtilis* and *E. coli* in chloroform extract and for *C. albicans* in the methanolic extract. Susceptibility differences between Gram-positive and Gram-negative bacteria may be due to cell wall structural differences between these classes. Work carried out independently by different research groups have reported similar findings with the fruit aqueous extract displaying the maximum antimicrobial activity [21, 27]. The maximum antimicrobial activity of fruit extract could be attributed to the presence of flavonoids, tannins and phenol/ polyphenols. This signifies its importance towards the development of novel chemotherapeutic agents.

Conclusion

The study evidenced the presence of active phytochemical compounds within the leaf, bark and fruit extracts of *T. arjuna*. The phytoconstituents present in these plant extracts were responsible for the variation in antimicrobial activity (zone of inhibition) against the pathogenic

Table 1: Phytochemical screening of *T. arjuna* (leaf, bark, and fruit).

S. No.	Solvents	Plant Part	Methanol Ether	Ethanol	Chloroform	Acetone	Petroleum	Aqueous
1.	Alkaloids	L	+	-	-	+	+	-
		B	+	+	+	+	+	+
		F	-	-	-	-	-	-
2.	Carbohydrates	L	+	+	+	+	-	-
		B	+	+	+	+	+	+
		F	-	-	-	-	-	-
3.	Cardiac Glycosides	L	-	-	-	+	+	-
		B	-	-	-	-	-	-
		F	-	-	-	-	-	-
4.	Proteins	L	+	+	+	+	+	+
		B	+	+	+	+	+	+
		F	+	+	+	+	+	+
5.	Phytosterols	L	-	-	-	-	+	-
		B	-	-	-	-	+	+
		F	+	-	-	+	+	+
6.	Flavonoids	L	+	+	+	+	+	+
		B	+	+	+	+	+	+
		F	+	+	+	+	+	+
7.	Tannins	L	+	+	+	-	-	-
		B	+	+	+	+	+	+
		F	+	+	+	+	+	+
8.	Terpenoids	L	+	-	+	-	+	-
		B	+	-	+	-	+	+
		F	-	-	-	-	-	-
9.	Saponins	L	-	-	+	+	-	-
		B	+	+	+	+	+	+
		F	-	-	-	-	-	-
10.	Phenols/ polyphenols	L	+	+	+	+	+	+
		B	+	+	+	+	+	+
		F	+	+	+	+	+	+

Where, “+”: Presence; “-”: Absence, “L”: Leaf, “B”: Bark and “F”: Fruit.

Table 2: Antimicrobial activity of different extracts of *T. arjuna* against pathogenic microbes.

S. No.	Extract (mg/disc)	Solvents	Plant Parts	Inhibition zone (in mm) against pathogenic microbes after 24 hrs incubation						
				B. subtilis	*E. coli*	*S. aureus*	*P. aeruginosa*	*A. niger*	*A. flavus*	*C. albicans*
1.	(10 mg/ disc)	Methanol	Leaf	9 ± 0.21	8 ± 0.1	8 ± 0.2	6 ± 0.2	5 ± 0.2	4 ± 0.1	Nil
			Bark	14 ± 0.1	14 ± 0.1	13 ± 0.1	10 ± 0.2	9 ± 0.2	9 ± 0.2	5 ± 0.2
			Fruit	10 ± 0.1	12 ± 0.2	13 ± 0.1	11 ± 0.2	7 ± 0.1	4 ± 0.2	Nil
2.		Ethanol	Leaf	8 ± 0.2	8 ± 0.1	7 ± 0.2	Nil	6 ± 0.2	4 ± 0.2	5 ± 0.1
			Bark	13 ± 0.1	10 ± 0.1	9 ± 0.2	9 ± 0.1	7 ± 0.2	7 ± 0.1	6 ± 0.2
			Fruit	13 ± 0.2	13 ± 0.1	12 ± 0.2	14 ± 0.2	10 ± 0.2	10 ± 0.2	5 ± 0.1
3.		Chloroform	Leaf	7 ± 0.2	8 ± 0.1	7 ± 0.2	5 ± 0.2	6 ± 0.2	Nil	Nil
			Bark	8 ± 0.1	8 ± 0.2	7 ± 0.1	Nil	Nil	Nil	Nil
			Fruit	Nil	Nil	3 ± 0.2	4 ± 0.2	11 ± 0.2	9 ± 0.2	3 ± 0.1
4.		Acetone	Leaf	8 ± 0.1	7 ± 0.1	7 ± 0.1	8 ± 0.2	5 ± 0.1	6 ± 0.1	Nil
			Bark	15 ± 0.1	14 ± 0.1	14 ± 0.2	10 ± 0.1	9 ± 0.1	7 ± 0.1	4
			Fruit	12 ± 0.2	14 ± 0.2	14 ± 0.2	14 ± 0.1	8 ± 0.1	7 ± 0.1	4
5.		Petroleum ether	Leaf	9 ± 0.2	8 ± 0.2	5 ± 0.1	Nil	Nil	Nil	Nil
			Bark	13 ± 0.1	12 ± 0.1	9 ± 0.2	6 ± 0.1	10 ± 0.1	8 ± 0.1	Nil
			Fruit	4 ± 0.2	6 ± 0.1	2 ± 0.1	2 ± 0.1	8 ± 0.2	10 ± 0.2	3 ± 0.2
6.		Aqueous	Leaf	10 ± 0.1	10 ± 0.2	8 ± 0.2	9 ± 0.2	9 ± 0.1	7 ± 0.1	5 ± 0.1
			Bark	16 ± 0.2	15 ± 0.1	15 ± 0.2	10 ± 0.2	11 ± 0.1	10 ± 0.2	8 ± 0.1
			Fruit	16 ± 0.1	19 ± 0.2	19 ± 0.2	15 ± 0.1	18 ± 0.2	16 ± 0.1	10 ± 0.2

Table 3: Antimicrobial potential of extracts against standard antibiotics.

Antibiotic	Dose (mcg)	Zone of inhibition (mm) against pathogenic agents						
		B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. niger*	*A. flavus*	*C. albicans*
Gentamicin	10	30 ± 0.15	18 ± 0.21	22 ± 0.1	25 ± 0.18	-	-	-
Ketoconazole		-	-	-	-	17 ± 0.22	20 ± 0.21	15 ± 0.22

microbes. Among the different extract of leaf, bark and fruit the antibiogram assay of aqueous and methanolic extract displayed significant ($p < 0.01$) antibacterial potential against test microbes. The present investigation has revealed the broad spectrum antibacterial and antifungal activity of polar constituents of *T. arjuna* plant parts against bacterial strains. The maximum antimicrobial activity of the aqueous extract indicated its potential use as a feed for livestock's with the dual benefit of protection from infection and strengthening the immune system. Further studies are required to isolate and characterize the bioactive principles for developing novel antimicrobial drugs in near future.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Potential health risk assessment of heavy metal accumulation in the selected food fishes from Krishna Esturine region of Southern Deltaic Region of India

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Abstract:

Rapid development of industries result in the production of high amounts of solid or liquid wastes in the form of effluents, which are usually discarded into the nearby water bodies causing contamination of ecosystem. These industrial effluents released can pose great health hazards to aquatic organisms particularly fishes. A study was conducted between June 2016 and May 2017 to assess the concentration of Zn, Cu, Mn, Pb and Mn in muscle and liver tissues of selected food fishes namely *Mugil cephalus* and *Sillago sihama* from Krishna estuarine region of Andhra Pradesh in the Southern Deltaic Region of India. The investigation was aimed at revealing the differences in accumulation pattern of heavy metals in fishes inhabiting estuaries with varying metal bioavailability. The concentration of metals was higher in liver than that of muscle. The highest metal concentrations found in fishes can cause oxidative stress resulting in shortening of life span. Most of the effluents are contributed by sewage water and pollutants from local industries and shipping activities in Machilipatnam and Nizampatnam area.The Estimated Daily Intake (EDI), and Target Hazard Quotient (THQ) indicated that health risks-associated with heavy metal exposure via consumption of the fish's muscles were insignificant to human health. The present study suggests that metals present in the muscle tissues of both fishes are found to exceed the permissible limits of WHO. Furthermore, the potential health risk was observed in both fish samples showing the heavy metal contamination in this area.

Key words: Heavy metals, Krishna estuarine region, *Mugil cephalus, Sillago sihama*.

Introduction

Heavy metals are considered as critical toxic contaminants of aquatic ecosystem because of their high potential to enter and bio-accumulate in food chain. The main source of contamination may be either due to natural processes or domestic, industrial and anthropogenic activities. Liao et al (1) reported that the heavy metal pollutants find their way into creeks,canals and river water bodies through emissions from industry and aquaculture, agriculture, as well as wastewater generated by town and cities, rainfall, wind-derived dust deposition, etc. Moreover, heavy metals can also be released from the surface sediments of the coastal water itself. Regardless of their origin, the contamination of such heavy metals in the water is a serious threat to aquatic animals. Some metals like Zn and Cu, which are required for metabolic activity in animals and the other heavy metals like Cd and Pb may exhibit extreme toxicity even at low levels under certain conditions, thus necessitating regular monitoring of sensitive aquatic environment. From an environmental point of view, coastal zones can be considered as the geographic space of interaction between terrestrial and marine ecosystem that is of great importance for the survival of a large variety

of fauna (2). The discharge of wastes without adequate treatment often contaminate the coastal and estuarine water with conservative pollutants (heavy metals), many of which accumulate in the tissues of resident organisms like fishes, oysters, crabs, shrimps(3). In many parts of the world, especially in coastal areas and on smaller islands, fish and fishery products are major part of food, which supplies all essential elements required for life processes in a balanced manner. Hence, it is important to investigate the levels of heavy metals in these organisms to assess whether the concentration is within the permissible level and will not pose any hazard to the consumers (4).

The distribution of heavy metals in water, sediments and fish play a key role in detecting sources of heavy metal pollution in aquatic ecosystem. Metals occur in less than 1% of the earths crust, with trace amounts generally found in the environment and when these concentrations exceed a stipulated limit, they may become toxic to the surrounding environment and when these concentrations exceed a stipulated limit, they may become toxic (5). The coastal zones receive a large amount of metal pollution from agricultural runoff, aquaculture chemicals, and other industrial activities. The discharge of these wastes without adequate treatment often contaminate the estuarine and coastal waters along with resident organisms like fishes and other aquatic organisms(6).

Fish, as human food, are considered source of protein, polyunsaturated fatty acids particularly omega-3 fatty acids, calcium, zinc and iron (7). And it is considered as one of the highest nutrient sources for humans that contribute the lower the blood cholesterol and reduce the risk of stroke and heart diseases (8,9). Among the aquatic biota, fish is the most susceptible organism to heavy metal contamination than any other fauna. It is also well known that fish are good indicators of chemical pollution and as a result they have long been used to monitor metal pollution in coastal and marine environment. Therefore, fishes were considered as better specimens for use in the investigation of pollution load than water sample because of the significant levels of metals they bioaccumulate. In aquatic ecosystem, deposition of contaminants including heavy metals can lead to elevated sediment concentrations that cause potential toxicity to aquatic biota (10). Hence, harmful pollutants like heavy metals released by anthropogenic activities will be accumulated in marine organisms through the food chain and as a result, human health can be at risk because of consumption of fish contaminated by toxic chemicals. Keeping in view of the potential toxicity nature of heavy metals as well as the environmental pollution, it is deemed necessary to have the base line environmental data on potential metal contamination so that pollutants can be monitored in the environment. This paper presents the data on heavy metal (Zn, Pb, Mn, Cu, Cr) concentration in fish, *Mugil cephalus and Sillago sihama* from Nizampatnam coast.

Materials and Methods

Fish *(Mugil cephalus and Sillago sihama)* samples collected from fish landing centres, at Krishna estuarine region (Fig-1) of Southern Deltaic Region of India. The fish samples were transported to the laboratory in iceboxes and stored at -10°C until subjected for future analysis. The fishes were dissected and care was taken to avoid external contaminated to the samples. Rust free stainless steel kit was sterilized to dissect the fishes. Double distilled water was used for making of the muscle sample and for analysis in the Atomic Absorption Spectrophotometer (ASS). The gut content, gill and muscles were separated and dried to constant weight and both wet and dry weight recorded. 25% was used as blank samples accompanied every run of the analysis. Each sample was analyzed in triplicate to ensure accuracy and precession for the analytical procedure.

Health risk assessment:

Estimated daily intake (EDI):

$$EDI = \frac{E_F \times E_D \times F_{IR} \times C_f \times C_m}{W_{AB} \times T_A} \times 10^{-3}$$

E_F = The exposure frequency 365 days/year

E_D = The exposure duration, equalent to average life time (65 years)

F_{IR} = The fresh food ingestion rate (g/person/day) which is considered to be India 150/g/person/day (11).

C_f = The conversion factor = 0.208

C_m = The heavy metal concentration in food stuffs mg/kg d-w)

$W_{AB=}$ average body weight (bw) (average body weight to be 60kg)

TA = Is the average exposure of time for non carcinogens (It is equal to ($E_F \times E_D$) as used by in many previews studies (12)

Target hazard Quotient :

$$THQ = \frac{EDI}{RfD}$$

Rfd: Oral reference dose (mg/kg bw/day)

A THQ below 1 means the exposed population is unlikely to experience obviously adverse effects, whereas a THQ above 1 means that there is a chance of non-carcinogenic effects, with an increasing probability as the value increases.

Results and Discussion: The annul mean concentrations of heavy metals in fishes *Mugil cephalus and Sillago sihama* are presented in Fig 2 & 3. The order of heavy metal concentration was Zn > Mn > Cu > Cr > Pb. This data indicated zinc accumulation in higher levels in both the fishes. Marine and brackish water organisms including fish accumulated metals through direct absorptions or via food chain pass them to human beings through consumption causing acute and chronic disorders (14). The fishes have been widely employed as good bioindicator of metal pollution in the aquatic ecosystem because they possess several advantages (13). Fishes are likely to cover all components of aquatic ecosystems because their foods include both aquatic and terrestrial origins that are influenced by anthropogenic impacts.

Zinc (Zn):

Zinc is an essential element in animals' diet but it is regarded as potential hazard for both animal and human health (15). Insignificant seasonal variation was observed with slight higher concentration during monsoon season. Zinc was present in natural water only as a miner consultant because lack of solubility of free metal and its oxides (16). It's high concentration may cause some toxic effects. A normal human body contains 1.4 to 2.3 g of zinc. Recommend daily dietary in take of zinc is about 15mg for adults and 100mg for children over a year old. The average diary intake of zinc is India is about 16.1 mg (3). It is relatively non toxic and concentrations of zinc up to 25mg/l have shown few adverse effects (17). Zinc may be toxic to aquatic organisms but the

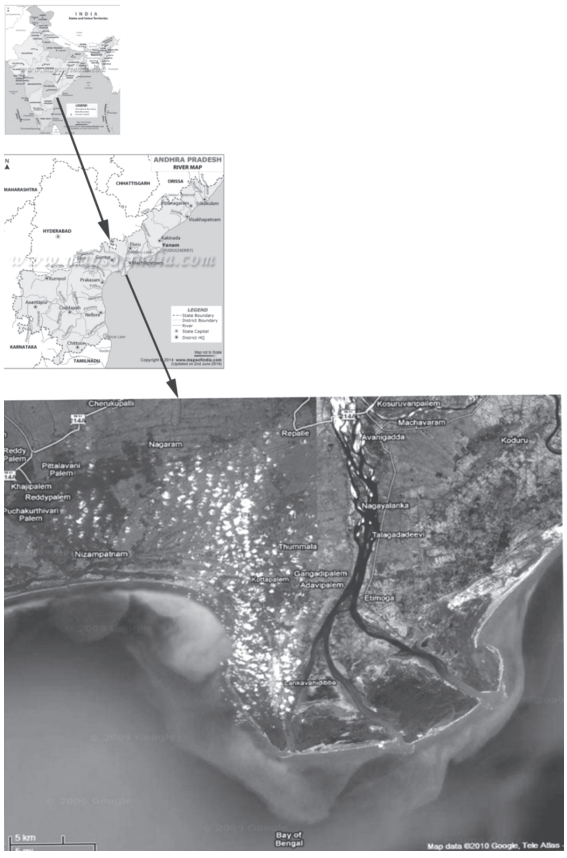

Fig 1 : Map Showing Krishna Estuarine Region

degree of toxicity varies greatly, depending on water quality characteristics as well as species being considered (18). The present study shows that the average concentration (32mg/kg) of Zn was slightly higher than standards of WHO (19).

Lead (Pb): Lead is considered as a toxic but non-essential metal implying that it has no known function in the biochemical processes (20). Lead enters the aquatic environment through soil erosion and leaching gasoline combustion, municipal and industrial wastes and runoff. Pregnant women exposed to lead were found to have high rates of still births and miscarriages (21). Lead causes mental retardation among children. Hyper tension caused by Pb exposure has also been reported (22). Lead poising is accompanied by symptoms of intestinal cramps, peripheral nerve paralysis anemia, and fatiage (21). The concentration of lead in natural water increases mainly through anthropogenic sources which include base metal mining and lead containing pesticides. In the present study, Pb concentration ranged from 2.3 to 3.5/kg in the muscle whereas in liver, its concentration was between 2.8 and 3.9 mg/kg in both the fishes. According to WHO (19), the maximum accepted limit is 2mg/kg for food fish. The present results indicated that the concentration levels of Pb was slightly higher than the permissible limits set for human consumption by various regulatory agencies and therefore indicated possible health risks associated with consumption of these fish. High levels of Pb exposure can cause deleterious effects to almost all organ systems, most importantly the central nervous system, kidneys, and blood, culminating in death, if levels are excessive. At low levels, haeme synthesis and other biochemical processes are affected and psychological and neurobehavioral functions are impaired (25, 26).

Manganese (Mn): Manganese is an essential micro nutrient and it functions as a co factor for many enzyme activities (27). Mn is an essential element for human beings and is involved in the formulation of bone and metabolism of protein, lipid, and carbohydrates in biological systems (28). High Mn concentration interferes with central nervous system of vertebrates by inhibiting dopamine formation as well as interferering with other metabolic pathways such as Na regulation which ultimately can cause death. High Mn levels are a matter of concern as the consumption of Mn contaminated fish could result in the Mn related disorders in the consumers. In the present study manganese ranged from 4.3 to 5.2 mg/kg in the fish muscle which is higher than the permissible limits of 0.5 mg/kg set by WHO (19).

Copper (Cu): Copper in aqueous systems received attention mostly because of its toxic effects on biota. Excess of Cu in human body is toxic and causes some disorders including hypertension. Cu also produces pathological changes in brain tissues (3). Cu is an essential part of several enzymes and is necessary for the synthesis of hemoglobin. However, high intake of Cu has been recognized to cause adverse health problems (29). The average concentration of cu in the present study ranged from 4.0 to 4.5mg/kg in fish muscle who is above permissible limits (2 mg/kg).

Chromium (Cr): Chromium concentration in natural waters is usually very small. Elevated concentration was due to industrial and mining processes (18). Fishes are usually more resistant to Cr than other aquatic organisms, but they can be affected sub-lethally if exposure concentration increases. In the present study, Cr was also above permissible levels of 0.5 mg/kg set by WHO (19).

The increasing demand of food safety has accelerated research regarding the risk associated with food consumption contaminated by heavy metal (30). Long term intake of contaminated sea food could lead to toxicity of heavy metals in human beings. There are reports of high levels of heavy metals in natural components of food stuffs because of environmental contamination and contamination during processing (31). Industrial effluents, agriculture runoff, aquaculture chemicals and drugs, animal and human excretion, and geological weathering and domestic wastes contribute to the heavy metals in the water bodies

(32). With the exception of occupational exposure, fishes are acknowledged to be the single largest source of mercury and other heavy metals (lead and chromium) affecting human beings. Lead poisoning in children causes neurological damage leading to reduced intelligence, loss of short-term memory, learning disabilities and coordination problems. The threat of heavy metal to human and animal health is aggravated by their long-term persistence in the environment (33). Further concern is that the heavy metals may be transferred and accumulated in the bodies of animals or human beings through food chain, which will probably cause DNA damage and carcinogenic effects due to their mutagenic ability (34). Heavy metal exposure may also cause neurobehavioral disorders such as fatigue, insomnia, decreased concentration, depression, irritability, sensory and motor symptoms **(**35**).** Exposure to heavy metals has been linked to developmental retardation, various types of cancer, kidney damage, autoimmunity and even death in some instances of exposure to very high concentrations **(**36). In some cases, fish catches were banned for human consumption because their heavy metal concentrations exceeded the maximum limits recommended by the World Health Organization (5). Among sea foods, fishes are commonly consumed and hence, are a connecting link for the transfer of toxic heavy metals in human beings. Bhuvaneshwari *et al.,* (37) concluded that the metals are inherent components of the environment that pose a potential hazard to human beings and animals. The effluents from the textile factory, tannery, and floriculture farm probably contain harmful contaminants such as dye stuffs, bonzothiozole, sulphonate polyphenols and pesticides. These compounds could bioaccumulation and affect the health of aquatic organisms and subsequently the health of humans that consume these fish (38). In the present study, Krishna estuarine region is also contaminated with pollutants particularly dyes factory, agriculture, and aquaculture chemicals. Kularatne et al., (39) discussed bioaccumulation and temporal variation of heavy metals in three edible lagoon fish species with references to gender. However, Cd and As were undetected in these three fish species. Generally, gills are the major route of metal ion exchange or adsorption from the water as they have very large surface areas to facilitate rapid diffusion of toxic metals. Therefore, in gills having higher surface areas, it is expected that there will be more effective facilitation of metal entry (irrespective of the heavy metal provided, it is available in the most bio-available form) due to the availability of more metal ion exchange or adsorption sites in the gills.

All heavy metals exist in surface waters in colloidal, particulate and dissolved phases, although dissolved concentrations are generally low (40). The solubility of heavy metals in surface waters is predominately controlled by the water pH, concentration and type of ligands on which the metal could adsorb, and the oxidation state of the mineral components and the redox environment of the system (41). The differences between the metal levels in the two fish species might have been due to differences in metabolic activities (42). The THQs values of *M. cephalus* were: Mn-1.02; Pb-0.7; Cu-0.9; Zn-6.9; Cr-0.8; and for *S.sihama*, Mn-0.9; Pb-0.4; Cu-0.8; Zn-6.8; and Cr-0.6. The THQ was higher than1 for some metals like Zn and Mn in *M. cephalus* whereas in *S. sihama* for Zn only. The analyzed metal concentrations are potentially toxic if they enter the food chain. Since their toxicity for human beings is given by the ingestion rate, data were obtained on THQs (above 1) indicating a risk for human health (3). Of course, it is just a preliminary step and fish contamination levels should be carefully monitored on a regular basis to detect any change in their patterns that could become a hazard to human safety. Similar results were observed by Ambedkar and Maniyan (43) and they concluded that the heavy metal concentrations were above the maximum levels recommended by regulatory agencies and, depending on daily intake by consumers it might represent a risk for human health. Every water body receives the effluents containing heavy metals either from point or from nonpoint sources. Worst thing about heavy metals is their

persistence in environment due to their non-biodegradable nature. It is for this reason that aquatic fauna particularly fish tend to bioaccumulate them, and thus, they remain in the tissues of the fish for long time. Fishes are the important source of protein and PUFA. Therefore, American Heart Association (AHA) recommended fish twice a week to the human adults. Unfortunately, fishes are now becoming the major source of heavy metals due to the pollution caused by industries. These metals generally cause two types of health effects. One is carcinogenic and other is non-carcinogenic effects. Both these effects can be measured in terms of target hazard quotients (THQ) and they worked on the amount and frequency of fish consumed.

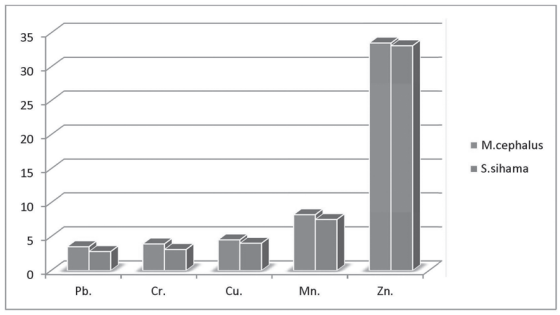

Fig. 2. Average concentrations of heavy metals in muscle of *M.cephalus* and *S.sihama* (mg/kg, dry weight)

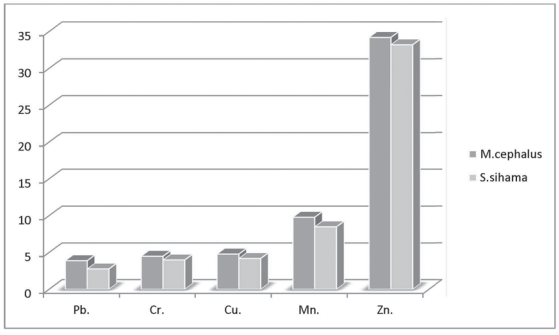

Fig.3. Average concentrations of heavy metals in Liver of *M. cephalus* and *S. sihama* (mg/kg, dry weight)

Conclusions

The international official regulatory agencies like WHO have set permissible limits for heavy metals above which the fish and fishery products are unsuitable for human consumption. However in the Indian subcontinent, there are no safe levels of heavy metal in fish tissues although the Indian population is the major fish consumers in the tropics. Finally it is recommended to have a long-term continuous monitoring to check metals pollution in order to minimize the metal accumulation in fish at Krishna estuarine region. In addition, guidance should be given to people and farmers about the use of pesticides, chemicals, drugs in agriculture and aquaculture and also about the control of house wastewater spreading in rivers and crops. Nobody will deny that industries are necessary for development, but on the other hand they should not creat any damage to the livelihood of human beings and this can be done only by treating their effluents thorougly before releasing into the environment.

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The Emergence of Blockchain Technology and its Impact in Biotechnology, Pharmacy and Life Sciences

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Abstract

The emergence of blockchain technology is regarded as 4th industrial revolution i.e., the integration of cyber-physical systems since the advent of Internet with far reaching applications in Banking, Insurance and Government, may impact other sectors especially life sciences. The fourth revolution is characterized by fusion of technologies that is blurring the lines between the physical, digital, and biological spheres. Therefore, it is vital to understand the opportunities and threats of adopting this technology in life science/ pharmaceutical research. The proprietary databases of today's institutions interact with some form of interface either human or otherwise. Integrating blockchain with life science/ pharmaceutical applications will decentralize the interface as well as the data exchange, resulting in high efficiency, greater speeds, low marginal cost, and infinite scalability.

Keywords: Blockchain, Agriculture, Pharmacy, Genomecoin, Cryptography, Smart contract

Introduction

The computing paradigms underwent through many changes since the first main frame. There is a birth of a new paradigm in every decade. Personal Computer (PC) was regarded as revolutionary over mainframes and later on Internet revolutionized everything (1, 3). It can be seen that mobile phone and social networking have established their place in the computing world over the last decade and now, the current decade may be dominated by blockchain and Internet-of-Things (IoT). Over the years, the companies or institutions stored the data in the proprietary database where they have complete control over it. The structure of database of an institution is different from other institutions due to its proprietary nature. The access of data and operation of the database are localized within the institution or company with restrictions/permissions. With the advent of new technologies, data exchange between different databases has become a necessity. The inter-communication between databases was achieved through broker or intermediaries and recently by Application Programming Interface (API). Even with the best of API available today, a transaction must be coordinated among stakeholders before final delivery or closing. Regulations, selective enforcement and efficiencies are some of the key issues encountered in modern-day commerce. In centralization (10), if two databases communicate directly with some commonality, it can be regarded as merger where data are combined under a central authority. Such centralization may suffer from the fear of legislation, monopoly, inefficiencies etc. In decentralization (11), everyone can share the same database with no controls and central authority. It may be faster and cheaper but suffers from other anomalies like cheating, fraud etc. But, the blockchain technology solves the problems

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encountered in decentralization. Blockchain may serve as the main backbone for the future computing world in terms of connecting wearable devices, IoT sensors, smart phones, tablets, laptops etc.

Blockchain technology enables direct interactions between users : Blockchain technology is a distributed, shared, encrypted, chronological, irreversible and incorruptible database and computing system (public/private) with a consensus mechanism (permissioned/ permission less), that adds value by enabling direct interactions between users (4). Or blockchain is a technology for shared databases between multiple non-trusting writers, yet can be modified and authenticated without a trusted intermediary (9). Blockchain-based authentication model allows users to truly identify and confirm each other's public keys. This eliminates the man-in-the-middle threat and any kind of manipulation attempts from the server and third parties' sides. A blockchain is a shared and secured database, which is not controlled by a single user but by the network as a whole. Schwab (2) defined blockchain as a technology that represents a "distributed database or ledger or a registry, which uses a secure protocol where a network of computers collectively verifies a transaction before it can be recorded and approved. Therefore, blockchain can be used to create trust, by enabling people who do not know each other (and thus have no underlying basis for trust) to collaborate without going through a central authority". Blockchain (the technology behind Bitcoin and other digital currencies) would permit multiple parties to share a single database with no central authority where access and controls are managed with software. Blockchains are very difficult to tamper with once the information is registered thereby making it less error prone and more efficient. Therefore, blockchain can be thought of as an application layer over Internet enabling transactions, digital currency payments (crypto currency), asset management etc.

Listed below are some of the characteristics of blockchain technology (5)

1. As a public ledger system, blockchain records and validates each and every transaction made, which makes it secure and reliable.
2. All the transactions made are authorized by validators, which makes the transactions immutable and prevent it from the threat of hacking.
3. Blockchain technology discards the need of any third-party or central authority for peer-to-peer transactions.
4. Decentralization of the technology.

Any new technology such as blockchain now and Internet in earlier era needs trust and ease of use for main stream adoption. In the financial world, the wallet companies such as Circle Internet Financial and Xapo developed applications for mainstream adoption of Bitcoin. Bitcoin refers to three layers in the technology stack of blockchain viz., platform (i.e. decentralized ledger), protocol (i.e. software) and Crypto currency. In blockchain technology, the user initiates the action and pushes the information to the network as opposed to pulling the information from a centrally authorized data store which may be vulnerable to thefts, attacks etc. Hence, blockchain technology operates on push model ensuring no dependency on centralized data store. Blockchain may become the "Internet of Money", connecting finances in the way that the IoT connects machines. In currency and payments (7), the blockchain adoption may reduce the transaction fee below 3% from 7 to 30% (8). A key outgrowth of the ledger is the idea of "smart contracts", which "provide security superior to traditional contract law and reduce other transaction costs associated with contracting". The decentralized computer-code can be run on a blockchain database and these pieces of computer codes are called smart contracts (6). In the absence of blockchain, if a person sends a contract over e-mail, each party would hold an identical copy that could be easily manipulated, whereas with blockchain only the receiving party would hold a valid copy.

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Bitcoin and e-wallets are related to money. Trust becomes a major factor in currency exchange as well as the sensitivity to security with regards to crypto currency, digital currency backup, privacy etc. Blockchain and Bitcoin are used interchangeably though being different. Bitcoin is digital currency where as blockchain is a distributed database. Currencies that use a blockchain (and thereby these cryptographic algorithms) are referred to as Cryptocurrencies. After the introduction of blockchain technology in Bitcoin, other cryptocurrencies emerged. The flow of money transfer in blockchain is depicted in the figure 1.

Blockchain has three types of users like (1) users that read data (2) users that write data (users, transaction users), and (3) users that validate data (miners, full nodes/users, validator nodes/users). Beyond currency, blockchain applications cover various other types of transactions some of which are bonded contracts, bonds, business licenses, copyrights, death certificates, derivatives, driver's licenses, identity cards, land and property titles, marriage certificates, mutual funds, passports, patents, registrations, reservations, stocks, third-party arbitrations, trademarks, vehicle registrations, voter lists etc. There are many blockchain projects that are currently being developed. Ethereum (http://ethereum.org/) is a general-purpose crypto-currency platform which is being widely used. Some of the wallet projects are ChromaWallet, CoinSpark, Counterwallet etc., built over blockchain protocol. Wallets help to store and exchange bitcoin in secure way.

Utility of blockchain in life sciences and pharmacy

(*a) Blockchain and genomics :* Blockchain is allowing to record genomics data that can combat counterfeit pharmaceuticals and protect intellectual property rights. Medicinal Genomics Corporation (https://www. medicinalgenomics. com) is a molecular information company that applies state-of-the-art life science technology to *Cannabis* plant genetics and devoted to sequencing of genomes of the patients with seizure disorders such as epilepsy, developmental and intellectual disabilities (including those on the autism spectrum), and mitochondrial diseases. It has developed novel DNA purification technologies and Next-Generation DNA Sequencing technologies which have been widely adopted by major scientific research institutions and commercial enterprises. It is using blockchain to counter claims on patents as there is no legitimate prior proof of it in the field and also using it to get strains digitally notarized by verification of blockchain technology. Blockchain is being used to fight the mistrust in genomic research by

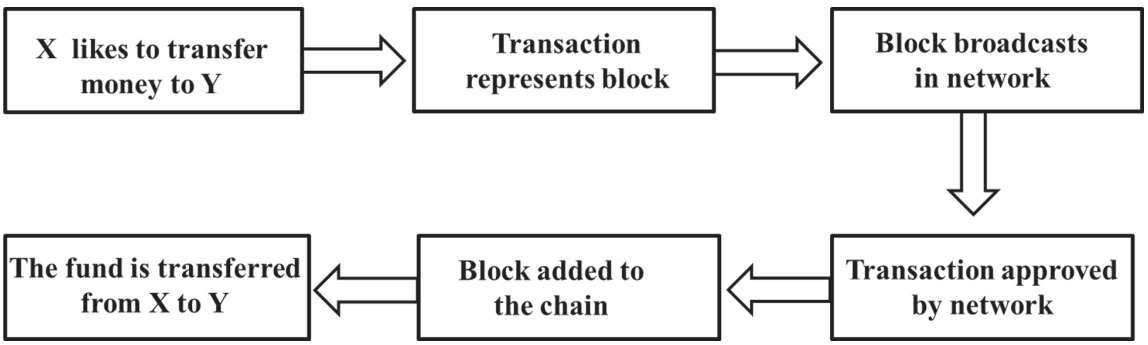

Fig. 1. Figure displaying flow of money transfer in blockchain

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sharing data. Data silos (a repository of fixed data) and centralization is causing suspicion and mistrust in genomic research which necessitates decentralization of data like ledgers in blockchain. Global Alliance for Genomics Health (GA4GH) (https://www.ga4gh.org) is planning to use blockchain for internationally sharing genomics data. Decentralization and sharing of data through blockchain will lower the cost and enable poorer countries to access the data. Due to privacy restrictions of data, it may be possible to implement private blockchain, however, everything in private blockchain will be subjected to the supervision of an independent central authority. Private blockchain may be a necessity in some situations in genomics like when dealing with the entire genome. Of late, patients are demanding more assurances that their genomic data are being secured appropriately, accessed as per their wishes which comes into reality with blockchain. In future, patients are empowered in terms of their data access and let companies access some blocks of their data for research purpose. Therefore, it is crucial for stakeholders to understand blockchain usage in life science R&D and especially in pharma R&D and its potential disruption in near future.

The data inserts in blockchain are immutable. So, information stored on a blockchain cannot be subsequently amended or deleted. Therefore, attentions is required if the sensitive data stored in blockchain is to comply with data protection laws, which may be challenging due to specific regulations in terms of individual rights in deleting data or correcting the data. A Russian startup called Zenome.io is looking to put consumers in control of their genomic data. The blockchain-based, peer-to-peer technology will automate collection and sharing of genomic data in part by allowing individuals to upload their own DNA profiles control which researchers may access and add to each secure store of information (12). This may be a platform for buying and selling genomic data. EncrypGen, a startup from Coral Springs, Florida is getting ready to beta test a blockchain-based system to allow patients to store and share genomic data through what it calls a "gene-chain."

The right to one's own genetic information must be seen as a basic human right, and migrating the transactional organizations to decentralized blockchain is a necessity in view of protecting the personal rights. In the United States of America, prominent genomic researchers have tried to make a public case that the Food and Drug Administration is overcautious on consumer genomics (13) and established in studies that there is no detrimental effect to individuals having access to their own genomic data (14). Blockchain-based genomic services could be an idea for providing low-cost genomic sequencing to individuals, making the data available via private key.

Genomecoin and Genomic Research Coin : Genecoin (www.genecoin.me) samples one's DNA, turns it into data and stores it in the Bitcoin network. Genecoin developed a tool to spread the DNA across the globe by collecting DNA sample and converting it into the data before loading the genome data into Bitcoin network. The genome data are stored and the network is used as a permanent back up of the data.

DNAnexus (https:/www.dnanexus.com), a cloud-based platform, provides a global network for sharing and management of genomic data and tools to accelerate genomics research. The DNAnexus solution is the largest current data store of human genomes and is ever expanding. As the future of human health is in genomics and only 300 worldwide preapproved genomic researchers have permission to use it, it is important to add coin functionality or blockchain functionality to DNAnexus to make it more open to public use. The Genomic Data Commons is a US-government-funded large-scale genomic research project which is accessible only to USA based researchers. This has to be expanded on large scale to be accessible to an individual worldwide by adding genomecoin. Organizing genomic data into a standard unified repository and allowing access to it at least to a limited research group/population is ideal. A further step in this direction could be using an appcoin

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like Genomecoin to expand its accessibility to a wider population.

(b) Blockchain and agriculture : Blockchain has a potential to transform agricultural industry in the three key sectors namely (1) provenance (the place of origin) and radical transparency (2) mobile payments, credits, and decreased transaction fees (3) real-time management of supply chain transactions and financing. While agriculture is the most significant sector in rural areas and provides a livelihood for 70 per cent of the world's poor, it is also the industry that provides the biggest disconnect between supplier and retailer. Using blockchain, however, a more direct link can be established, ensuring that farmers receive fair payment for their produce and enabling retailers to verify that they are getting what they have paid for (16). Consumers are always willing to pay for organic food, and clean food if proper information is provided. Despite many regulations are in existence as of today, consumer remains in utter confusion over it. Blockchain may be useful in solving this problem by effectively monitoring the food supply chain. Practical applications of blockchain technology in the agriculture sector also include minimizing unfair pricing, product origins, and reducing multinational agricultural influence in favor of more localized economies. In the future, platforms could also help with remittances to rural regions as well as other rural farming finance solutions (17). The digital tokens based on blockchain can be exchanged for fertilizer for small farmers. Because the tokens are on a blockchain, they cannot be misused or imitated, ensuring that the government-allocated funds are creating maximum impact where intended. Blockchain can be used to create immutable land titles to prove ownership and protect farmers from widespread corruption and digitization of paper contracts into smart contracts to improve efficiency and minimize costs. Farmers always look for technologies that deliver value and solve significant problems. The future of blockchain in agriculture solely depends how it can help and take agriculture forward by connecting this technology to viable business models.

(c) Blockchain and pharmaceuticals : The disruptive nature of blockchain technology caught the imagination of technologists as it makes markets more efficient and the technologists continue to fuel the hype over the possibilities. According to Gartner Hype Cycle for emerging technologies, blockchain technology started going down the trough of disillusionment after hitting the peak (19). However, the majority of leaders in the life sciences and pharmaceutical industries expect a broad adoption of blockchain in life sciences and pharmaceuticals in the next five years and most of the companies are experimenting with blockchain and the storage and all access tools may go through a rapid change as to leverage previously unavailable data sources. The creation of immutable and auditable records in clinical trials with blockchain is important in meeting regulatory requirements. The blockchain should enable the companies to collect the clinical data more securely. Clinical trial data in real-time on an immutable basis will also make it hard for subsequent manipulation of the results by researchers. Blockchain enables Food and Drug Administration (FDA) approvals faster in new drug discovery process due to diverse sampling of data, auditable trial of supply chain and minimized fraud because of consensus of different parties in the network. Supply chain security is one aspect that has recently won attention, when the Drug Supply Chain Security Act (DSCSA) has been implemented in the USA. The act has been implemented amongst other things to fight the counterfeit drug problem. Counterfeit drugs are drugs that do not contain the active ingredients that they are supposed to have and consequently can harm patients from all sectors (15). Blockchain may be seen as an answer to handle the illegitimate drug problem as the blockchain platform ensures drug identification, tracing, verification and notification, trust and transparency between parties during ownership changes in supply chain.

Blockchain and challenges ahead : Some claim that in a few years' time, blockchain will no longer be a buzzword but some others believe that

blockchain is all hype and it is an untested technology with huge risks and little upside. There are potential threats (18) for adopting blockchain technology in every country, some of which are listed below.

1. Powerful Incumbents
2. Ideological Pushback
3. Privacy Concerns
4. Off-Chain Transactions
5. Loss of Discretion and Arbitration Challenges
6. Distrust of the Technology Due to Lack of Adoption

The lack of widespread understanding of blockchain and the difficulty of integrating the technology into existing infrastructures and bureaucracies is blocking the adoption. However, once the value is recognized and trust is built over privacy, the threats may not be major challenges but surmountable. The adoption of blockchain technologies would limit the means and methods for illicit activities.

Conclusions

It is the technology which drives science always forward. Blockchain technology is a kind of mechanism that needed to achieve the next orders-of magnitude progress in vital areas such as human healthcare/genome research, agriculture, pharmacy and well beyond. It is time to adopt such innovative and impending technologies which will take science forward and change the lives of common man in the society.

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NEWS ITEM

Researchers from TIFR examined liver regulation of fat secretion

Lipid droplets accumulate in the liver during fasting, as in during sleep. Yet, the liver controls the release of these lipid droplets into the blood, thereby preventing excessive deposition of very low density lipids (VLDL) into the blood stream which could lead to heart disease. While this role of the liver is well known, the question of how it happens has not been addressed until recently. This question was taken up by Roop Mallik's group at the Tata Institute of Fundamental Research, Mumbai.In a paper published in Proceedings of the National Academy of Sciences, US (PNAS), the researchers led by Dr Mallik show that the motor protein kinesin transports lipid droplets to the endoplasmic reticulum within the liver cells, from where it is secreted into the blood. During fasting, kinesin is removed from the lipid droplets, and this ensures they do not reach the endoplasmic reticulum, nor are they secreted into the blood. This tempers the secretion of lipids from the liver during fasting and protects the organs.In their first experiment, the group of researchers extracted lipid droplets from cells and watched them move in a test tube. There they found that when they added specific compounds that blocked the motor protein kinesin, the droplets stopped moving. This is how we found for the first time that kinesin is a key factor,says Dr Mallik. They published these results in the journal Nature Methods. Following this study, they experimented with rats and found the connection between regulation of lipid transport within the liver cells and that of the motor protein kinesin.

IGIB's novel approaches may lower the Tuberculosis induced tissue damage

It is generally believed that TB bacteria make the host cells accumulate triglyceride and become lipid-rich as bacteria prefer lipids for their nutrition. Now, using human macrophage cells researchers at the Institute of Genomic and Integrative Biology (CSIR-IGIB) have shown that when TB-infected macrophages undergo necrosis (where the cell ruptures when it dies) lipids and bacteria contained in the cells are released. The neighbouring cells — both healthy and TB-infected — take up the lipids thus leading to lipid accumulation.Our study brings a new facet to the way the field has been thinking about pathogenesis where it was believed that because the bacteria prefer lipids for their nutrition, they make the host cell become lipid-rich. Our work points to the relevance of the incident pathology — necrosis in a granuloma result in the development of lipid-rich foamy macrophages [presence of cells with large lipid-filled vacuoles], says Dr. Sheetal Gandotra from the Cardio Respiratory Disease Biology Unit at IGIB and corresponding author of a paper published in the journal Frontiers in Immunology. The ability to induce necrosis is peculiar to virulent TB bacteria. The avirulent Mycobacterium bovis strain used in BCG vaccine is unable to cause necrosis; it triggers a programmed cell death (apoptosis) instead. Like the BCG strain, When macrophages encounter TB bacteria they mount an inflammatory response wherein certain factors are secreted to help recruit other cells of the immune system to kill the bacteria. As a result of the inflammatory response more macrophages are recruited to the site of infection thus exposing them to infection. Human blood monocyte-derived macrophages, too, showed increased inflammatory response when triglyceride accumulation was increased. Central to the storage of triglycerides in macrophages is the DGAT1 enzyme (diacylglycerol o-acyltransferase). When the DGAT1 gene is silenced in the macrophage cell lines, the macrophages' ability to accumulate triglycerides is compromised.

Bacteria develop resistance even without exposure to antibiotics

The IISER Pune team found E. coli coped better when exposure to complex, unpredictable environment continued for 100 daysThe environment where bacteria such as E. coli thrive can be complex with different stresses being present at the same time and changing unpredictably at different time scales — daily or seasonal. Researchers at Pune's Indian Institute of Science Education and Research (IISER) found exciting results when they replicated these conditions in the lab — E. coli developed resistance to antibiotics and heavy metals even when the bacteria were not exposed to them.When exposed to a combination of stresses — salt, pH and oxidative stress — that varied unpredictably on a daily basis, E. coli did not show statistically

significant adaptability at the end of 30 days (170 generations). But bacteria did show improved fitness and better growth rate at the end of 100 days. Thirty days produce 170 generations of E. coli while 100 days produce 900 generations.To make the environment complex, the researchers led by Sutirth Dey from IISER Pune's Biology Division exposed the bacteria to one or two factors (says pH and salt) that were kept normal while one or two factors were abnormal and caused stress. Unpredictability was brought in by randomly changing the three factors that caused stress on a daily basis. Totally, 30 combinations that make the environment complex and unpredictable were used for the study.At the end of 30 days when compared with ancestors [or controls], the bacteria exposed to complex, unpredictable environment did not develop statistically significant advantage in terms of improved fitness," says Shraddha Karve from IISER Pune's Biology Division and first author of a paper published in the Journal of Evolutionary Biology.But what came as a surprise was that the bacteria evolved the ability to tolerate novel stresses that they were not exposed to, such as antibiotics (norfloxacin) and heavy metals (cobalt and zinc)," says Dr. Karve. "We repeated the experiment all over again as the result was so surprising, and we got the same outcome."

Entire genomic sequencing of Koala bear unearthed

The koalas of Australia are not just famous for their cuteness. They have been an unsolved mystery among scientists for their strange eating habits — they enjoy the leaves of eucalyptus that would be toxic or even fatal to most mammals — and their exceptional parental care. Now an international team of scientists has successfully sequenced the marsupial's whole genome and answered burning questions about the critter. The study published in Nature Genetics was authored by 54 scientists from seven countries and the whole genome was found to consist of over 26,000 genes. The genome provides a springboard for the conservation of this biologically unique species," said co–lead author Katherine Belov from the University of Sydney in a release. Researchers found expansions within a particular gene family (P450 gene) and reported that these genes help the koala detoxify the eucalyptus leaves. These genes were found to be expressed in many tissues, especially the liver, indicating its role in detoxification. The koala has evolved an excellent toolkit to deal with eating highly toxic eucalyptus, one made up of lots of copies of the same (or very similar) tools, explained Dr. Will Nash, one of the authors from Earlham Institute, UK, in the release. Researchers also found novel lactation proteins in the koala bears. They reported that these proteins protect the young ones in the pouch and help it develop a strong immune system. We characterised the main components of the mothers' milk – which is crucial for koala joeys — born the size of a jellybean and weighing half of one gram, said Dr. Belov. "We identified genes that allow the koala to fine-tune milk protein composition across the stages of lactation, to meet the changing needs of their young.

Pivotal role of proteins in implantation of embryo examined

The team led by Dr. Deepak Modi from ICMR's National Institute for Research in Reproductive Health (NIRRH) has discovered that the endometrium is not a passive tissue which readily promotes embryo implantation but undergoes extensive remodelling brought about by the embryo at the time of implantation.They discovered that a protein OVGP1 is induced in the endometrium precisely at the time when the embryo has to implant. That the embryo can implant only during a narrow window is well known. Studies carried out in mouse models showed that OVGP1 protein is expressed for a brief period that coincides with the time of implantation. It is known that the pregnancy hormone — human chorionic gonadotropin (hCG) produced and released by embryos is crucial for the implantation process.The role of hCG hormone in inducing the endometrium to produce the OVGP1 protein became clear when the researchers studied the role of different hormones on endometrial cell lines. The cell lines were exposed to progesterone, estrogen and human chorionic gonadotropin hormone. We found the hCG hormone induced the expression of OVGP1 protein. Progesterone and estrogen, too, had a role, though minor, whereas hCG had a major role in the expression of OVGP1. This tells that embryos signal the endometrium to express OVGP1, says Dr. Modi, who is the corresponding author of a paper published in the Journal of Assisted Reproduction and Genetics.To study how the protein helps in the implantation process, the researchers silenced the expression of the protein in the endometrium cell lines. They found that the expression levels of integrin

proteins, which are essential for implantation, were significantly reduced in OVGP1 silenced cells.

Amazon forests found to discover 7 novel wasp species

An international team of researchers have discovered seven new wasp species belonging to the genus Clistopyga from Peru, Venezuela and Colombia. A recent report published in Zootaxa describes their morphology, such as colouration, wing size and other distinct features.The most notable among them is Clistopyga crassicaudata, named after its distinctly thickened ovipositor. The ovipositor is a tube-like organ present in many insects that helps in laying eggs and also in injecting venom. The researchers say that these new species could be parasitoid wasps, which lay their eggs near a host, which the larvae then feeds on and, eventually, kills. The biology of these seven new species is really unknown but other species of this genus, and other genera of Ichneumonidae lay their eggs into spiders or egg-sacs, explained Dr. Santiago Bordera from the University of Alicante, Spain, in an email to The Hindu. The female wasps inject venom into spiders, paralyse them, and then lay their eggs on them. The hatching larvae feed on the paralysed spiders and their eggs.

Drugs for multiple sclerosis have the potentiality to fight pancreatic cancer

An FDA-approved drug currently used for treating multiple sclerosis has been found to be effective for pancreatic cancer. Researchers from Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, in collaboration with Regional Cancer Centre in the city and NIMHANS, Bengaluru, found that the drug was also able to increase the efficacy of gemcitabine, the current standard drug for pancreatic cancer. The results of the study have been recently published in Theranostics.The drug used to treat multiple sclerosis was found to act through a receptor called S1PR1 that is involved in lipid signalling and which regulates numerous cellular events such as cell growth, migration and vascular integrity.The precise role of the receptor in pancreatic cancer is still not clear and our study has brought out its importance. We found that the [multiple] sclerosis drug can bind to the receptor and alter the key cellular events and prevent the progression of pancreatic cancer, explains Dr. K.B. Harikumar, from the Cancer Research Program at RGCB and corresponding author of the paper. The sclerosis drug was also found to be a potent inhibitor of NF-kappaB, a transcription factor that helps in tumour progression.The effectiveness of the multiple sclerosis drug when used together with the current pancreatic cancer drug was checked in mice models. The combination drug treatment was able to control various signalling molecules, thereby decreasing cancer cell proliferation and increasing apoptosis. It also helped produce higher levels of reactive oxygen species and inhibited the migration of the cancer cells. They also studied the genes involved in inflammation and immunity in pancreatic cancer and found that the combination drug regime activated a tumour-suppressor gene and downregulated another that is involved in drug resistance and decreased immunity.

Goa Mushroom's Pigments as a potential source against cancer

The mycological laboratory of the Department of Botany, Goa University on Wednesday reported the discovery of a new pigment from local wild mushrooms.We are proud to report a new suphur-rich melanin biopigment of immense bioindustrial, biomedical (anti-cancer, anti-tumour) and biotechnological potential from local Roen alamis (wild variety of Goan mushrooms that grows on termite hills) or Termitomyces species, said Dr. Nandakumar Kamat, Assistant Professor of Botany, department of Goa University here on Wednesday.Our paper was published on July 9 in Mycology: International Journal Of Fungal Biology affiliated to Mycological Society of China, and published by Taylor and Francis, U.S.. This discovery shows the chemical nature of the brown or black colour that you see in these wild edible mushrooms, explained Dr. Kamat, with more than two decades of research experience on mushrooms. More than 50 teams are working on these mushrooms, globally, but we in Goa got the lead now. People of Goa who consume these mushrooms are actually eating sulphur-rich melanin. We declare it as world's first sulphur-rich edible melanin. Its structure is similar to black pigment found in human hair,said an excited Dr. Kamat.

Evolution of Insect immune shield to counter bacterial attacks?

Much like humans, insects too develop an immune-memory in response to infection, a team at the National Centre for Biological Sciences (NCBS), Bengaluru has found. In humans, for

instance, natural infection or vaccination can lead to the formation of important immunological memory in the human immune system. In other words, once infected, the immune system becomes ready to deal with that particular antigen because of immune-memory. For long, it has been a point of debate whether insects have such a memory that can protect them against future infections. The present study shows that such a memory can evolve over generations in red flour beetles (Tribolium castaneum) infected with Bacillus thuringiensis (Bt). The results of the study were recently published in Proceedings of the Royal Society B.The study was conducted in Deepa Agashe's lab at NCBS and the experiment was designed by Imroze Khan, first author of the paper, and Dr. Agashe, the principal investigator. The team infected nearly 5,000 to 6,000 beetles in every generation. "Every insect had to be pierced at the right point and injected with a standard number of bacterial cells. It took a year to standardise this process," says Dr. Khan, who is now a faculty at Ashoka University, Delhi. With a generation being approximately 45 days long, the study of 10 generations stretched over two years. "Every day Arun Prakash [one of the authors] and I had to infect 1,000 insects," he adds when asked what was the most challenging part of the study. The beetle populations were exposed to a single large dose of live Bt antigens or exposed to dead bacteria followed by live infection.In the past few years, multiple studies showed that insects do show some form of immune-memory, but how such memory evolves remained a puzzle. "We now have some clues about how fast and how reliably memory could evolve, what might be the mechanisms involved and when might immune-memory versus resistance be favoured by natural selection, says Dr Agashe.

SCIENTIFIC NEWS

NASA braces to pull out data from energy deficit Kepler

Scientists at NASA are preparing to download the latest bit of data stored in its plant-hunting Kepler space telescope as the spacecraft is now running very low on fuel.The US space agency has placed the spacecraft in a no-fuel-use safe mode to save the remaining fuel so that data extraction can be completed, NASA said on Friday.On August 2, the Kepler team will command the spacecraft to awaken from its no-fuel-use state and manoeuvre the spacecraft to the correct orientation and downlink the data.Once the data has been downloaded, the expectation is to start observations for the next campaign with any remaining fuel.But as of now, returning the data back to Earth is the "highest priority" for the remaining fuel.Since May 12, Kepler has been on its 18th observation campaign, staring at a patch of sky towards the constellation of Cancer it previously studied in 2015.The data from this second look will provide astronomers with an opportunity to confirm previous exoplanet candidates and discover new ones.Launched in 2009, the Kepler mission is specifically designed to survey our region of the Milky Way galaxy to discover hundreds of Earth-size and smaller planets in or near the habitable zone and determine the fraction of the hundreds of billions of stars in our galaxy that might have such planets.

IISc soon inceptionalizing science incubation centres

The Indian Institute of Science, founded in 1909 by Jamsetji Tata and former Maharajah of Mysore Krishnaraja Wadiyar IV, plans to open a research park at its Bengaluru facility within the next three years to incubate sci-tech companies.Currently the Society for Innovation and Development is incubating about 15 companies,said Prof. G.K. Ananthasuresh, chairman of the Centre of Biosystems Science and Engineering. "We want to scale it up ten times and the tenders for setting up the facility has already been issued."The institute has collaborations with companies such as Tata Consultancy Services, Volvo, Google Inc., General Motors, Microsoft Research, IBM Research, Boeing, Robert Bosch Foundation and Pratt & Whitney. It also works with the Indian Space Research Organisation, Aeronautical Development Agency and Centre for Development of Advanced Computing.Of about 12 companies incubated by the Society for Innovation and Development arm, an inter-disciplinary body, include simulators used for endoscopy, microsatellites to access the Internet at lower costs, a medical diagnostic kit and a superwave technology to extract oil from sandalwood.Many of these companies employ core technology. There are deep science and deep technology involved and the impact they can create is big, Prof. Ananthasuresh said in an interview.An air-conditioned blanket invented by scientists in the institute enables one to cool "in cycles," he said. "It is a layered blanket and one does not have to cool the whole room. It is a personalised air-conditioner," he said.

Neanderthal Humans hunts in groups, speared down prey in short range, examined study

Neanderthals were capable of sophisticated, collective hunting strategies, according to an analysis of prehistoric animal remains from Germany that contradicts the enduring image of these early humans as knuckle-dragging brutes.The cut marks — or "hunting lesions" — on the bones of two 1,20,000-year-old deer provide the earliest "smoking gun" evidence such weapons were used to stalk and kill prey, according to a study the journal Nature Ecology and Evolution.Microscopic imaging and ballistics experiments reproducing the impact of the blows confirmed that at least one was delivered with a wooden spear at low velocity. "This suggests that Neanderthals approached animals very closely and thrust, not threw, their spears at the animals, most likely from an underhand angle," said Sabine Gaudzinski-Windheuser, a researcher at Johannes Gutenberg-University Mainz, Germany.Neanderthals lived in Europe from about 300,000 years ago until they died out 30,000 years ago, overtaken by our species.It was long thought that these evolutionary cousins — modern Europeans and Asians have about 2% of Neanderthal DNA — were not smart enough to compete, and lacked symbolic culture, a trait supposedly unique to modern humans.3,00,000-to 4,00,000-year-old wooden staves found in England and Germany are the oldest known spear-like implements likely used for killing prey. But there was no physical evidence as to their use, leaving scientists to speculate.They have also turned up thousands of stone artefacts, attesting to a flourishing Neanderthal presence in what was a forest environment during an interglacial period 135,000 and 115,000 years ago. The old deer bones examined for the study were unearthed more than 20 years ago, but new technologies helped unlock their secrets: which injuries were lethal, what kind of weapon was used, and whether the spears were thrown from a distance or thrust from close up.

Central University of Hyderabad enhances harpin biopesticide bioavailability

Researchers at the University of Hyderabad have found that harpin biopesticide brought about 80-90% reduction in severity of fungal infection in tomato plants when it is encapsulated in chitosan nanoparticles. The fungal infection was caused by Rhizoctonia solani. The reduction in disease severity is only about 50-55% when the biopesticide is used without loading it in nanoparticles. The results were published in the journal Carbohydrate Polymers.Though harpin is used against several bacterial, fungal and viral infections, poor bioavailability is a major hurdle when harpin protein, taken from the bacteria Pseudomonas syringae pv. syringae, is just sprayed on the leaves like any other pesticide.To address the issue of poor bioavailability of harpin arising from the inability to permeate into plants, the researchers led by Prof. Appa Rao Podile from the Department of Plant Sciences turned to nanotechnology. They used the biocompatible and biodegradable chitosan in nanoparticle size to encapsulate the biopesticide. Chitosan nanoparticles are capable of getting into the plant through the stomata (pores on the leaves through which gas exchange takes place) and then diffuse through the cell wall to enter the cells. The team found that chitosan nanoparticles containing harpin pass through the cell wall and end up in the chloroplast of tomato plants.As a result, bioavailability of harpin inside tomato plants increases sharply when loaded in chitosan nanoparticles. Also, less amount of harpin will have to be sprayed on leaves when it is contained in nanoparticles.Chitosan by itself has another advantage. "Chitosan's antifungal property and its role in triggering plant defence responses are already well known. Laboratory studies found harpin was released from the nanoparticles in two phases.The biopesticide adsorbed on the nanoparticles gets released in a burst in the first 48 hours followed by slow release up to 120 hours,says Dr. Sandhya Rani Nadendla from the Department of Plant Sciences at UoH and first author of the paper.The team is planning to test harpin-containing chitosan nanoparticles on a large-scale on four different crops and at least two pathogens per crop. Two of the crops to be tested will be grown in fields and two others will be greenhouse crops.

Mini Neanderthal brains grown in U.S. lab

Scientists have successfully grown pea-size versions of Neanderthal brains, an advance that may help better understand the species that went extinct about 40,000 years ago.Cultivating and studying these mini brains may reveal why Neanderthals died out and Homo sapiens went on to conquer much of the planet, researchers said.Genetic differences between Neanderthal and human brains may explain their demise and our success, said Alysson R. Muotri, director of the University of California, San Diego,U.S. Researchers compared the genome of

Neanderthals with that of modern humans. Out of 200 candidate genes that showed significant differences between the two species, the researchers focussed on a gene expression regulator known as NOVA1.To grow mini Neanderthal brains, they used the gene-editing tool known as CRISPR to Neanderthalise human pluripotent stem cells that can develop into any cell in the body.Then, using their in-house protocol, we coaxed the stem cells to become a brain organoid,, Mr. Muotri said.

IIT-Delhi, IIT-Bombay and IISc Bangalore get Institution of Eminence status

The Union Human Resource Development Ministry has granted the Institution of Eminence (IoE) status to IIT Delhi, IIT Bombay and IISc Bangalore today. Along with them, in the private sector, the Manipal Academy of Higher Education, BITS Pilani and Jio Institute granted institution of eminence tag. The UGC had received 103 applications including JNU and Delhi University for IoE status.The Union HRD Minister, Prakash Javadekar, tweeted the names of IIT Bombay and IIT Delhi who will receive government funding as the private sector institutes which are granted the status of Institutes of Eminence will get the government grants of Rs 1000 in next five years.The IoEs are proposed to have greater autonomy in comparison to other higher education institutions. For instance, they will be free to decide their fee for domestic and foreign students and have a flexible course duration and structure. Moreover, their academic collaborations with foreign institutions will be exempt from approvals of government or UGC except institutions based in MEA and MHA's list of negative countries.Once identified, the target for the IoEs would be to break into the top 500 bracket in one internationally reputed ranking framework in 10 years and come up in the top 100 over time.

POST DOC OPPORTUNITIES:

1. **50 Postdoc Research Fellows: ICMR CENTENARY** – Scheme - Indian Council of Medical Research (ICMR) and Division of Human Resource Planning and Development (HRD) invite applications under ICMR CENTENARY – Scheme.For correspondence - **Dr N.C. Jain, Scientist-G & Head,Email: drencejain@gmail.com**

2. Post Doctoral Fellowships at IIT Palakkad - Post Doctoral Positions are available in the areas of Civil Engineering, Computer Science and Engineering, Electrical Engineering, Mechanical Engineering, Chemistry, Mathematics, Physics & Humanities and Social Sciences. Please refer **iitpkd.ac.in**

3. **Institute for Stem Cell Biology and Regenerative Medicine** - The primary interest of the Raghavan lab is to elucidate how epithelial homeostasis is maintained in development and perturbed in disease states. Please refer **instem.res.in**

4. **IIT Ropar** invites applications for Institute Post Doctoral Fellowship in the various Departments/ Centres. Please refer **http://www.iitrpr.ac.in**

5. **IISER Pune** Postdoctoral Research Associate - Applications are invited for Postdoctoral Research Associate (PRAs) positions at the Indian Institute of Science Education and Research (IISER) Pune, India. Please refer **www.iiserpune.ac.in**

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The 12th Annual Convention of ABAP &
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BIODIVERSITY, ENVIRONMENT AND HUMAN HEALTH : INNOVATIONS AND EMERGING TRENDS
(BEHIET - 2018)

12 - 14 November, 2018
Auditorium, Mizoram University
Aizawl, Mizoram, India

THEMES

* Biodiversity and Environmental Protection
* Pharmacology and Drug Development
* Omics of Food, Water, Health and Environment
* Environmental Pollution

IMPORTANT DATES

Abstract (Oral and Poster) Submission	31st July, 2018
Notification of Acceptance/Rejection	3 August, 2018
Full paper submission	15 August, 2018
Registration deadline	31 Aust, 2018
Nomination for Awards	15 September, 2018

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