Characterization of Potential Probiotic bacterial strains isolated from traditionally fermented fish and their effect on cancer cell lines

Dissertation submitted in fulfillment of the

Requirements for the degree of

Master of Philosophy

In Biotechnology

By

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Declaration of the Candidate

I, **Sambanduram Samarjit Singh**, hereby declare that the subject matter of this dissertation entitled "***Characterization of Potential Probiotic bacterial strains isolated from traditionally fermented fish and their effect on cancer cell lines***" is a record of work done by me, that the contents of this dissertation did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and the dissertation had not been submitted by me for any research degree in any other University/Institute.

This is being submitted to Mizoram University for the award of the degree of Master of Philosophy in Biotechnology.

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(Prof. N. Senthil Kumar)
(Supervisor)

CERTIFICATE

This is to certify that the dissertation entitled **"*Characterization of Potential Probiotic bacterial strains isolated from traditionally fermented fish and their effect on cancer cell lines"*** submitted to Mizoram University for the award of Master of Philosophy in Biotechnology by **Sambanduram Samarjit Singh** Registration No. ***MZU/M.Phil/355 of 26.05.2017***, Research scholar in the Department of Biotechnology, is a record of research, based on the results of the experiments and investigations carried out independently by him during the period from 2016 to 2017 of study, under my guidance and supervision and has not been previously submitted for the award of any Indian or foreign University.

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Place: **Prof. N Senthil Kumar**

Date: **(Signature of the supervisor)**

.....Earnestly dedicated to my parents S. Ajit Singh (L) and S. Sumati Devi and my family whose hand always rise for me for their spiritual inspiration in motivate me to the highest ideals of life carrying out my work to achieved my goal.....

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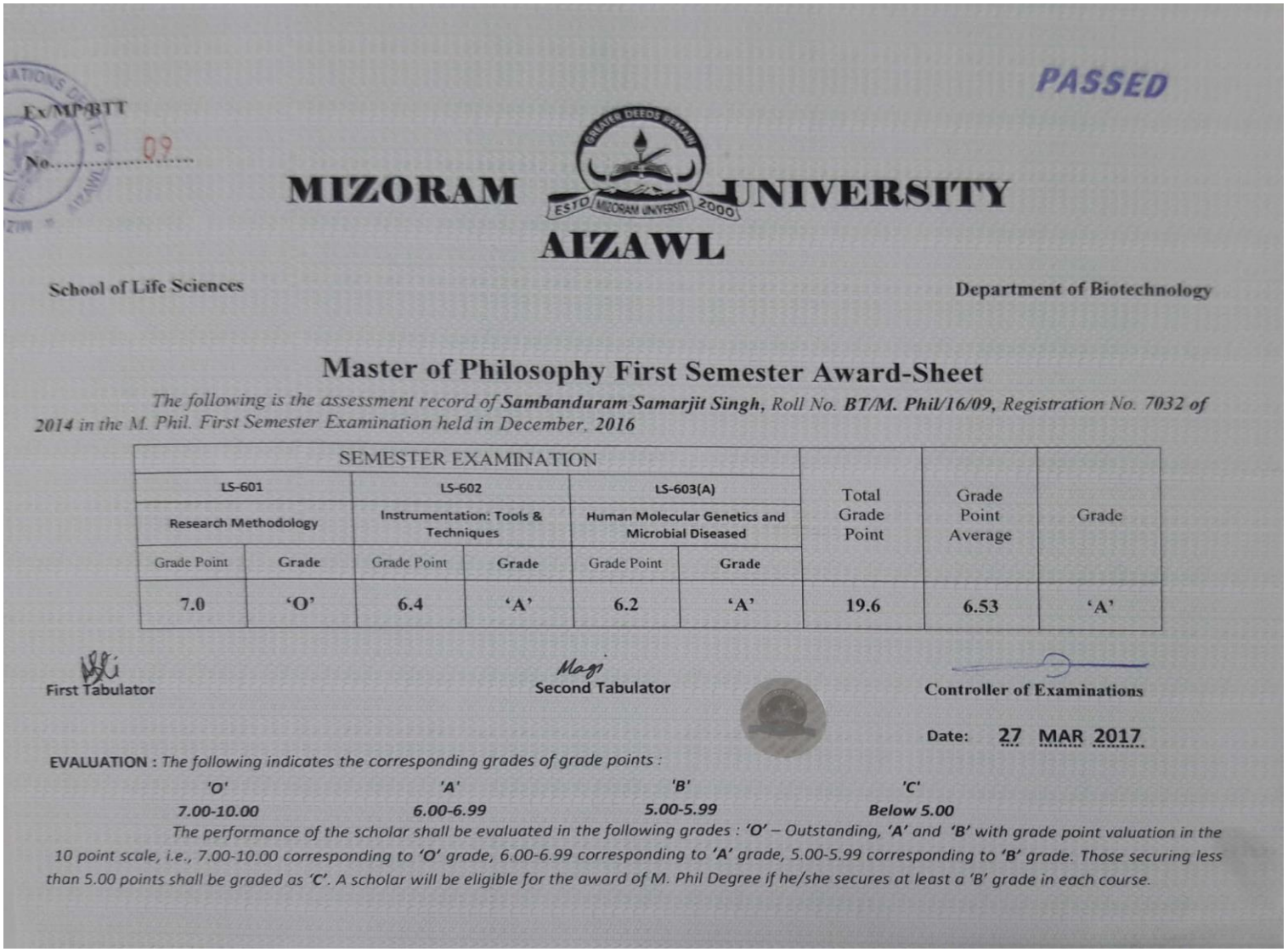
(Sambanduram Samarjit Singh)

COURSE WORK CERTIFICATE



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SEMESTER EXAMINATION						Total Grade Point	Grade Point Average	Grade
LS-601		LS-602		LS-603(A)				
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Grade Point	Grade	Grade Point	Grade	Grade Point	Grade			
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'O'	'A'	'B'	'C'
7.00-10.00	6.00-6.99	5.00-5.99	Below 5.00

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TABLE OF CONTENTS

ABBREVIATIONS

LAB **–** Lactic Acid Bacteria

HeLa – Henriatta Lacks

MTT – 3 - (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide

IL – Interleukin

UK – Utonga-kupsu

MRS – De Man Rogosa and Shape

S.A – *Staphylococcus aureus*

P.A – *Pseudomonas aeruginosa*

E.C – *Escherichia Coli*

CV – Crystal violet

PBS – Phosphate buffer saline

CFU – colony forming unit

OD – Optical Density

DNA – Deoxyribonucleic acid

PCR – Polymerase Chain Reaction

BLAST – Basic Local Alignment Search Tool

MEGA – Molecular Evolutionary Genetics Analysis

BSA – Bovine Serum Albumin

AOAC – Association of Official Analytical Chemists

CFTRI – Central Food Technological Research Institute

SFA – Saturated Fatty Acid

MUFA – Mono Unsaturated Fatty Acid

PUFA – Poly Unsaturated Fatty Acid

NCBI – National Center for Biotechnology Information

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

Northeast part of India is associated with east India through a thin passage sandwiched amongst Nepal and Bangladesh and popularly known as Seven Sister states of India: Assam, Arunachal Pradesh, Meghalaya, Manipur, Mizoram, Nagaland, and Tripura. This place is also regarded as the center of a various nourishment food culture involving fermented and non-aged ethnic foods and various alcoholic beverages (Sathe and Mandal, 2016). These indigenous food also plays a critical role in their traditional heritage and also helps in distinguishing one ethnic group from the other (Asati and Yadav, 2003; Medhi et al., 2013). It is an inherent part of routine nutrition of the ethnic tribes being the earliest and most monetary techniques for advancement of a decent variety of fragrances, flavors, and textures, food preservation and natural improvement of nourishment by the control of various microbial populations (Sekar and Mariappan, 2007).

Traditional fermented foods have been consumed by the ethnic individuals of Northeast India since 2500 years ago and all these foods are specific according to their location and have remarkable substrates and preparation strategies (Das and Deka, 2012; Tamang et al., 2012). Locally accessible materials such as milk, vegetable, bamboo, soybean, meat, fish, and oat are commonly used substrate for traditional fermentation process (Das and Deka, 2012). More than 250 unique sorts of natural and less-recognizable fermented foods and alcoholic beverages are consumed by the distinctive ethnic individuals of Northeast India such as fermented soybean and non-soybean vegetable, fermented vegetable (*Gundruk*, *Sinki*, *Anishi*) and bamboo shoot (*Soibum*, *Mesu*), fermented grain and pulses (*Kinema*, *Bhatootu*, *Marchu* and *Chilra*, *Tungrymbai*), fermented and smoked fish items (*Ngari*, *Hentak*) preserved meat items, milk beverages (*Kadi*, *Churpa* and *Nudu*) and alcoholic beverages (*Ghanti*, *Jann*, *Daru*) (Tamang et al., 2012).

These indigenous foods contribute to an extensive proportion of every day food intake of the local people of North East India. Different tribes of this region bears their own strategies for fermenting food materials with the goal of preservation and taste improvement according to the tradition and culture found in the diverse geographical territories (Sekar and Mariappan, 2007). The preparation processes of such fermented foods is not well known since most of them are homemade and the tradition is passed from one generation to next generation (Lee and Kim, 2016)

These fermented foods have been consumed since ancient times because of their extended shelf life, shorter cooking period and better nutritive value as compared with the non-fermented foods (Tamang et al., 2016). It also has its specific taste, therapeutic properties, strong appetizing nature and contains several beneficial activities such as probiotic potential by introducing beneficial bacteria into the digestive system and helps in balancing of gut bacteria and food preservation by facilitating the growth of desired microorganism (Caplice et al., 1999). Each fermented food has a unique group of the micro flora and their role in medicinal values have been documented (Chettri and Tamang, 2015; Sarojnalini and Singh, 2009).

Fermentation is one of the most seasoned and efficient technique for preserving the foods and helps in improving the dietary quality, flavor, and aroma by enhancing the quantity of vitamins, unsaturated fats, amino acids, and protein solubility. The word "Fermentation" means 'to boil' which is derived from the Latin word '*fervere'* meaning a procedure for the generation of a product by the mass culture of microorganisms to separate complex mixes compound to produce a novel taste and aroma (Stanbury, 1999). Fermentation also enhances digestibility by separating proteins inside the food products and allowed the generation of organic acids, dietary improvement, lessening of endogenous toxins, produces valuable compounds such as Omega-3 fatty acids and decrease cooking period (Sekar and Kandavel, 2002; Sathe and Mandal, 2016).

The preparation of indigenous fermented foods uses variety of substrates and non-pathogenic microorganisms as a starter. Lactic acid bacteria (LAB) take part a principal role in the output of a large quantity of the fermented foods (Mokoena et al., 2016). Breakdown of food proteins by the activity of microbial or indigenous protease catalysts brought about the improvement of various bioactive peptides, leading to considerable enrich in the organic properties of the food (Steinkraus 2004). LAB isolated from various fermented foods generate organic acids and different bioactive compounds with antimicrobial properties which helps to enrich the final product in respect to its nutritive content (Mokoena et al., 2016; O'sullivan et al., 2010). Fermentation may also aid the pulverization or detoxification of certain unwanted compounds, for example, phytates, polyphenols, and tannins. Other beneficial advantages such as anticancer impacts, cholesterol control, enhancing immunity, anti-hypertensive impact, and against diabetic impact are also reported (Sekar and Kandavel, 2002).

Utonga-kupsu is commonly prepared by the Manipuri (meetei) People settling in Assam, Cachar Northeast India for daily consumption. Similar kind of fermented fish preparation

(*Hentak*) has been practice by the Manipuri people in Manipur. It is believe that the tradition for the preparation of *Hentak* has been carried away and the preparation of the *Utonga-kupsu* has been developed by this local people in the small scale household level. *Utonga-kupsu* is prepared by the mixture of small fish (*Esomus danricus*, *Puntius sophore*, *Amblypharyngodon mola*, *Channa punctata*, *Mystus vittatus* etc.) with *Alocasia macrorhiza* (*Hongu*) as well as a little amount of mustard oil and kept in an earthen pot for a period of one month. The plant *Alocasia macrorhiza* has a good medicinal value. Traditionally local people used this particular plant for the treatment of the Gout disease. Mainly the seed of this plant is used for the treatment of this disease. The phenolic compound produced by the plant *Alocasia macrorhiza* is much prompted to allergic reaction but after fermentation with fish it loses it's the allergic activity.

Since the fermented fish have been contributing a large portion of regular food intake and its popularities have been growing day by day in Northeast India. It is already been proved that addition of probiotics in foods makes it more health beneficial (Berner and Donnell, 1998; McNaught and MacFie, 2001; Saarela et al., 2002), Therefore a proper scientific investigation is needed to identify and characterize the microbes involved in the enhancement of the nutritional value of these fermented fish. However, limited amount of data are available concerning the traditional procedure and scientific values of fermentation fish in Northeast India (Thapa et al., 2004; 2006). Previous studies that have evaluated the bacterial communities in *Ngari*, *Hentak* and *Tungtap* have isolated bacterial species such as *Lactococcus lactis*, L. *plantarum*, *Enterococcus faecium*, L. *fructosus*, L. *amylophilus*, L. *coryniformis*, L. *plantarum*, *Bacillus subtilis* and B. *pumilus* and *B. Cereus* population (Thapa et al., 2004; 2016).

Although *Utonga-kupsu* has high significance being a regular food ingredient, no scientific study has been performed till date. The present study was designed with an objective to isolate, identify and characterize the probiotic and anticanceral potential of the potential LAB species of *Utonga-kupsu* using both biochemical and molecular approaches. Furthermore, we performed nutrient profiling in order to better understand this indigenous food.

2. REVIEW OF LITERATURE

Probiotic bacteria are widely used for humans as well as to organisms like fish, poultry and other live stock for its multifactorial benefits (Gaudana et al., 2010). In addition to different sources of isolation, various recent investigations related to probiotic microorganism from traditionally fermented sources like *Kimchi*, *Meju*, *Dongchimi* and *Doenjang* has been documented (Lim et al., 2009). Likewise, this traditional fermented fish might serve as a source and a reservoir of potential probiotic microbes but the number of food borne illness still prevail a public concern. Now a days, the ability of the pathogenic food spoilage microorganism has been rising rapidly in terms of food wastes even after the food is stored in refrigerator, such type of scenario is a major risk in food industry. Subsequently, the utilization of naturally derived anti-microbial compounds with no unfavorable impact on human health to suppress the expansion of pathogenic microorganisms in food has to be taken as a primary concern (Tharmaraj and Shah, 2009). Nevertheless, there are very little efforts made in exploration of probiotic microorganisms from fermented fish sources.

Previous studies have also evaluated the bacterial communities in *Ngari*, *Hentak* and *Tungtap* and have isolated bacterial species such as *Lactococcus lactis*, L. *amylophilus*, L. *coryniformis*, L. *fructosus*, L. *plantarum*, *Enterococcus faecium*, *Bacillus subtilis* and B. *pumilus* and B. *cereus* population. Most of the identified strains of LAB characterized for future applications as probiotics (Thapa et al., 2004; 2016). LAB such as *lactobacilli* and *pediococci* and a small number of coagulase-negative *staphylococci* also isolated from fermented foods in Thailand. Among them *Staphylococcus carnosus*, and *Staphylococcus piscifermentans* were isolated identified from fermented fish and soy sauce mash (Tanasupawat et al., 1992). These two has also been reported from the Malaysian traditional fermented shrimp product known as *Cincaluk*, which is commonly consumed by the local people as dishes. The practice of the starter culture in variety of fermented foods has been well known for the production of different compounds such as anti-microbial agent, bacteriocian and other useful enzymes. Seven strains were isolated successfully from this fermented foods and it was identified as *Staphylococcus spp*. It is reported that all the strains possessed anti-bacterial properties against *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Bacillus subtilis*. Among them two strains identified as *Staphylococcus piscifermentans*, which is a rare stain specifically isolated exclusive from fish sources (Hajar et al., 2013).

In view of the role of diet in human diseases such as cancer, the potential of dietary bacteria to prevent cancers is a matter of great interest. LAB has been reported for various anticancer properties (Kim et al., 2002; Lee et al., 2004). LAB has become the most studies area and has concentrated on the impacts of LAB with respect to the decrease of cancer cell viability or tumor estimation. Choi et al. (2006) demonstrates that the inhibitory impacts of Lactobacillus on different human cancer cell lines and found the LAB strains L. *acidophilus* 606 and L. *casei* ATCC 393 showed the most significant inhibitory impacts on malignancy cell development. The inhibition of tumor cell development by *Lactobacillus* has likewise been reported in different studies (Kim et al., 2002). Probiotic strain E. *lactis*IW5 was isolated from human gut and characterisation of was studied. This strain was highly impervious to low pH and high bile salt and it was found that this microorganism has strong ability to adhere on Caco - 2 human epithelial colorectal cell lines. The supernatant of these strains strongly hindered the growth of a various pathogenic microorganisms and reduced the number of cell viability of different cancer cells, for example, HeLa, HT-29 however on the other hand did not repress the viability of ordinary FHs - 74 cells. It is also reported that the strain did not secrete any lethal enzymes, including β-*glucosidase*, β -*glucuronidase*, and N-acetyl- β -*glucos aminidase* and was highly susceptible to ampicillin, clindamycin, chloramphenicol, gentamycin, penicillin, vancomycin and sulfamethoxazol. Therefore E. *lactis*IW5, as a bioactive therapeutics, should be subjected to other relevant tests to confirm the remedial suitability of this strain for clinical applications (Nami et al., 2014).

Many cancer therapy agents are limited in their use because of their toxic effects on normal cells and tissues. Therefore such strains will be useful source for key determinant of drug activity agents. The cancer cells are vulnerable due to defects in checkpoints that control the function of other checkpoints, which disturbed the overall response to the cell cycle. The checkpoints are capable to repair any damage during the cell cycle, or to activate the apoptotic (programmed cell death) machinery. G1 and G2 checkpoints plays an important role in induction of death response to cancer cells. Therefore, the anticancer agents have the advantage to modulate these pathways not only to increase the activity to programmed cell death, but also increase in their specificity (Damia and Broggini, 2004). The LAB strains, *Lactobacillus acidophilus* 606 and L. *casei* ATCC 393 shows the most significant inhibitory phenomena in the treated cell lines. L. *acidophilus* 606 was known to possess less cytotoxicity to human embryo fibroblasts (hEF cells). The polysaccharides extracted from L. *acidophilus* 606 possessed the most effective anticancer activity, but inhibited growth of hEF cells by only 20%. Whereas the

polysaccharides extracted from L. *acidophilus* 606 were observed to possess less activity to activate apoptosis in the HT-29 cells by DNA fragmentation. Therefore, the probiotic microorganism may be the best target and safely used as natural anticancer drug discovery. The probiotic microbes exhibit little or no cytotoxicity, because the compound or polysaccharides generate by them specifically target cancer cells have the selective mechanism of inhibiting the growth of the cancer cell (Choi et al., 2006). The effect of the membrane components of LAB strains on cancer cell lines activities such as *Bifidobacterium Lactobacillus, Lactococcus* and *Streptococcus,* have also been evaluated. LAB strains were tested for in vitro cytotoxicity on diverse cancer cell lines. The fractionated portions of peptidoglycans and cytoplasm, as well as the whole cells of LAB which is pretreated to heat, had significant mechanism of inhibiting the proliferation several cancer cell lines. Specifically, the cytoplasm fractions possessed direct antiproliferative activities to colon and gastric cancer cell lines, on the other hand peptidoglycans slow down the growth of colon and bladder cancer cell lines. The cytoplasm fractions obtain from *Bifidobacterium longum* and *Lactococcus lactis* shows good antiproliferation activity against two cancer cell lines SNUC2A (colon cancer) and SNU-1 (gastric cancer) (Kim et al., 2002).

The crude protein and lipid extracts from the same probiotic strain, alternatively, shows no effect in the function of cell growth and proliferation of cancerous cells. This proved that soluble polysaccharides, fractionated portion of peptidoglycans and cytoplasm have the principal component to target the cancer cells induced the apoptosis. Since the different fractionated portion of the LAB strains shows the high degree of effectiveness, therefore it may be used as good adjuncts for anticancer in the food industry (Choi et al., 2006). The cytotoxicity induced by probiotic bacterial strain on cancer cells such as HeLa, MCF-7, AGS, HT-29 etc. can be determine by MTT colorimetric assay (Nami et al., 2014). This technique is based on the ability of the cells to metabolize the yellow tetrazolium salt MTT to a blue crystalline formazan product (Prinsloo et al., 2013). The manner in which a cell dies determines the nature of the response by the surrounding tissue. Death by necrosis acts induces oxidative stress and production of numerous pro-inflammatory cytokines. The potential phagocytosis of apoptosis cells by macrophages decreased the efficiency for an inflammatory response by monitoring the dying cells are lysed before their intracellular components are excreted. By contrast, cell death through apoptosis is a monitored event, this process is assisted by loss of membrane integrity until the later stage or secondary necrosis. the in vivo processed of Apoptosis is a natural phenomena which is followed by rapid uptake of the targeted cell into the adjacent phagocytic

cells, which is a critical process in remodeling of tissue, triggering of the immune response, or resolution of inflammation. This type of cell death is frequently associated with phagocytosis by resident tissue macrophages and the release of anti-inflammatory cytokines. The processed of phagocytosis by macrophages to eliminate the apoptotic cells has been proposed to be a calm process that does not produced any inflammatory mediators. In contrast to immunoglobulin G susceptible to phagocytosis of apoptotic cells, the response of neutrophils to apoptotic cells for initiating phagocytosis is actively inhibited by the released several interleukin, colony-stimulating factor, granulocyte macrophage and leukotriene C4. Therefore, it can be said that the mechanism of inflammation depends on active response to suppression of inflammatory mediator not only on the removal of apoptotic cells (Fadok et al., 1998).

3. METHODOLOGY

3.1. Traditional procedure for the production of Utonga-kupsu

Utonga-kupsu, a fish paste obtained after fermented, is very commonly consumed by the Manipuri people in the North-East State of India. Varieties of small fish are selected and sun dried properly until all the water is vaporized. The dried fish are crushed into powder along with same quantity of *Alocasia macrorhiza*'s petioles as well as a little amount of mustard oil. The mixture is then fermented for a one month (Fig. 1).

3.2. Sample collection

Samples of *Utonga-kupsu* were collected in sterile vials from different villages of Sonai, Cachar district of Assam (24.7326° N, 92.8889° E), Northeast-India and transported to the laboratory within 24 hrs for further analysis. All the samples were mixed into a single composite vile. The area of the sample collection site is shown in Fig. 2.

3.3. Isolation of Lactic acid Bacteria from the fermented fish Utonga-kupsu

The sample was 5-fold serially diluted in sterile distilled water and mixed with acidified De Man Rogosa and Sharpe (MRS) broth (pH 2.5) for 2 hrs. in order to select acid-tolerant LAB. After acid treatment, 100 µl of culture is spread on MRS agar plates and incubated at 37°C for 48 hrs (Jena et al., 2013).

3.4. Gastric acid tolerance

The tolerance to the gastric juice was performed as described by Kumar et al. (2011) with some modifications. All the forty isolates were inoculated in MRS broth (5 ml) and incubated overnight and the pellets were collected by centrifugation. Further the pellets were mixed with MRS broth supplemented with simulated gastric juice (1.28 g NaCl, 0.239 g KCl, 6.4 g NaHCO3, 0.3% bile salts, 0.1% (w/v) pepsin, pH – 2) and incubated at 37°C for 24 hrs. The numbers of survival strains were evaluated by spreading 100µl of cultures on MRS agar plates and incubated at 37ºC 24 hrs.

3.5. Bile salt tolerance

After all the forty isolates were grown in MRS broth, the pellets were collected by centrifugation and the tolerance of bacterial isolates to bile salts was assayed in 10 ml sterile MRS broth containing with 0.3 % bile salts (Perelmuter et al., 2008). The collected pellets were

mixed with sterile MRS broth with bile salts and incubated at 37°C for 24 hrs. The numbers of survival strains were evaluated by spreading 100µl of cultures on MRS agar plates and incubated at 37ºC 24 hrs.

3.6. Phenol tolerance

Tolerance to phenol was carried out according to Xanthopoulos et al. (2000). The over grown bacterial cultures were centrifuged and the pellets were collected. MRS broth containing 0.4% phenol were mixed with the pellets and incubated for 24 hrs at 37°C. After incubation, the numbers of survival strains were evaluated by spreading 100µl of cultures on MRS agar plates and incubated at 37ºC 24 hrs.

3.7. Biofilm production

Thirteen bacterial strains were selected for solid-surface-associated biofilm formation by the crystal violet (CV) staining method (O'Toole et al., 1999). 100 ml of each bacterial isolates were inoculated in freshly prepared MRS broth (5 ml) and incubated at 37°C overnight in shaking condition. After the incubation, the cultures were further incubated again at 37°C for 10 hrs. in non-shaking condition. CV solution 1% (1 ml) were mixed with the cultures and kept for 25 min. at room temperature, followed by washing with distilled water. The appearance of violet color on the walls of the test tube was recorded for positive activity.

3.8. Antimicrobial activity test

Antimicrobial activities of the selected thirteen bacterial strains were tested against three pathogenic strains: *Staphylococcus aureus* (S.A) (ATCC 25923), *Pseudomonas aeruginosa* (P.A) (ATCC 27853) and *Escherichia Coli* (E.C) (ATCC 27859) using agar diffusion method in Muller-Hinton agar media according to (Angmo et al., 2016).

3.9. Co-aggregation assay

The selected six strains were estimated for co-aggregation ability according to the method described by Collado et al. (2007). Overnight grown bacterial culture at 37°C in MRS broth was centrifuged and pellets were collected. It is washed twice with PBS (pH 7.0) and resuspended in PBS. The standard bacterial concentrations (10^8cfu/ml) were adjusted to 0.25 ± 0.05 by taking OD of the bacterial suspension at 600 nm. Bacterial cell suspensions were mixed with the various suspensions of pathogenic bacteria (SA ATCC 25923, PA ATCC 27853 and

EC ATCC 27859) in 1:1 ratio and vortex it for at least 10 sec. and incubated for 4 hrs at 37°C. The co-aggregation ability was expressed as follows:

$$\text{Co-aggregation (\%)} = \{(A_P+A_L)/2 - A_{mix} / (A_P+A_L)/2\} \times 100$$

Where A_L represents the absorbance (A600 nm) of a bacterial mixture at t = 0, A_{mix} represents the absorbance (A600 nm) of a bacterial mixture after 4 hrs of incubation and A_P represent the absorbance of bacterial pathogen at t = 0.

3.10. Molecular characterization of the bacterial isolates

3.10.1. DNA isolation from the six potential bacterial isolates

Bacterial isolates were inoculated in the MRS broth and incubated at 37°C overnight. The cultures were centrifuged at 5000 rpm for 5 minutes and the pellets were further subjected to DNA isolation following CTAB method (Wilson, 1987). The quality of the DNA was checked by running on 0.8% agarose gel electrophoresis and the DNA was stored at -20°C for further analysis.

3.10.2. PCR of 16s rRNA gene

The 16s rRNA gene was amplified by Polymerase Chain Reaction (PCR) using primers 27-F (5'-AGAGTTTGATGMTGGCTCAG-3') and 1492-R (5'-GGTTACCTTGTTACGACTT-3'). PCR was carried out in 25 µl total reaction volumes. The reaction condition was 94°C for 5 minutes followed by 35 cycles each consisting of 40 seconds, denaturation at 94°C for 1 minutes, annealing at 54°C for 30 seconds, 3 minutes of extension at 72°C. The PCR product is further purified and sequenced using Applied Biosystems® 3500 Series Genetic Analyzer.

3.10.3. Sequence analysis

Online similarity searches were performed using the BLAST algorithm of GenBank. Sequence alignment was carried out with the CLUSTALW program by MEGA6 software (Tamura et al., 2013). The taxonomic studies and properties of bacterial isolates were determined.

3.11. Biochemical characterization

Six isolates were selected based on the result of antibiotic and antimicrobial activity and further examined for cell morphology and by performing Gram staining, catalase and oxidase tests according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

3.12. Estimation of soluble Protein

The quantity of soluble protein can be determined by colorimetric method introduced by (Bradford, 1976) with slight modification. 500 mg of the fermented fish sample were crushed with 10 ml cold phosphate buffer saline (PBS 1X, pH 7.5, 0.1M) using a chilled mortar and pestle which give concentration of 50 mg/ml. The homogenate was centrifuged at 10,000 rpm for 10 min in a centrifuge. The temperature of the centrifuge was maintained at 4°C. The supernatant was transferred into a graduated tube and its volume was raised to 10 ml with the same buffer. The supernatant were used for protein estimation at 595 nm. The standard BSA was prepared 1mg/ml concentration.

3.13. Fat estimation

Estimation of the total fat was done by AOAC method. 200g of the samples were sent to Central Food Technological Research Institute (CFTRI), Mysuru for the estimation of Saturated Fatty Acid (SFA), Mono Unsaturated Fatty Acid (MUFA), Poly Unsaturated Fatty Acid (PUFA) and Total fat content. (AOAC Official Method, 2006)

3.14. Preparation of secondary metabolites and crude protein extract

The selected bacterial strains were cultured in 500ml of MRS broth for 10 days in shaking condition. The broths were transferred to the rotor evaporator to extract the secondary metabolites. After extraction 20mg of the crude extracted were weighted into the eppendorp tube and the volume was makeup to 1ml using milli-pore water. The prepared crude extracted were filtered and used to treat cancer cells (Rajan and Kannabiran, 2014).

The liquid culture of the isolates, at the log growth phase was centrifuged at 10,000 rpm for 1min to obtain cell precipitates .The pellet was subjected to sonication to extract the membrane and intracellular protein (5 cycle with 30 sec interval). The extracted crude proteins were further evaluated for protein concentration using Bradford protein estimation. The prepared crude proteins were filtered and used to treat cancer cells. (Moirangthem et al., 2014)

3.15. Cytotoxicity against Different Cancer Cells

The cytotoxicity effect of the isolates on cancer cell lines was evaluated through tetrazolium [MTT, 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide] assay (Mosmann, 1983). HeLa (cervical cancer) and HT-29 (Colorectal cancer) as well as L-132 (Normal lung cell) cells were seeded in each well of a 96-well micro plate with DMEM growth medium. Once 50% confluence was reached 24h after the cells were seeded, the cells were treated with the filtered secondary metabolites extract and crude intracellular protein extract of the isolated strain for 24h. After the treatment, 50 µl of MTT solution (2mg/mL in PBS) were added to each well along with 200 µl of fresh medium and incubated for another 4h at 37 C. After incubation was completed, the MTT mixture was removed; dimethyl sulfoxide (200 µl) of and Sorenson's glycine buffer (25 µl) (0.1Mg lycine and 0.1M NaCl at pH 10.5) were added to each well and incubated for 30 min. The absorbance was determined after 5sec of shaking by using a micro plate reader (Spectro max M 2e molecular devices) at 570nm. Doxorubicin (anticancer drug as a reference) served as positive controls, respectively.

3.16. DNA fragmentation assay

The induction of apoptosis was determined by DNA fragmentation analysis, observing the biochemical changes. HT-29 cell line was treated with the crude protein extract of the isolates (107 cells/well, 50 ug/ml) and incubated for 24 hours. Untreated cells were used as control .The cells were washed thrice using PBS and trypsinized the cells prior to harvest and resuspended in 300 ul lysis buffer [25 mM EDTA,50 mM Tris-HCl, pH 8.0, 0.9% (w/v) sodium dodecyl sulphate (SDS),30 mg/ml proteinase K, 1 mM DTT, 1 mM phenyl methyl sulfonylfluoride (PMSF), 1% Triton-X-100]. The samples were incubated at 65ºC for 1 h. Total DNA was extracted and 70% ethanol washed was done twice. DNA was air-dried and resuspended in 50 µl sterile water. The total DNA was detected on 1% agarose gel (Kalinina et al., 2002).

4. RESULTS

4.1. Bacteriological analysis of Utonga-kupsu

Results show that the number of bacterial count in MRS agar was 100cfu/g *Utonga-kupsu* respectively. A total of 40 colonies were randomly selected which is showing healthy growth morphology and streaked onto MRS agar plates and stored in refrigerator for further analysis.

4.2. Tolerance to Gastric acid, Bile salt and Phenol tolerance and biofilm production

All the strains were having the capacity to tolerance in bile salt and phenol. But thirteen strains form *Utonga-kupsu* - UK2, UK3, UK4, UK19, UK20, UK12, UK10, UK29, UK30, UK32, UK25, UK26, and UK27 have the resistance capacity to gastric juice. Among all the strains UK12 was highly resistance to gastric juice (Fig. 3 and Table 1). Biofilm formation were observed in all the thirteen strains (Fig 4).

4.3. Antimicrobial activity test

Most of the bacterial isolates had the antimicrobial properties. Table 2 shows the list of bacterial isolates having the antimicrobial properties against the three pathogens viz. S.A, P.A and E.C. Among the isolates, UK2, UK10, UK12, UK20 and UK25 identified as genus *Staphylococcus* shows antimicrobial activity against to all the three pathogens and rest of the isolates also shows good activity against P.A and E.C (Fig 5).

4.4. Co-aggregation assay

All the tested isolates were able to co-aggregate with pathogens. Among the isolates, UK12, UK 10 and UK2 were better able to co-aggregation with pathogens (Table 3). UK10 had a high co-aggregation percentage with *P. aeruginosa* (38.50%), followed by UK12 with *S. aureus* (24.6%) and UK2 with *E. coli* (21.8%).

4.5. Biochemical characterization

Biochemical characterizations of isolates were shown in Table 4. All the six pure culture bacterial isolates were shown positive in gram staining, catalase and negative to oxidase. Thus, it indicates that the strains belong to LAB.

4.6. DNA isolation, PCR analysis and Sequence analysis

The results of the DNA isolation and PCR product is shown in Fig. 6. All the sequences were subjected to BLAST in the NCBI database and the nearest representative is shown in Table 5. Based on the sequence similarity all the six bacterial strains were identified as *Staphylococcus piscifermentans, S. condiment, S. carnosus* and *Staphylococcus sp.*

4.7. Estimation of soluble Protein and Fat

The value of the standard BSA was generated 5.37µg. Therefore, the actual O.D of 20µl sample is 4.16. Hence it can be concluded that 1g of fermented fish sample contain 416mg of proteins (Table 6). Result shows that the total fat content is 13.75% where as the SFA content is 6.31%, MUFA content is 5.11% and PUFA content is 2.34% (Table 6).

4.8. Cytotoxicity

The cytotoxicity effects of the metabolites secreted by the isolates as well as crude protein extracts are determine by performing tetrazolium assay on cancer cell lines such as HeLa and HT-29 along with L-132. After 24h of incubation, the EC50 values were calculated accordingly (Fig.'s 7, 9, 11 and 13). The effectiveness of the crude protein extract and metabolites on the evaluated cancer cells significantly differed from that of the un-treated cell lines. The metabolite does not have any cytotoxicity to HeLa (Fig. 8). But the crude protein extracted show a significant cytotoxicity against HeLa and HT-29 (Fig.'s 10 and 12). However, the crude protein extract showed insignificant cytotoxicity to the L-132 (normal cell line) (Fig. 14).

4.9. DNA fragmentation assay

DNA fragmentation was tested by agarose gel electrophoresis and the results indicated a significant DNA fragmentation of HT-29 cells (Fig. 15). After the treatment of the crude protein extract (50µg/ml), the DNA was extracted from cells which exhibit characteristics of apoptosis. The fragmented sizes of the DNA were observed form approximately 1000 bp to 100 bp.

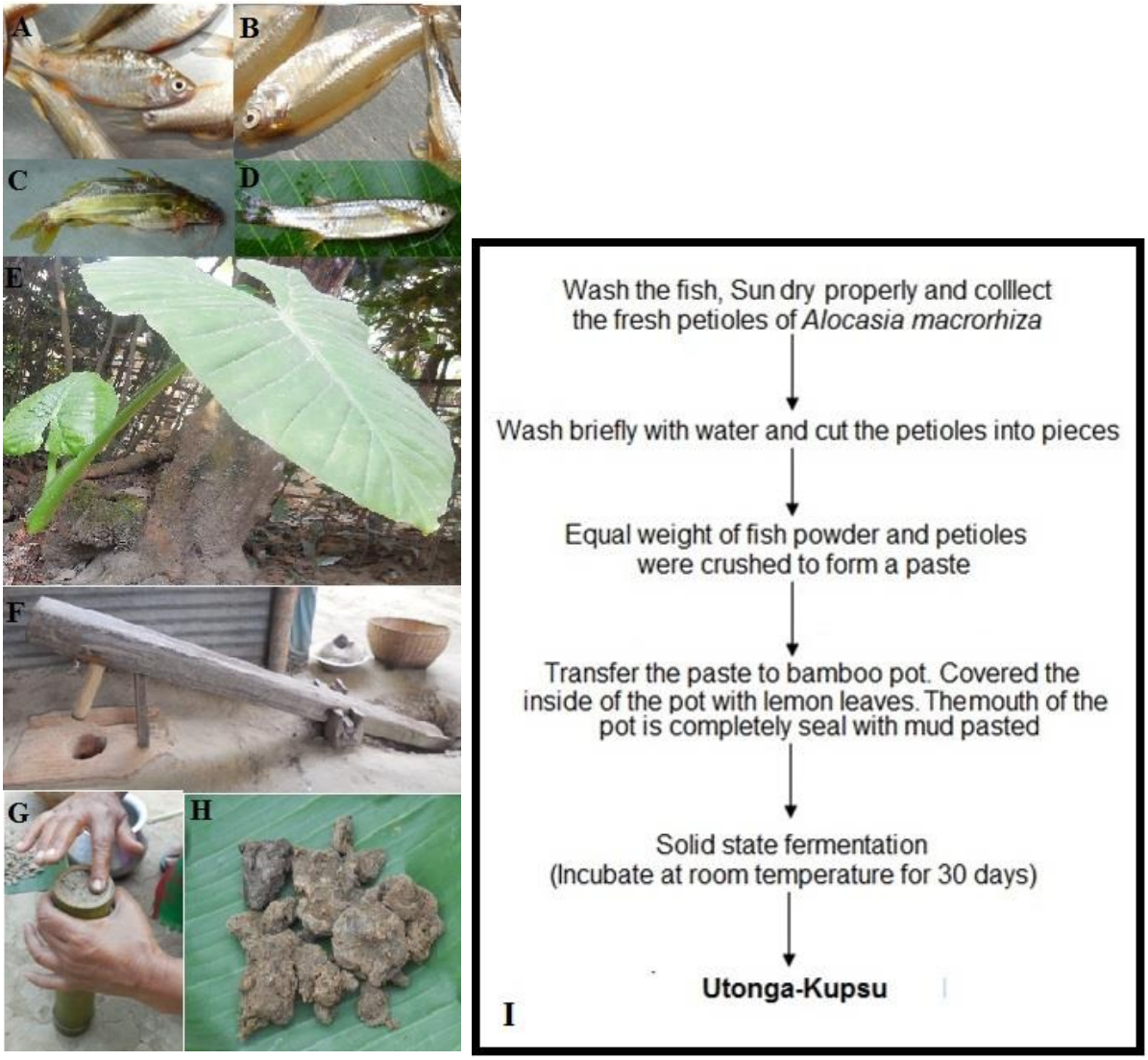

Fig. 1 Raw materials and the preparation procedure of *Utonga-kupsu* (A) *Puntius sophore* ((Phabounga or Puthi)) (B) *Amblypharyngodon mola* (Mukanga) (C) *Esomus danricus* (ngasang) (D) *Mystus vittatus* (ngasep) (E) *Alocasia macrorhizat* (Hongu) (F) Traditional instrument for crushing the dried fish and petioles (G) Sealing for fermentation (H) *Utonga-kupsu* (I) Steps for the preparation o *Utonga-kupsu*.

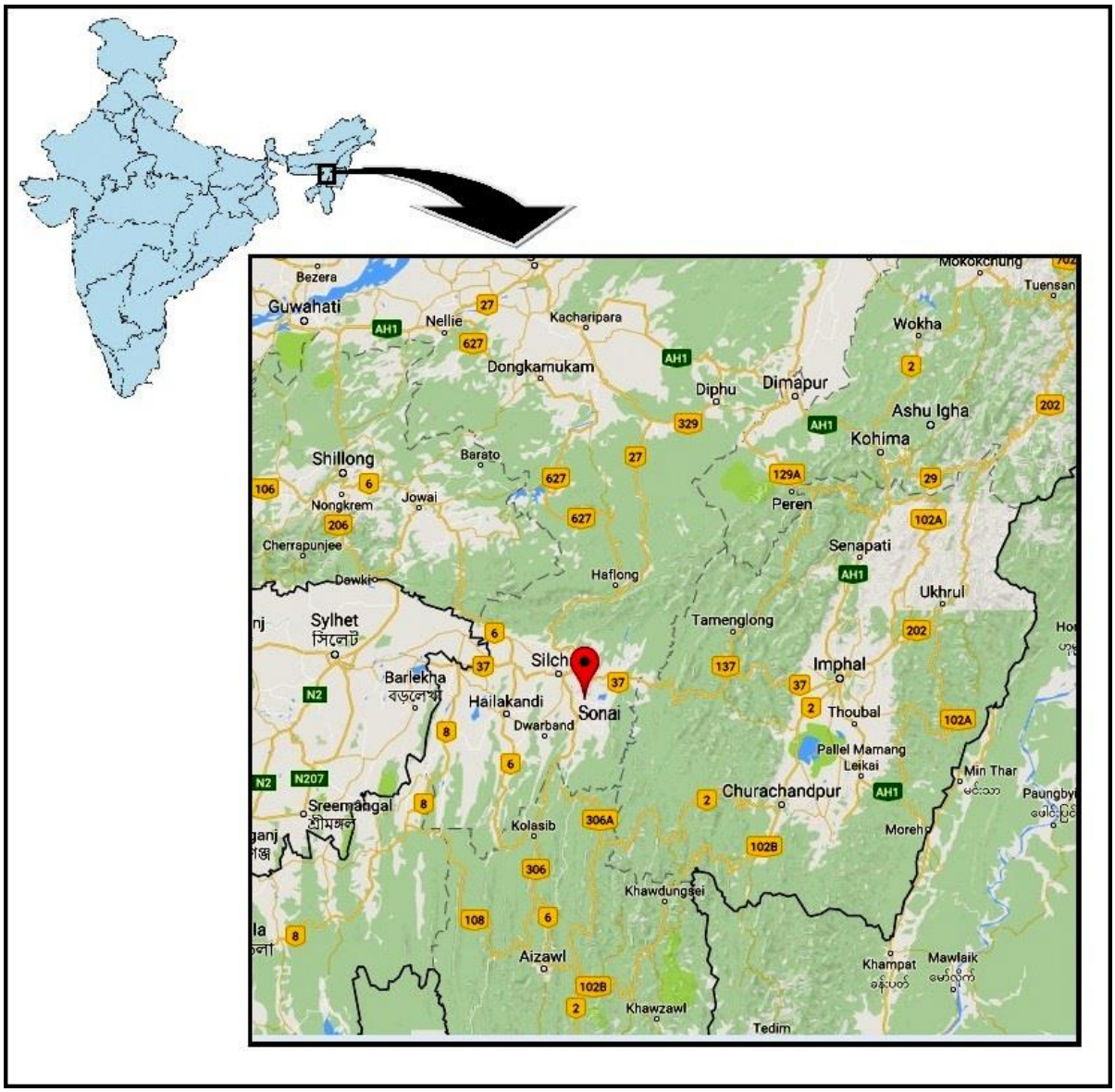

Fig. 2 Geographical location of the sample collection site

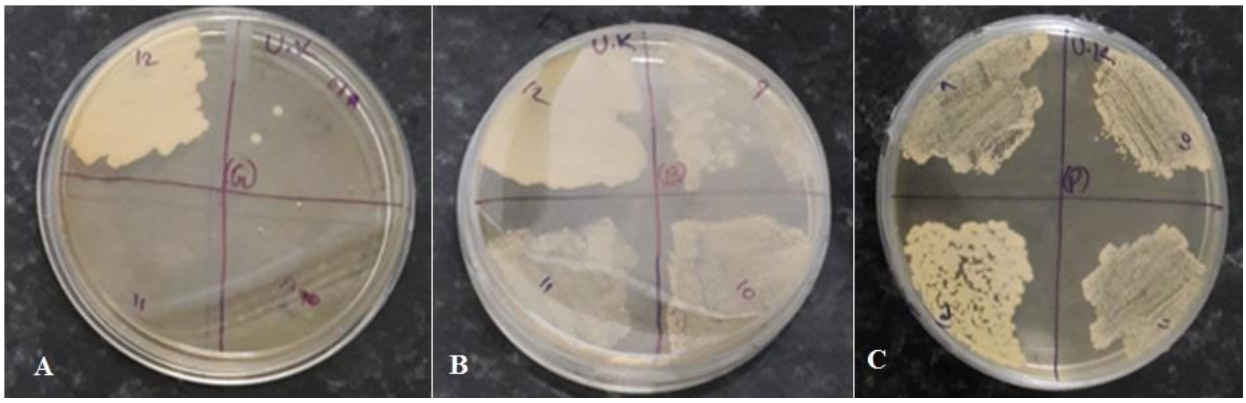

Fig. 3 Results of the tolerance to (A) Gastric acid (B) Bile salt (C) Phenol.
UK12 shows the strongest resistance to all the test.

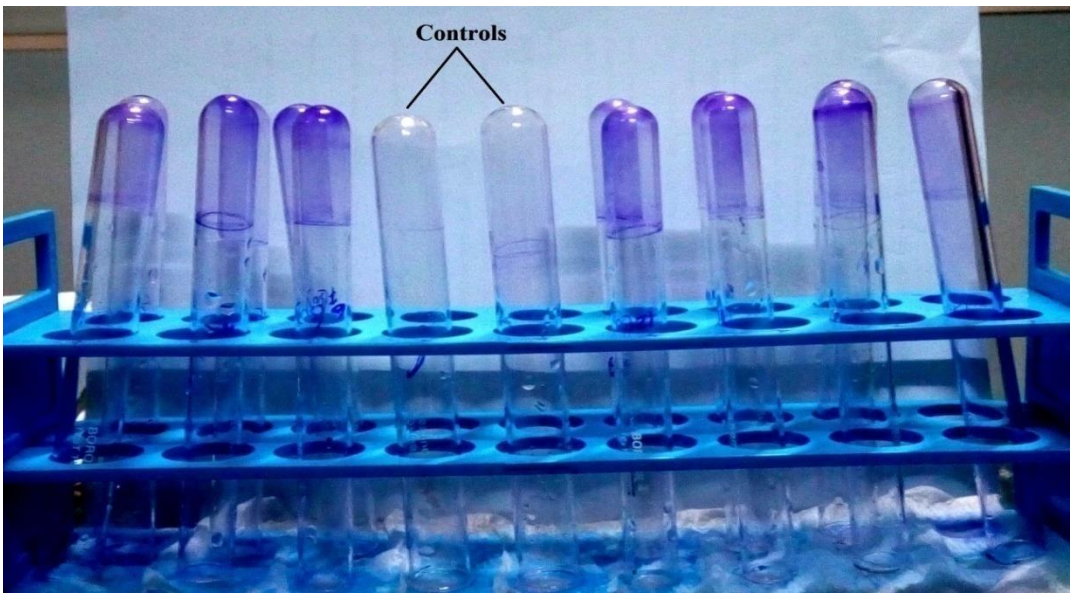

Fig. 4 Biofilm formation on the wall of the test tubes. The colorless tubes served as control.

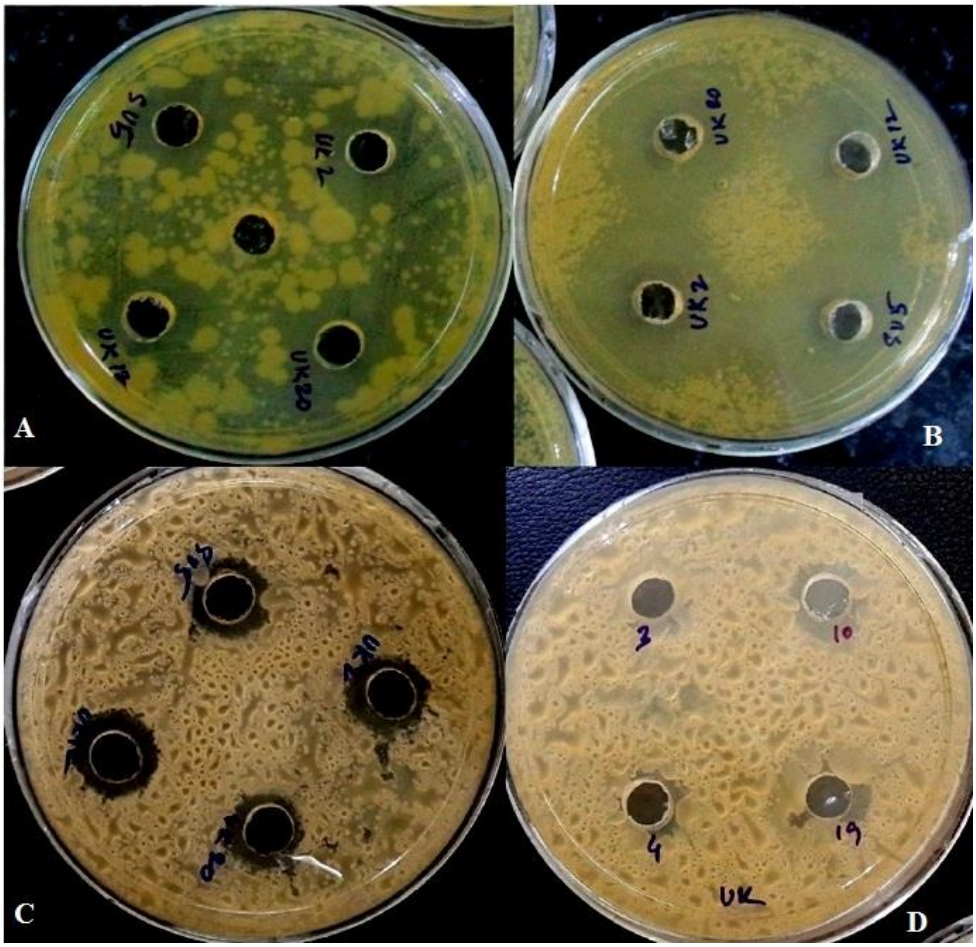

Fig. 5 Antimicrobial Activity against (A) P.A (ATCC 27853) (B) E.C (ATCC 27859) (C) S.A (ATCC 25923) (D) Controls.

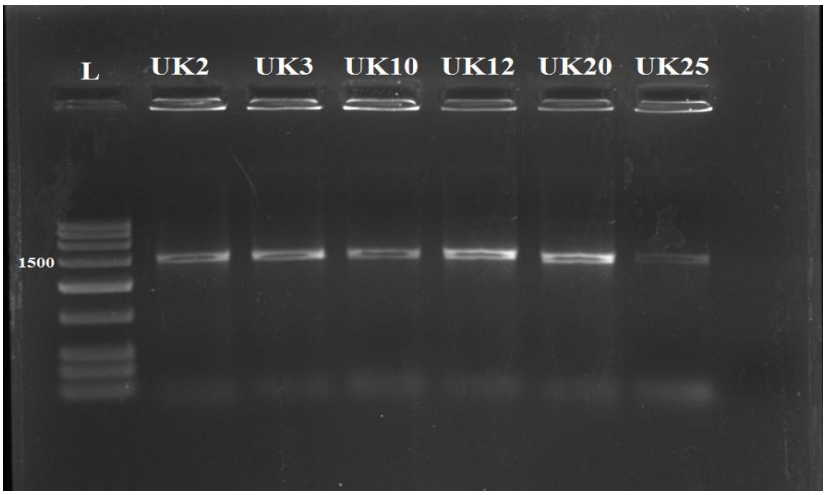

Fig. 6 Results of amplified PCR product for 16s rRNA gene.

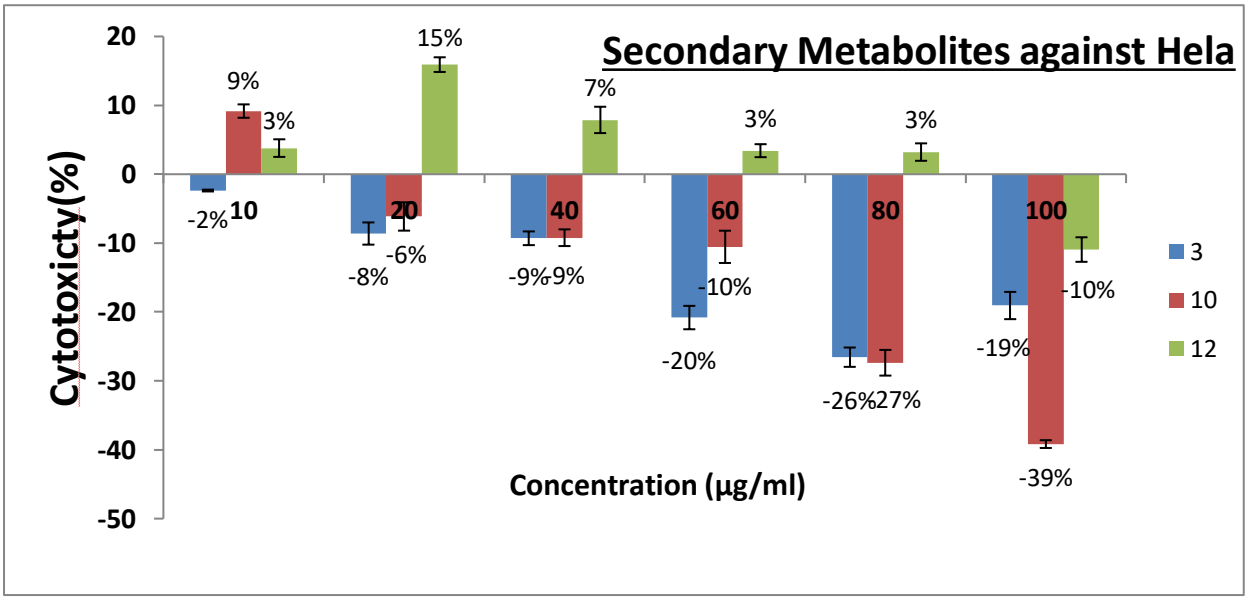

Fig. 7 Cytotoxic activities of secondary metabolites on **HeLa cell lines.**

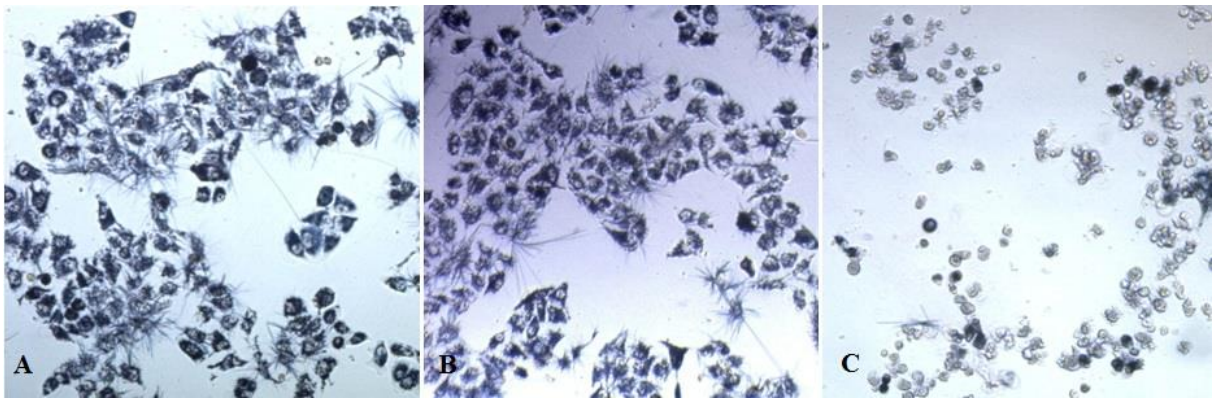

Fig. 8 Cell morphology of HeLa cell line (A) Control (B) Treatment (C) Doxorubicin.

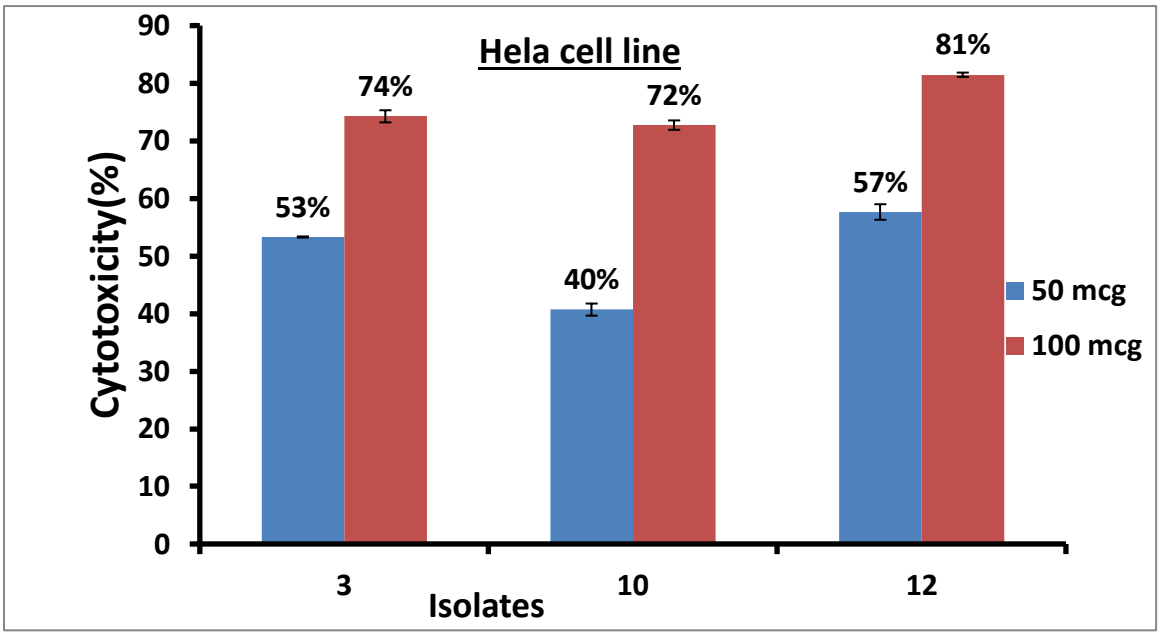

Fig. 9 Cytotoxicity activity of crude protein extract of the isolates on **HeLa cell lines**.

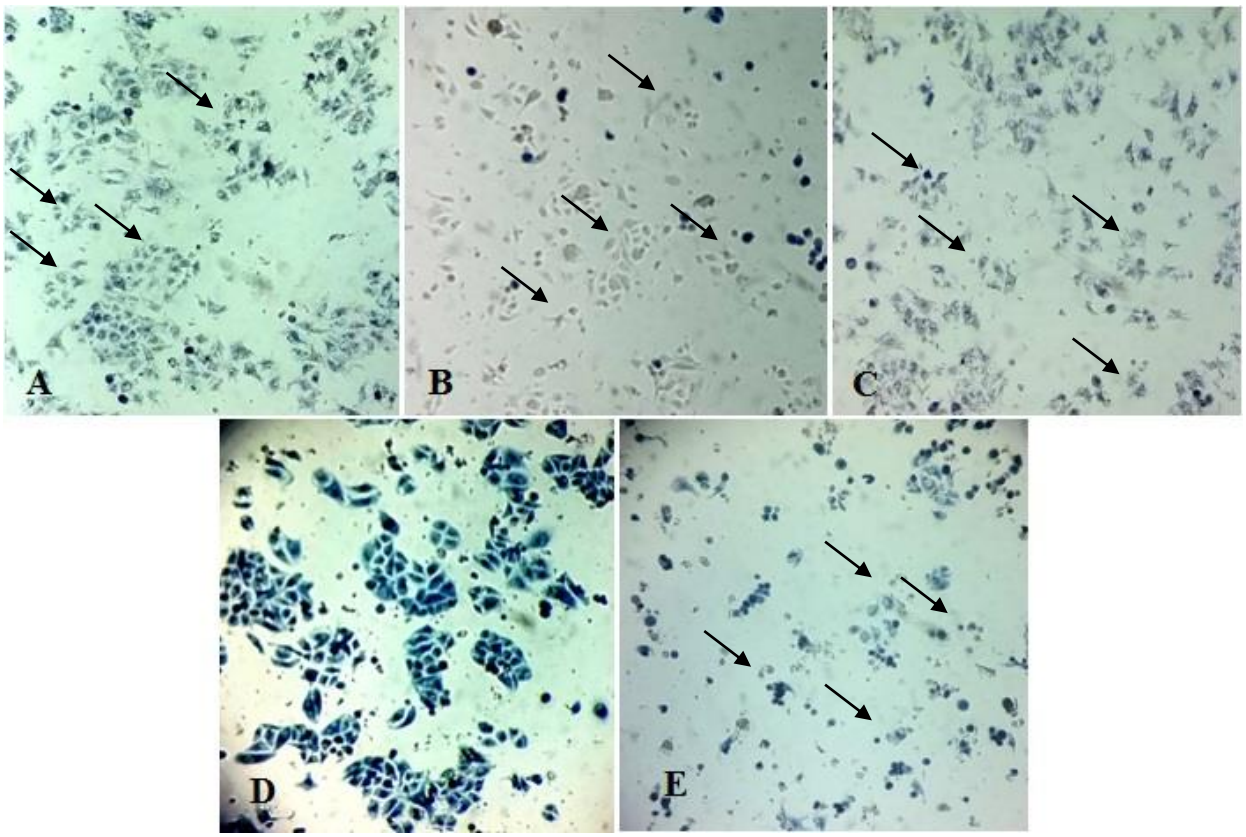

Fig. 10 Changes in cell morphology of **HeLa cell line** (A) UK3 (B) UK10 (C) UK12 (D) Control (E) Doxrubicin. Arrows indicate the dead cells.

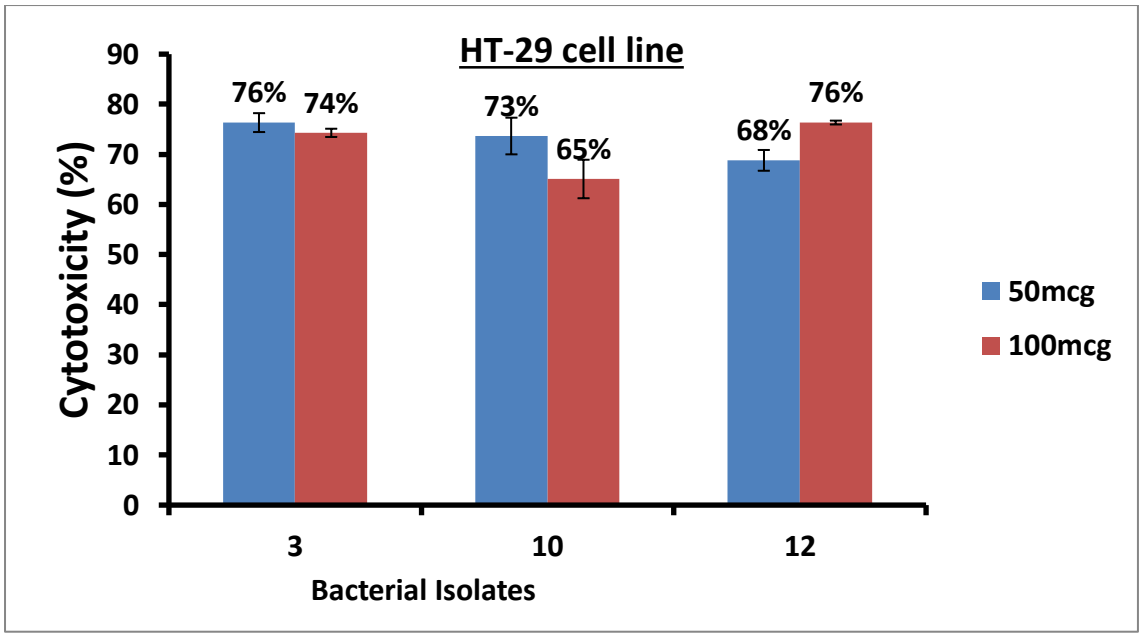

Fig. 11 Cytotoxic activity of crude protein extract of the isolates on **HT-29 cell lines.**

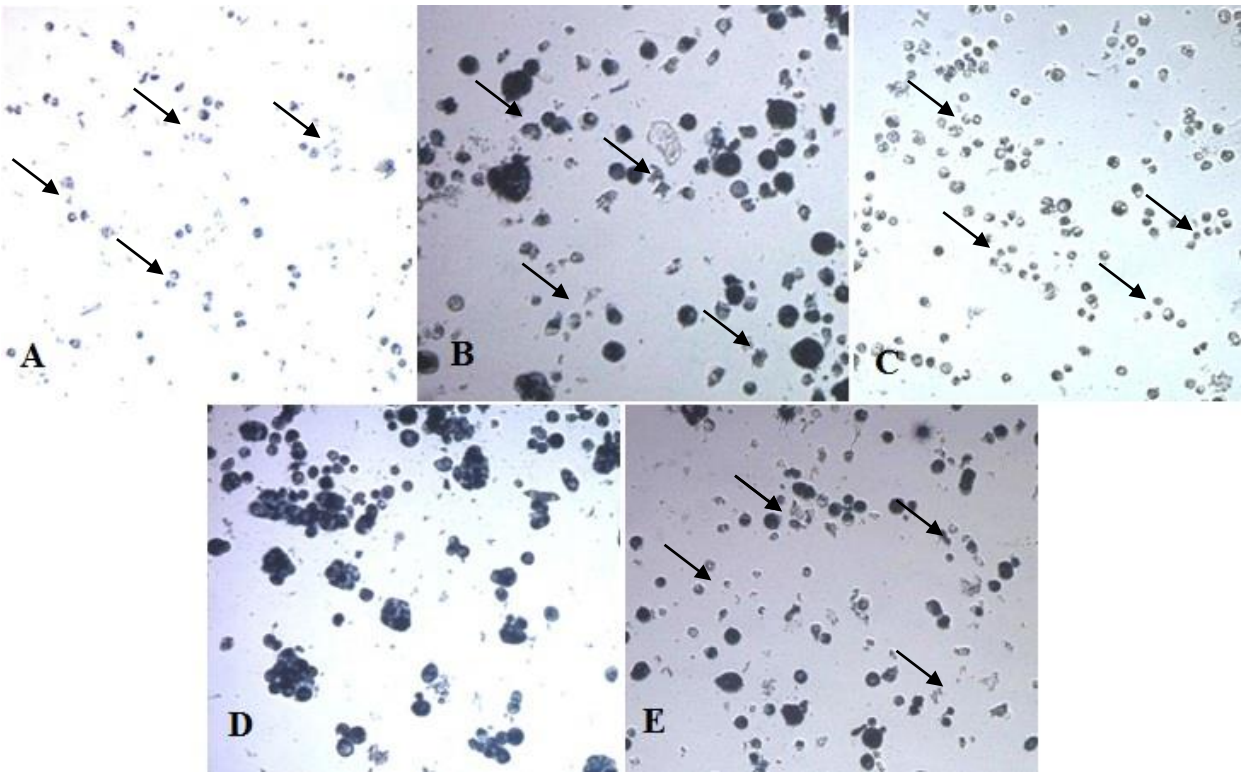

Fig. 12 Changes in cell morphology of **HT-29 cell line** (A) Control (B) Doxorubicin (C) UK3 (D) UK10 (E) UK12. Arrows indicate the dead cells.

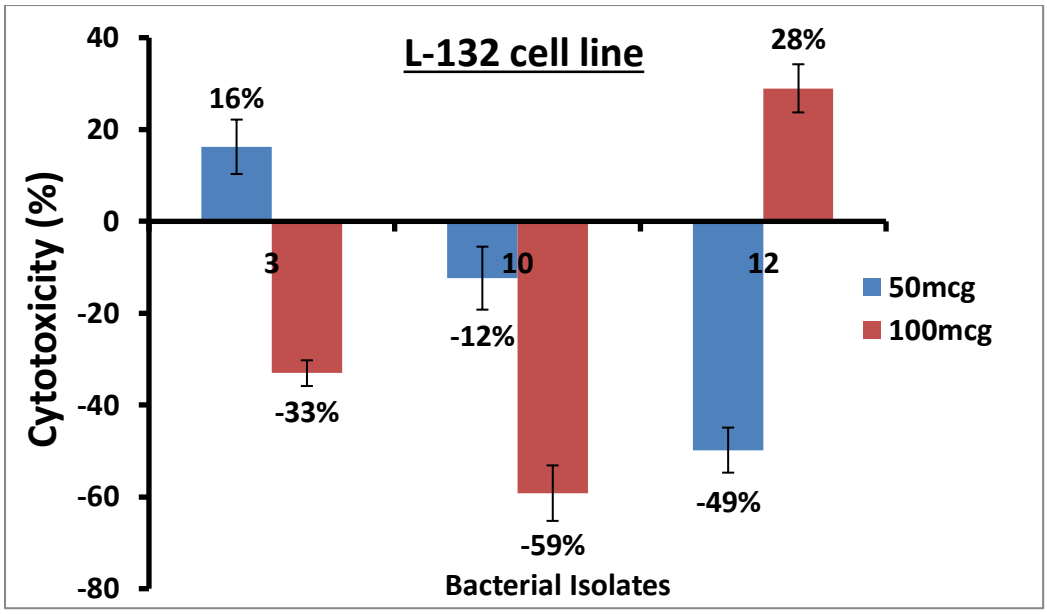

Fig. 13 Cytotoxic activity of crude protein extract of the isolates on **L-132 normal cell lines**.

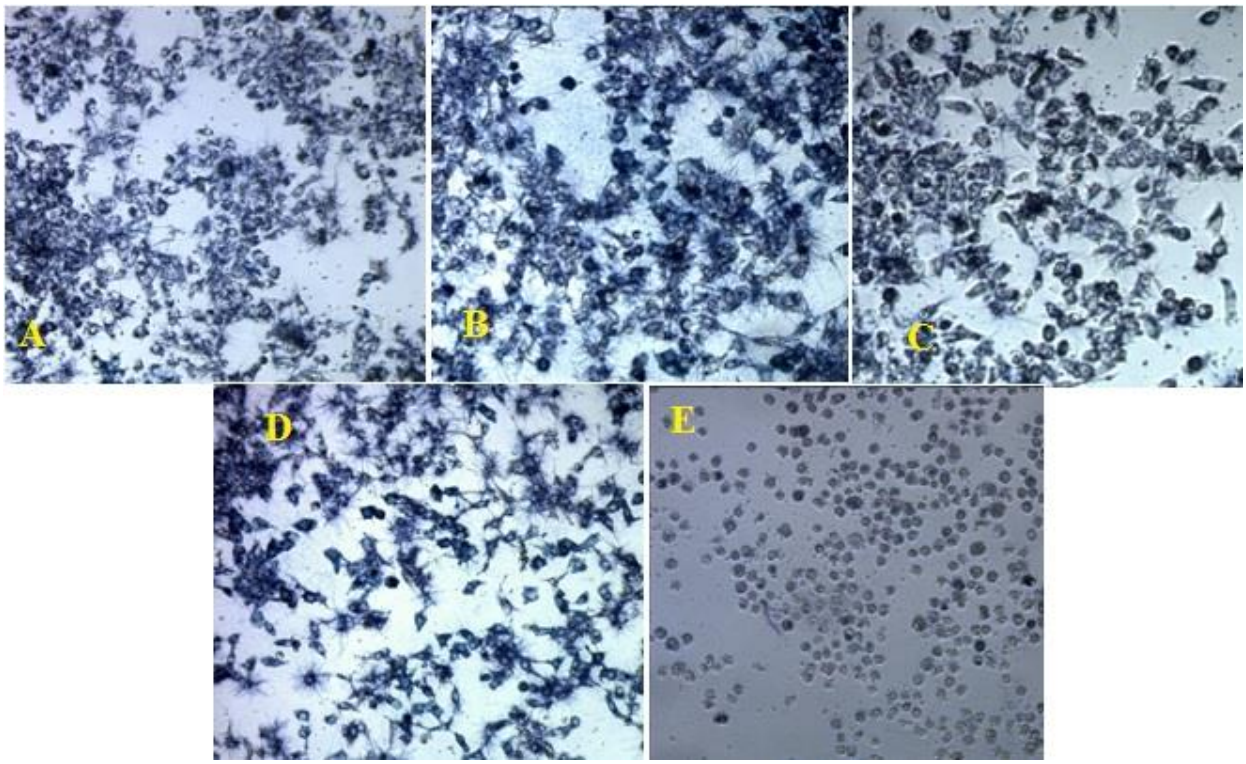

Fig. 14 Cell morphology of **L-132 normal cell lines** (A) UK3 (B) UK10 (C) UK12 (D) Control (E) Doxorubicin.

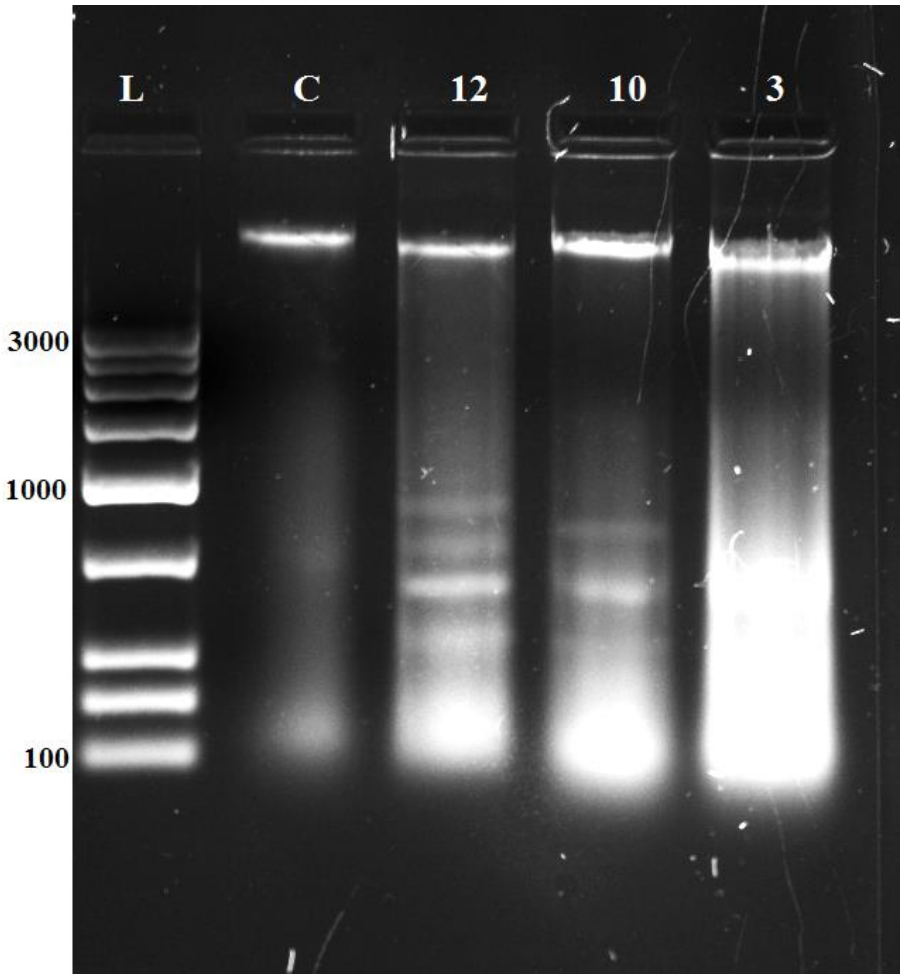

Fig. 15 DNA fragmentation analysis of HT-29 cell line in 1 % agarose gel.

Table 1 Tolerance activity against Bile salt, Gastric acid and Phenol

Bacterial isolates from *Utonga-kupsu*	Bile salt tolerance	Gastric Acid tolerance	Phenol tolerance
UK1	+	-	+
UK2	+	+	+
UK3	+	+	+
UK4	+	+	+
UK5	+	-	+
UK6	+	-	+
UK7	+	-	+
UK8	+	-	+
UK9	+	-	+
UK10	+	+	+
UK11	+	-	+
UK12	++	++	++
UK13	+	-	+
UK14	+	-	+
UK15	+	-	+
UK16	+	-	+
UK17	+	-	+
UK18	+	-	+
UK19	+	+	+
UK20	+	+	+
UK21	+	-	+
UK22	+	-	+
UK23	+	-	+
UK24	+	-	+
UK25	+	+	+
UK26	+	+	+
UK27	+	+	+
UK28	+	-	+
UK29	+	+	+
UK30	+	+	+
UK31	+	-	+
UK32	+	+	+
UK33	+	-	+
UK34	+	-	+
UK35	+	-	+
UK36	+	-	+
UK37	+	-	+
UK38	+	-	+
UK39	+	-	+
UK40	+	-	+

(+) indicates the survival of the isolates to Bile salt, Gastric acid and Phenol
(-) indicates the dead of the isolates to Bile salt, Gastric acid and Phenol
(++) indicates the strongest resistance of the isolates to Bile salt, Gastric acid and Phenol

Table 2 Antimicrobial activity test against the bacterial pathogens.

Samples	S.A (mm)	P.A (mm)	E.C (mm)
UK2	**14**	**17**	**32**
UK3	No zone	No zone	24
UK4	No zone	16	30
UK10	**13**	**18**	**31**
UK12	**15**	**17**	**27**
UK19	No zone	19	31
UK20	**13**	**17**	**28**
UK25	**14**	**15**	**35**
UK26	No zone	17	30
UK27	No zone	20	34
UK29	No zone	15	29
UK30	No zone	18	31
UK32	No zone	No zone	32

Staphylococcus aureus (S.A) (ATCC 25923), *Pseudomonas aeruginosa* (P.A) (ATCC 27853) and *Escherichia Coli* (E.C) (ATCC 27859)

Table Result of Co-aggregation assay (%).

Samples	S.A (%)	P.A (%)	E.C (%)
UK2	**17.7**	**13.5**	**21.8**
UK3	11.1	9	21.5
UK10	**12.7**	**38.5**	**15.3**
UK20	19.5	14.4	13.2
UK25	15.8	12.2	14.8
UK12	**24.6**	**18.4**	**12.7**

UK2, UK10 and UK12 possessed highest Co-aggregation activity

Table 4 Biochemical characterization of six Bacterial isolates.

Samples	Morphology	Gram staining	catalase	Oxidase
UK2	Cocci	+	+	-
UK3		+	+	-
UK10		+	+	-
UK12		+	+	-
UK20		+	+	-
UK25		+	+	-

Table 5 Nearest representative of the bacterial isolates based on NCBI Blast result.

Isolates	Nearest representative	Identity (%)	Accession no.
UK2 (MF581274)	*Staphylococcus condimenti*	99	CP015114.1
UK3 (MF581277)	*Staphylococcus sp.*	99	KU644345.1
UK10 (MF581275)	*Staphylococcus carnosus*	99	CP016760.1
UK12 (MF581273)	*Staphylococcus piscifermentans*	99	NR_116436.1
UK20 (MF581276)	*Staphylococcus sp.*	99	JX442510.1
UK25 (MF581278)	*Staphylococcus sp.*	99	JX442510.1

Table 6 Total fat and fatty acids content and estimation of soluble proteins.

Fat estimation	Fat (%)	Protein estimation	Result
Saturated Fatty Acid	6.31	Standard BSA (4 µl) generated 5.37µg. O.D of 20µl sample is 4.16	1g of fermented fish sample content **416mg of proteins**
Mono Unsaturated Fatty Acid	5.11		
Poly Unsaturated Fatty acid	2.34		
Total fat	13.75		

5. DISCUSSION

Within North-east India, fermented foods have been consumed widely as daily human diet, thereby introducing probiotic bacteria into the digestive system which helps in stabilizing the gut microbiota (Majumdar et al., 2016). Recently, several studies have shown that foods supplemented with LAB improved several health benefits such as strengthen immune system, improved the function of gastro intestine and reduction of serum cholesterol (Kim et al., 2002; Lee et al., 2004). The Present study concerned with the identification of selected probiotic bacterial isolates from fermented fish (*Utonga-kupsu*) as well as their antimicrobial activity and co-aggregation assay. In any fermented food, presents of LAB are dominant which has the ability to tolerant to hazard condition such as gastric juice, bile salt and phenol. Such bacterial strains have the capacity producing the valuable compound which inhibits the growth of the unwanted microbes in the gut environment (Majumdar and Basu, 2010).

In the present study, all the six LAB isolates were classified under the genus *Staphylococcus* which is a Gram-positive, coagulase-negative and consisting of single, paired, and clustered cocci. It has been identified previously in traditionally fermenting fish and soy sauce in Asia (Tanasupawat et al., 1991). *S. carnosus* and *S. piscifermentans* in food exert desirable components which enhance fermentation flora whereas other species such as ***S. aureus*** are known for food poisoning organisms or potential pathogens (Resch et al., 2008). Strains under this species showed positive result for catalase, urease, arginine dihydrolase, nitrate reductase, beta-galactosidase, and phosphatase activity.

To understand the effectiveness of probiotic properties of the bacterial strains, the isolates must be able to survive in gastric acidic, Bile salt and phenol environments. In this study, all the identified six strains which were able to tolerate in stimulated gastric juice (pH 2). Strain no. UK12 and UK3 possessed strong resistance against stimulated gastric juice. Therefore these strains have the ability to proliferate and colonize in the acidic environment of the stomach (Jena et al., 2013). Bile plays a fundamental role in the gut environment; it acts as a bio-surfactants that helps in the digestion and nutrients absorption as well as control the transport of soluble lipid. Bile salts concentration determined the primary strength of its inhibitory effects (Succi et al., 2005). In the present study, all the isolated strains were able to tolerate bile salt. Phenols were released in the gut environment by deamination of several aromatic amino acids derived from dietary or endogenously produced proteins (Gilliland and Walker, 1990). Among

all the six strains, UK12 and UK3 have the strongest resistance to phenol. The ability to sustain in stimulated gastric juice, good bile tolerance and phenol tolerance of all the six strains suggests that these isolates have the capacity to survive in the human gastrointestinal tract (GIT) and can likely survive the passage through the stomach (small and large intestine) and provide health benefits.

Biofilm formation is an important factor for colonizing themselves in the large intestine and can directly involve in the breakdown of complex insoluble polymeric substances. This also allows an added advantage for competing for nutrients with non-adherent organisms (Gómez et al., 2016). All the six strains have the ability to form biofilm which indicate that they can adhered to the mucosal layer for the stomach as well as the intestinal tract and prevent the colonization of unwanted bacteria. Antimicrobial activity is also another important property of probiotic. The supernatant of the ideal probiotic bacterial strains must have hindered the growth of pathogenic microorganisms (Nami et al., 2014). The isolates UK2, UK10, UK12, UK20 and UK25 also possessed antimicrobial properties against the three human pathogens S.A (ATCC 25923), P.A (ATCC 27853) and E.C (ATCC 27859). This indicates that those strains can play an important role in maintaining the immune system and improve the gut environment.

The probiotic bacteria able to co-aggregation with other bacteria which are genetically different indicate the ability to interact closely with other bacteria (Malik et al., 2003). In the present study, UK2, UK10 and UK12 possessed high percentage of co-aggregation with the tested pathogens S.A (ATCC 25923), P.A (ATCC 27853) and E.C (ATCC 27859). Those bacteria having the ability to co-aggregate also have the mechanism to form biofilm and adhere to the mucosal layer of the host and thus inhabit the functions of the pathogenic bacteria. Co-aggregation mechanism of LAB evident the involvements of proteins and lipoproteins expressed on the cell surface (Reniero et al., 1991). The ability of UK2, UK10 and UK12 to co-aggregate with pathogens shows that the probiotic bacteria may enable them to form a barrier that prevents colonization by pathogenic bacteria (Collado et al., 2007).

The fermented fish product *Utonga-kupsu* (1g) content 416 mg of protein. While fermenting the fish, the degradation of proteins from complex to simpler substances helps it to storage at room temperature (Boruah et al., 2017). Microorganisms and enzymes produced by them also play an important role in breakdown of proteins. Such microorganism could serves as an excellent probiotic so that they can degrade the complex protein component and enhance the

digestive system. Saturated fatty acid was found to be dominant in our sample followed by mono unsaturated fatty acid and poly unsaturated fatty acid. It is also reported that *punti* (Tripura fermented fish) also contains high percentage of saturated fatty acids and rich in omega-3 fatty acid, whereas, in case of *phasa shidal* (Tripura fermented fish) low content of saturated and omega-3 fatty acids were observed. Although not much work has been done related to fatty acid profiling on such fermented fish, the possibility of losing PUFA during fermentation cannot be ignored. Therefore, to increase the PUFA modification is needed in fermentation process (Majumdar et al., 2016).

The effect of probiotic bacteria on different cancer cell lines has been demonstrated. The inhibition of mammalian cell proliferation by probiotic bacteria takes places when the cell reached to primary leukocyte state. It has been reported that the induction of apoptosis to cancer cell lines Caco-2 and HT-29 cells is facilitated by various probiotic strains (Ouwehand, 2007). In the present study, anticanceral activity of the potential isolates were tested against two cancer cell lines such as HeLa (cervical cancer), HT-29 (colon cancer), and one normal cell line L-132 (Normal lung cell). The results show that the metabolites secreted by UK3, UK10 and UK12 do not possess any significant inhibition on HeLa cell line. On the other hand, the crude protein extracted from the three isolates possessed significant inhibition on the growth of the two cancer cell lines but did not show any cytotoxic affect on L-132 normal lung cells. Therefore, these strains belong to LAB and identified as *Staphylococcus sp.*, S. *carnosus and* S. *piscifermentans* were non-pathogenic to human. Furthermore, the strains possessed significant cytotoxicity against the tested cancer cell lines but no cytotoxic activity against L-132 normal lung cell.

DNA fragmentation for the HT-29 (colon cancer) by LAB strains was also demonstrated (Koller et al., 2008). Similarly DNA fragmentation of the HT-29 was observed when the crude protein extract of the three isolates UK3, UK10, and UK12 were treated. This indicates that the bacteria release particular protein component that specifically target the cancer cell and induce the apoptosis.

6. SUMMARY AND CONCLUSION

- The Present study was conducted with an aim to isolate, identify and characterize the LAB strains present in these fermented food and to test their tolerance to phenol, gastric juice and bile salt, antimicrobial activity, co-aggregation assay and anticancer activities.
- Bacterial pure cultures were isolated in acidic condition (pH 2.5) using serially diluted method. The number of bacterial count in MRS agar was 100 cfu/g. A total of 40 bacterial strains from *Utonga-kupsu* were isolated.
- Six strains showing good activity on probiotic assays, antimicrobial, co-aggregation were further identified by 16s rRNA gene sequencing.
- The six strains were identified as *Staphylococcus piscifermentans*, *S. condiment*, *S. carnosus* and three unknown *Staphylococcus* species and assigned under phylum Firmicutes.
- The effect of secondary metabolites for three isolates UK3, UK10 and UK12 on cancer cell line (HeLa) does not show any cytotoxic activity rather it enhanced the cell growth.
- But the crude protein extracted show good cytotoxic activity on cancer cell lines (HeLa and HT-29) and less or no cytotoxic activity against L-132, respectively.
- UK12 and UK3, identified as *S. piscifermentans* and *Staphylococcus* spp. possessed highest tolerance to gastric juice, bile salts and phenol. Five isolates (UK2, UK10, UK12, UK20, UK25) possessed good antimicrobial activity to all the tested pathogens.
- The crude protein extracted of the three isolates UK3, UK10 and UK12 shows good cytotoxicity against HeLa and HT-29 which can be further exploited for anticancer drugs.

This is the first scientific investigation of the fermented food *Utonga-kupsu* from North-East India. Analysis revealed the presence of important LAB species belongs to *Staphylococcus* in the fermented fish. And also shows good probiotic activity as well as anticancer against HeLa and HT-29 but show less or no anticancer activity against L-132. They can be further utilized in probiotic purpose. UK3, UK10 and UK12 identified as *Staphylococcus sp., Staphylococcus carnosus and Staphylococcus piscifermentans* possessed the highest activity in probiotic assays as well as on cancer cell lines. They can be further used for providing several health benefits such as strengthen immune system and improved gastrointestinal function as well as the probiotic bacteria may be safely used as natural cancer therapeutic agents because none of the strains are human pathogenic.

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List of Publications

1) Screening and Characterization of dominant bacterial isolates from traditional fermented fish of Manipur, North-East India. Accepted (Journal of Food Science and Technology).
2) Probiotic potential and anticancer properties of selected bacterial strains from traditional fermented fish. Communicated (Letters in Applied Microbology).

Conferences/ Seminars and Workshops Attended

- **57th Annual Conference of Association of Microbiologists of India** and **International Symposium** on "Microbes and Biosphere: What's New What's Next", November 24-27, 2016. Dept. of Botany Gauhati University (Poster Presentation).

- **National Level Workshop** on Biostatistics and Bioinformatics, September 01-07, 2016. Dept. of Biotechnology Mizoram University.

- **International Workshop** on "Cancer Epidemiology", November 29-30, 2016. Dept. of Biotechnology Mizoram University.

- **National Level Workshop** "Hands on Training on DNA Barcoding and Phylogenetics" March 20-25, 2017. Dept. of Biotechnology Mizoram University.

- One day **National Workshop** on *Science Communication* (*SciComm 101*), 6th June 2017. The Wellcome Trust/DBT India Alliance, Mizoram University.

- **GIAN International Workshop** on "Molecular Phylogeny and Next-Generation Sequencing and Molecular Entomology, June 19-28, 2017. Dept. of Biotechnology and Zoology, Mizoram University.

ABSTRACT

The process of fermentation is an economical and oldest method, used for food preservation (Tamang et al., 2016). Traditionally fermented foods have been consuming widely since it contains several beneficial activities. It is often used for probiotic purposes by introducing beneficial bacteria into the digestive system and helps in balancing of bacteria of the digestive system; for better absorption of the nutrients in the foods by maintaining the proper balance of gut bacteria and enough digestive enzymes and food preservation by facilitating the growth of desired microorganism.

Each fermented food has a unique group of the microbiome and the role of microbial activity and medicinal values of the fermented food products have been documented (Chettri and Tamang, 2015; Sarojnalini and Singh, 2009). The traditional secret behind the fermentation methodologies was generally confined within the knowledge of the local communities and was handed down from generation to generation. Generally, the desirable and organoleptically pleasing characteristics were associated with the specific handling and incubation duration of raw ingredients in a precise way (Caplice et al., 1999).

Utonga-kupsu is commonly prepared by the Manipuri (Meetei) People settling in Assam, Cachar Northeast India for daily consumption. Similar kind of fermented fish preparation has been practice by the Manipuri people in Manipur since time immoral known as *Hentak*. *Utonga-kupsu* is a fermented fish paste, which is commonly consumed by the Manipuri people located in the Cachar District of Assam North-East State of India. It is prepared by the mixture of small fish (*Esomus danricus*, *Puntius sophore*, *Amblypharyngodon mola*, *Channa punctata*, *Mystus vittatus* etc.) with *Alocasiamacrorhiza* (*Hongu*) as well as a little amount of mustard oil and kept in an earthen pot for one month.

Since the fermented fish have been contributing a large portion of regular food intake and its popularities have been growing day by day in North-east India, a proper scientific investigation is needed to identify and characterize the microbes involved in the enhancement of the nutritional value of these fermented fish. However, only no or limited amount of data are available concerning the traditional procedure and scientific values of fermentation fish in Northeast India (Thapa et al., 2004).

The Present study was designed with the aim to isolate, identify and characterize the LAB strains present in these fermented food and there tolerance to phenol, gastric juice and bile salt, antimicrobial activity, co-aggregation assay and anticancer activity.

Isolation of LAB was carried out according to Jena et al., 2013. Characterization of the potential isolates was carried out by evaluating their tolerance against phenol, gastric juice and bile salt. Further, probiotic potential was tested by performing antimicrobial activity, co-aggregation assay. Finally, cytotoxicity of the isolates were evaluated by performing MTT assay and DNA fragmentation assay.

Bacterial pure cultures were isolated in acidic condition (pH 2.5) using serially diluted method. The number of bacterial count in MRS agar was 100 cfu/g respectively. A total of 40 bacterial strains from *Utonga-kupsu* were isolated randomly. Six strains showing good activity on probiotic assays, antimicrobial, co-aggregation were further identified by 16s rRNA gene sequencing. The six strains were identified as *Staphylococcus pisciferm*entans, *S. condiment*, *S. carnosus* and three unknown *Staphylococcus* species and assigned under phylum Firmicutes. The effect of secondary metabolites for three isolates UK3, UK10 and UK12 on cancer cell line (HeLa) does not show any cytotoxic activity rather it enhanced the cell growth. But the crude protein extracted show good cytotoxic activity on cancer cell lines (HeLa and HT-29) and less or no cytotoxic activity against L-132 respectively. UK12 and UK3, identified as *S. pisciferm*entans and *Staphylococcus* spp. possessed highest tolerance to gastric juice, bile salts and phenol. Five isolates (UK2, UK10, UK12, UK20, UK25) possessed good antimicrobial activity to all the tested pathogens. The crude protein extracted of the three isolates UK3, UK10 and UK12 shows good cytotoxicity against HeLa and HT-29 which can be further exploited for anticancer drugs.

This is the first time scientific investigation of the fermented food *Utonga-kupsu* from North-East India. Analysis revealed the presence of important LAB species belongs to *Staphylococcus* in the fermented fish. And also shows good probiotic activity as well as anticancer against HeLa and HT-29 but show less or no anticancer activity against L-132. They can be further utilized in probiotic purpose. UK3, UK10 and UK12 identified as *Staphylococcus sp., Staphylococcus carnosus and Staphylococcus pisciferm*entans possessed the highest activity in probiotic assays as well as on cancer cell lines. They can be further used for providing several health benefits such as strengthen immune system and improved gastrointestinal function as well

as the probiotic bacteria may be safely used as natural cancer therapeutic agents because none of the strains are human pathogenic. Presents of such type of microorganism might responsible for the possible potential source of probiotics in this fermented food and thus can be further exploited for the discovery anticancer drugs.

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