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Cytotoxic effects and Identification of bioactive metabolites from crude extract of potential probiotic bacterium, *Bacillus amyloliquefacians* CS4, isolated from the gut of freshwater fish *Channa striata* (Bloch, 1793)

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Abstract— In the present study, the crude secondary metabolites were extracted from the potential probiotic bacterium, *Bacillus amyloliquefacians* CS4 using solvent ethyl acetate and the cytotoxicity of bacterial extract was assessed against HeLa cell line through MTT assay. The crude extract exhibited cytotoxicity effect on HeLa cell lines with the effective half maximal inhibitory concentration (IC_{50}) value of 121.65µg/ml. The brine shrimp toxicity assay of crude extract revealed half maximal lethal concentration (LC_{50}) value at 93.377µg/ml against *Artemia salina*. The zebra fish (*Danio rerio*) embryos were treated with different concentrations of extract upto 72 hpf and was found lowest survivability (36.6 %) at 200µg/ml concentration. The bioactive compounds and their functional groups of crude extract were identified using GC-MS and FTIR analyses. The secondary metabolites produced by *B. amyloliquefacians* CS4 have caused potential cytotoxic effects on cancer cell line and these compounds could be possibly used for the development of therapeutic agents, in relation to cancer drug discovery.

Keywords— *Anticancer activity, Bacillus amyloliquefacians, Secondary metabolites, Brine shrimp assay, GC-MS*

I. INTRODUCTION

The investigations for less toxic and more effective metabolites from non-infective organisms are most required at present to overcome the resistance exhibited against the existing antibiotics. The bioactive compounds having anticancer activity are extracted from different microorganisms, terrestrial plants and marine life forms [1]. During the past, many important bioactive compounds with high therapeutic value have been reported from various bacteria [2].

The fish gastro intestinal tract (GI) is populated with complex microbial community and it plays a vital role in promoting the health of the host through the production of secondary metabolites. Probiotic bacteria are known to produce bioactive substances that protect them against predators [3]. These microbial bioactive substances exhibit antibacterial, antiviral, anti-tumour and cardio protective properties. Currently, cytotoxicity testing of several bioactive compounds in cell lines are being carried out by MTT assay, which determines cell viability based on mitochondrial function by measuring the activity of mitochondrial enzymes [4]. The bioactive compounds may cause adverse effects on the organisms upon treatment (e.g., humans, animals, or plants) or, in the case of aquaculture which could negatively affect the other organisms involved in fish or shellfish culture such as algae or live prey (e.g., rotifers and *Artemia* sp.). Hence, the toxicity of bioactive compounds on the target organisms should be tested and any adverse effect be ruled out before they can be applied. A complete toxicology assessment is a significant task and therefore a pre-assessment determine the effect of the bioactive products in many model eukaryotic systems such as *Caenorhabditis elegans* and *Artemia* sp. [5]. Since, the extracts from microbes could be a source of many striking molecules that could form leads towards drug discovery [6]. Some *Bacillus* spp. can produce several types of active compounds, for example the *B. amyloliquefaciens* possess 8.5 % of the genome dedicated for the synthesis of secondary metabolites [7, 8].

In recent years, the roles of secondary metabolites in biological control, and the use of strains to produce metabolites as probiotics, have become an area of considerable research activity as they provide low technological and environmentally sustainable approaches for the inhibition of pathogens and improvements in the nutritional value of animal feeds [9]. Present investigation focusses on the therapeutic evaluation and identification of bioactive metabolites from *B.amyloliquefacians* CS4 isolated from gastro-intestinal tract of *Channa striata.*

II. MATERIALS METHODS

Strain isolation and culture condition

The potential probiotic strain, *B. amyloliquefacians* CS4 was previously isolated from the gut of snake head fish (*Channa striata*), and identified by 16S rRNA sequencing which was submitted on gene bank database (Genbank accession no. MK326902.1). Stock culture was stored in sterile LB broth containing 20% (v/v) glycerol at 70^{o}C and subculture was taken from the stock and then used for bioactive metabolites production.

Organic solvent extraction

An overnight broth culture of *B. amyloliquefacians* CS4 was inoculated into 250 ml Luria Bertani (LB) broth (25g/L) and incubated at 37^{o}C with shaking incubator for 2 to 3 days, the cells were removed by centrifugation at 10,000 rpm for 15 min at 4^{o}C. The supernatant was subjected to solvent extraction sequentially with equal amounts of solvent ethyl acetate using separating funnel. The obtained supernatant was extracted exhaustively with ethyl acetate and after evaporation of the solvent at 44°C under vacuum the concentrated residue was collected and used for toxicity analysis.

MTT Cytotoxicity Assay

Anticancer property of crude extract was carried out by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method against HeLa cell lines. The cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) was supplemented with 10% fetal bovine serum (FBS) and 100µg/ml streptomycin. The cell suspension 100µl was seeded into 96-well plates at a plating density of 10,000 cells/well and incubated to allow for cell attachment at 37^{o}C, 5% CO_2 95% air and 100% relative humidity. After 24 h, 100µl of the medium containing the crude extract at various concentrations (0, 12.5, 25, 50, 100 and 200µg/ml) was added and incubated at for 48 h. Triplicate was maintained, and the medium containing without extracts were served as control. After 48 h of incubation, 15µl of MTT (5 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^{o}C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using micro plate reader.

Brine shrimp toxicity assay

Cytotoxicity potential of the crude metabolites was tested against brine shrimp (*A. salina*) larvae according to brine shrimp lethality test (BSLT) method 24 well plates. The lethality bioassay was performed using 25 nauplii for each sample of the crude extract, which was tested with varied concentrations viz; 0, 12.5, 25, 50, 100 and 200µg/ml in artificial seawater and the assay was carried out in triplicate. The vials were maintained at room temperature for 24 h under the light and mortality of larvae was calculated, and their lethal concentration at 50% (LC_{50}) was estimated using probit analysis.

Embryo toxicity assay

Zebrafish embryo toxicity test (ZFET) for crude extract was performed according to the Organisation for Economic Co-operation and Development (OECD) guidelines. The embryos were exposed to different concentrations and the control was maintained without the treatment of extracts in the 24 well plates. The ten zebra fish (*D. rerio*) embryos were used per concentration of sample and also the control, where each test sample was performed in triplicates. The test wells treated with samples were incubated at 27 ± 1 °C. Toxic and teratogenic effects were examined using a light microscope at every 3, 6, 12, 24, 48 and 72 h post-treatment, respectively. The treated embryos were observed for the survivability (%) and also their mortality and malformations were recorded [10].

Gas chromatography-mass spectrometry analysis

The crude extract of *B. amyloliquefacians* CS4 was undergone for the detection of bioactive molecules by Gas chromatography-mass spectrometry (GC–MS). It was analysed using Perkin Elmer Clarus 500 gas chromatography equipped with an Elite-5 capillary column (5% Phenyl 95% dimethylpolysiloxane) (30m×250µm, 0.25µm film thickness) and mass detector turbomass gold of the company which was functioned in EI mode. The sample (10µl) injected into the column and Helium was the carrier gas at a constant flow rate of 1.0 ml/min. The oven temperature was initially set to 70^{o}C, and 4 min after injection, it was increased to 200^{o}C at a rate of 3^{o}C/min and gradually increased up to 300^{o}C final temperature. The mass spectrometry was operated at electron impact ionization mode at 70eV. Detection was achieved using an MS detector in an electron impact mode and a full-scan monitoring mode (m/z 30-600), with an acquisition rate of 20 spectra/sec. The identification of components was based on comparison of their mass spectra with NIST and WILEY library.

FT-IR spectral analysis

Fourier transform infrared spectroscopy (FTIR) analysis of extract was performed using Perkin-Elmer, Lamda 2000, and the sample was recorded in the mid-IR region (4000-400cm^{-1}) and the associated functional groups were determined.

III. RESULTS

MTT Cytotoxicity Assay

The cytotoxicity of crude extract against HeLa cell line treated with different concentrations (Fig. 1) was graphically represented in figure 2. The percentage of inhibition of HeLa cells after treatment with bacterial extracts were: 9.17% (12.5µg/ml), 22.79% (25µg/ml), 38.60% (50µg/ml), 47.56%

(100µg/ml) and 62.70% (200µg/ml). The crude extract of *B. amyloliqufacians* exhibited significant activity against the HeLa cell line with an IC_{50} (50% growth inhibition) value of 121.65µg/ml (Fig.2).

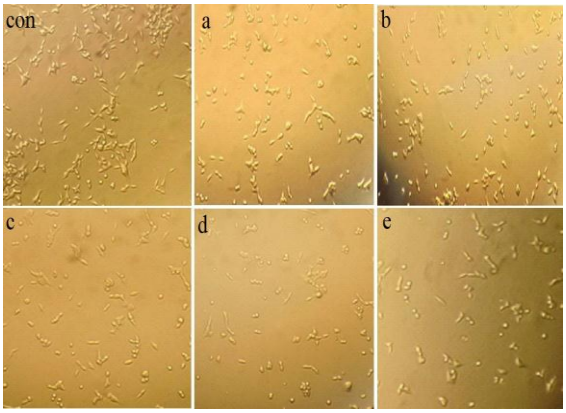

Fig. 1: *B. Amyloliquefaciens* crude extract treated HeLa cell lines at different concentration along with control, con) control, a)12.5µg/ml, b) 25µg/ml, c) 50µg/ml, d)100µg/ml, e) 200µg/ml

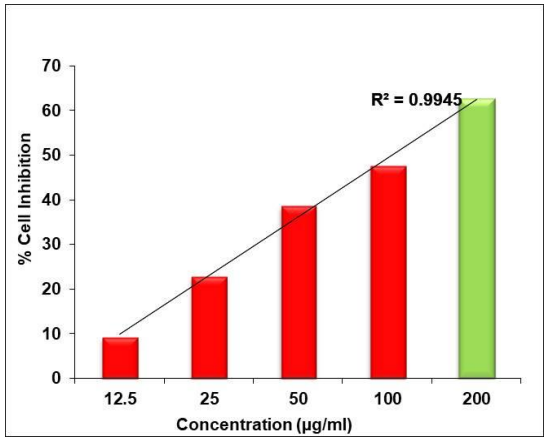

Fig. 2: The cytotoxic Growth Inhibition of *B. Amyloliquefaciens* crude extract on HeLa cell lines (IC_{50} =121.65 µg/mL, R^2 =0.994).

Brine shrimp toxicity assay

The cytotoxic effect of the crude metabolites was evaluated against brine shrimp (*Artemia salina*) larvae to assess its lethality profile and the result are shown in figure 3. The morphological changes in the treated (dead) and untreated (control) Artemia nauplii were observed under Stereomicroscope (Fig. 4). The crude extract showed 0.33, 14.6, 28.6, 49.33 and 77.33% mortality at 12.5, 25, 50, 100 and 200µg/mL concentration, respectively. The LC_{50} value was found to be 93.377µg/ml which was considered moderately toxic and no mortality found in negative control (DMSO) group.

Fig. 3: Effect of crude extract on brine shrimp after 24 h treatment, morphology of control and treated dead larvae of *Artemia salina*.

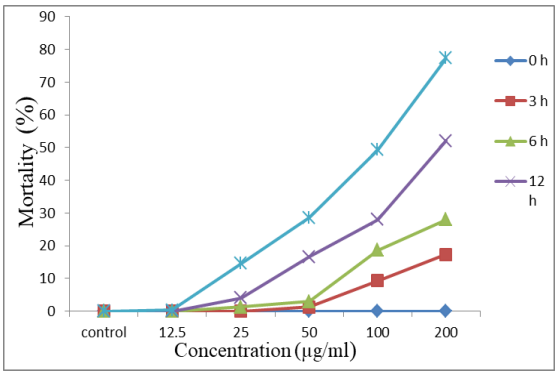

Fig. 4: Graphical representation of toxicity of brine shrimp at different concentration of bacterial extract after 24 h treatment, LC_{50} value= 93.37 µg/ml, LC_{90} value =369.54 µg/ml. Chi-Square ($\chi 2$) = 10.09 at $p > 0.05$ significant level.

Embryo toxicity

The survivability rate of zebra fish (*D. rerio*) embryos after 3, 6, 12, 24, 48, and 72 hpf of exposure in varying concentrations of crude metabolites are shown in figure 5. Survival rate and gross morphological changes were examined upto 72 hpf and the lowest survivability (36.6%) was found at 200µg/ml concentration. However, at 3, 6, 12 and 48 hpf, no mortality was observed in embryos exposed at lower concentrations (12.5, 25 and 50 µg/ml) and shows 100% survivability (Fig. 6). The increased death ratio was observed in embryos exposed at higher concentrations (100 and 200 µg/ml) after further exposure of 72 hours.

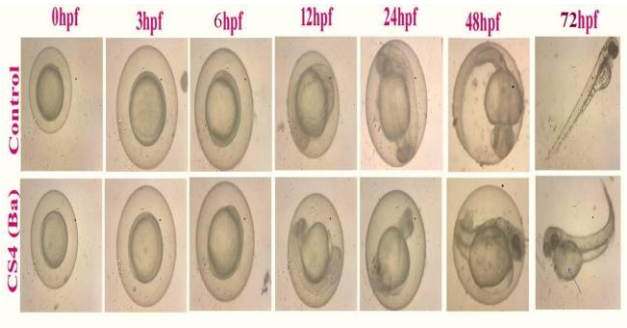

Fig. 5: The morphological changes in the crude extract treated Zebrafish embryos at different developmental stages.

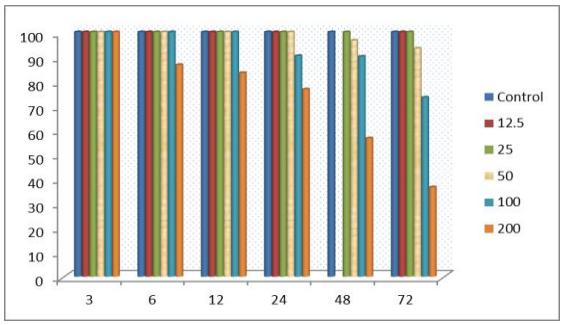

Fig. 6: Graphical representation of survivability (%) of zebrafish embryos treated with different concentrations crude extract up to 72 hpf

Gas chromatography-mass spectrometry analysis

In order to identify the structural features of bioactive compounds present in the ethyl acetate crude extract, the extract was subjected to GC-MS analysis and was identified in relation to their retention time using Standard NIST and WILEY library (Table 1). Totally 17 compounds were identified from ethyl acetate extract of *B. amyloliquefacians*. The major compounds present in the extract are (Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)-(71.32%), Naphthalene, decahydro-2,6-dimethyl (4.14%), 3-Pyrrolidin-2-yl-propionic acid (3.54%), 2-Cyclopentanedione, 3,3,5,5-tetramethyl (2.58%), Phenol, 3,5-dimethoxy- (2.29%), l-Proline-allyloxycarbonyl-, hexyl ester (2.71%), and Dibutyl phthalate (1.51%). The biological applications of the major compounds present in the crude extract were studied for their therapeutic significance.

FT-IR spectral analysis

To recognize the diverse functional groups found in bacterial extract, the extract was characterized under FT-IR. The major peaks were at 3343.54 (Dimer OH stretch; Carboxylic acids), 2946.40 (CH stretch; Alkanes), 2833.41 (Dimer OH stretch; Carboxylic acids), 2042.48 (N=C; misc), 1649.92 (C=C stretch; Alkenes), 1535.31 (NH out of place; amides), 1451.17 (CH2 and CH3; Alkanes), 1402.75 (C-O stretch; Carboxylic acids), 1258.75 (C-F stretch; Alkyl halides), 1113.22 (C-F stretch; Alkyl halides), 1022.16 (C-F stretch; Alkyl halides) and 680.33 cm^{-1} (C-H bend; alkanes) (Fig. 7).

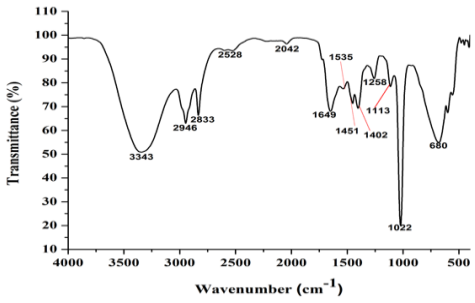

Fig. 7: The FT-IR spectrum of crude extract of *B. amyloliquefaciens*

Table 1: Bioactive compounds present in the crude extract were identified by GC-MS analysis.

Peak	RT	Compound name	Mol. formula	Area (%)
1	8.770	Naphthalene, decahydro-2,6-dimethyl	$C_{20}H_{38}$	0.53
2	8.929	Naphthalene, decahydro-2,3-dimethyl-	$C_{12}H_{22}$	1.07
3	9.223	Naphthalene, decahydro-1,5-dimethyl	$C_{18}H_{34}$	0.85
4	9.424	Naphthalene, decahydro-2,3-dimethyl	$C_{12}H_{22}$	1.17
5	9.625	2(1H)-Naphthalenone, octahydro-4a-methyl-,trans	$C_{13}H_{20}O_3$	0.82
6	9.701	Naphthalene, decahydro-1,5-dimethyl-	$C_{12}H_{22}$	0.52
7	12.268	Caryophyllene	$C_{15}H_{24}$	0.97
8	13.241	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{22}H_{30}O$	0.86
9	15.741	3-Pyrrolidin-2-yl-propionic acid	$C_7H_{13}NO_2$	2.43
10	16.026	3-Pyrrolidin-2-yl-propionic acid	$C_7H_{13}NO_2$	1.11
11	16.261	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro	$C_{10}H_{14}N_2O_2$	4.70
12	16.856	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)	$C_{11}H_{18}N_2O_2$	11.09
13	17.100	Hydantoin, 1-butyl-	$C_7H_{12}N_2O_2$	1.01
14	17.141	1,2-Cyclopentanedione, 3,3,5,5-tetramethyl	$C_9H_{14}O_2$	2.58
15	17.259	Dibutyl phthalate	$C_{16}H_{22}O_4$	1.51
16	17.838	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)	$C_{11}H_{18}N_2O_2$	7.42
17	18.031	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)	$C_{11}H_{18}N_2O_2$	20.66
18	18.123	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)	$C_{11}H_{18}N_2O_2$	3.67
19	18.173	Phenol, 3,5-dimethoxy-	$C_8H_{10}O_3$	2.29
20	20.153	2,5-Piperazinedione, 3-	$C_{12}H_{14}N_2O_2$	0.59

		methyl-6-(phenylmethyl)		
21	20.254	o-Butyl O,O-diethyl phosphorothioate	$C_8H_{19}O_3PS$	1.35
22	20.480	Cyclopentane, 1,2,4-trimethyl-	C_8H_{16}	0.89
23	20.891	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)	$C_{11}H_{18}N_2O_2$	1.92
24	21.034	Succinic acid, 2,6-dimethoxyphenyl ethyl ester	$C_{14}H_{18}O_6$	0.74
25	21.118	2,5-Piperazinedione, 3-benzyl-6-isopropyl	$C_{14}H_{18}N_2O_2$	1.09
26	21.755	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)	$C_{14}H_{16}N_2O_2$	4.00
27	22.133	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)	$C_{14}H_{16}N_2O_2$	18.47
28	22.619	Ethisterone	$C_{21}H_{28}O_2$	1.14
29	24.549	l-Proline, N-allyloxycarbonyl-, nonyl ester	$C_{18}H_{31}NO_4$	1.85
30	24.876	l-Proline, N-allyloxycarbonyl-, hexyl ester	$C_{15}H_2NO_4$	2.71

IV. DISCUSSION

The bacterial form of *Bacillus* genus are abundant source of antimicrobials compounds, since many species of this genus synthesize antimicrobial peptides. These bacteria in general represent a new and rich source of secondary metabolites that need to be explored [11]. In this present study, the MTT assay results shown that the crude extract of *B. amyloliquefaciens* CS4 showed higher inhibition rate against HeLa cells at high concentration (than of low concentration). In an earlier study, it has been proved that the crude extract of *B. amyloliquefaciens* AK-0, a recently isolated bacterium exhibited anti-proliferative activity against human colorectal cancer cells such as HCT116, SW480, LoVo and HT-29 [12]. Similarly crude extracts of *B. subtilis* and *B. thuringiensis* isolated from different marine environments showed cytotoxicity against established HeLa cell lines [13, 14]. The present findings are in line with the earlier research finding that documented the extract anti HeLa cell lines at IC_{50} value of 121.65µg/ml. Several studies have shown that brine shrimp assay has been an excellent method for preliminary investigations of toxicity of biologically active compounds. The resulted mortality or toxic effect of the extracts on brine shrimp larvae could be due to the toxic compounds that possess ovicidal and larvicidal actions either by affecting the embryonic development or that would slay the eggs [15]. Hence, the cytotoxic effects of the bacterial extracts were articulated for the further cytotoxicity assay in future as it correlated with activity against the brine shrimp nauplii [16]. Zebrafish embryo toxicity model has been proposed as the most promising alternative approach to classical acute fish toxicity testing with live fish [17]. Higher fecundity and rapid development have allowed testing a number of samples at shorter duration. Moreover, the transparent nature of embryos makes them easy to visualize morphological and developmental abnormalities during testing [18]. The crude metabolites of *S. bongori* possess active compounds that showed extensively toxic effects on zebrafish- embryo as well as brine shrimps-larvae [19]. When compared to the present work to previous reports, the presently extracted bioactive compounds from the bacterium, *B. amyloliquefaciens* have evidenced their role in several applications (Table 1). The active principle compounds in the crude extract of *B. amyloliquefaciens* were reported earlier for their biological properties (https://pubchem.ncbi.nlm.nih.gov/). Some of the applications of the major active compounds were; Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl) (Antibacterial agents, antifungal, antioxidative agent, anticancer activity), Naphthalene, decahydro-2,6-dimethyl (Antibacterial agents, Antineoplastic agents), 3-Pyrrolidin-2-yl-propionic acid (antipyretic or antiinflammatory agents) and Phenol, 3,5-dimethoxy- (Antiasthmatics, Drugs for disorders of the urinary system). The major compound Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl) isolated from marine sponge associated *Bacillus* species showed potential antioxidants and anticancer activity [20].

V. CONCLUSION

The present results suggest that the crude extract of *B. amyloliquefaciens* CS4 exhibited potential anticancer properties against HeLa cell line. The major 'pyrrol' derived compounds present in the crude extract could be responsible for the anticancer activity. The findings of this study revealed that probiotic bacterium, *B. amyloliquefaciens* CS4 has been found to produce bioactive secondary metabolites. The toxicity analysis on brine shrimps and zebrafish embryo result moderately toxic at increased concentration. In conclusion, the cytotoxicity exhibited by the crude extract was indicates the presence of potent bioactive compounds with potential application in pharmaceutical industry. This finding warrants further studies to isolate and elucidate the mechanism of bioactive components produced by *B. amyloliquefaciens*.

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