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Bacillus cucumis: A NEW SOURCE OF L-METHIONINASE ENZYME WITH POTENTIAL FOR CANCER THERAPY

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ABSTRACT

The existing cancer therapies usually target a single pathway, and the associated side effects and higher costs involved in such mono-target therapies have necessitated to find alternative treatment strategies. Methionine dependency is commonly seen in cancer cells whereas normal cells in absence of methionine can grow on homocysteine, as they possess intact methionine synthase. So reducing the level of cancer cell/plasma methionine with the help of L-methioninase enzyme looks a very promising therapy to manage the cancer. The present research was based on rapid screening of soil samples for L-methioninase producing microbes using selective media. The selected microbes were tested for their L-methioninase activity and total protein content. The results indicated a new strain of *Bacillus*, identified as *Bacillus cucumis* LAKHS12, exhibiting the highest L-methioninase activity. The cytotoxic activity on *in-vitro* cancer cell lines by MTT assay revealed that L-methioninase from *B. cucumis* exhibited highest cytotoxicity. The percentage viability of cervical cancer Hela cells was 35.7%, indicating a significant cytotoxicity of 64.3% for 48 h of treatment with L-methioninase at a low concentration. This finding was further supported by the microscopic observations of treated cancer cells. It can be concluded that the isolate has potential for further enzyme purification, characterization, and anticancer applications.

Keywords: *Bacillus cucumis*, cancer, L-methioninase, methionine dependency, MTT assay

INTRODUCTION

Cancer is a disease caused by uncontrolled and unregulated proliferation of cells in the body. It is a major cause of morbidity as well as mortality of people worldwide (Sharma *et al*., 2014). Among the various treatment modalities established, enzyme therapy is widely researched due to its therapeutic potential (Selim *et al*., 2015a). Amidst many enzymes, L-methioninase has received much attention as it has been found an ideal anticancer compound against many types of cancer cell lines including colon, lung, breast, glioblastoma and kidney (Sadraeian *et al*., 2013; Kavya and Nadumane, 2023).

L-methioninase (E.C 4.4.1.11, MGL), also known as L-methionine-γ-demethiolase, methioninase, L-methionine methanethiol lyase, and methionase, is the enzyme dependent on pyridoxal phosphate which mainly catalyses the α and β-elimination of L-methionine to methanethiol, ammonia, and α-ketobutyrate. (Kavya and Nadumane, 2020). L-methioninase is present in most of the organisms and was first identified in rumen bacteria (Salim *et al*., 2019). Methionine is one of the essential amino acids in mammals. Normal cells have active methionine synthase that makes them

adept at utilizing homocysteine to synthesize adequate levels of methionine (Sadraeian *et al*., 2013). Contrarily, cancer cells lack methionine synthase and are solely dependent on the supply of external methionine (Hoffman, 1984) as well as are incapable of growing on homocysteine (Cellarier *et al*., 2003; Kahraman *et al*., 2011). This is termed as methionine (MET) dependency or the Hoffman effect. Methionine dependency and its requirement by cancer cells have paved the way for a multitude of therapeutic applications (El-Sayed, 2010). Abnormalities and disturbances in transmethylation and in the metabolism of methionine are connected with many diseases such as Parkinson's disease, obesity, cancer, heart disease, and aging among humans (Al-Zahrani and Bukhari, 2019). Methionine restriction arrests tumour growth via S/G_2-phase cell cycle blocks *in vitro* and *in vivo* (Hoffman and Jacobsen, 1980; Guo *et al*., 1993; Kokkinakis *et al*., 1997). Consequently, this led to consider methionine as the main target in a tumour specific therapeutic approach. Use of L-methioninase as a treatment option to deprive cancer cells of external methionine has extensively been researched (Sundar and Nellaiah, 2013). L-methioninase has been extracted, purified and characterized from diverse bacteria such as *Pseudomonas putida* (Salim, *et al*., 2019), *Citrobacter freundii* (Manukhov *et al*., 2005), *Clostridium sporogenes* (Kreis and Hession 1973), *Hafnia alvei* (Alshehri, 2020), and *Pseudomonas extremaustralis* (Al-Zahrani and Bukhari, 2019), and fungi like *Aspergillus flavipes* (El-Sayed, 2009) and *Candida tropicalis* (Selim *et al*., 2015b). In view of the immense anticancer application potential of L-methioninase enzyme, the present study was aimed to isolate L-methioninase producing microbes from different soil samples, then purify the enzyme, analyse its cytotoxic potential to HeLa (cervical cancer) cell line, and to characterize the promising isolate for future anticancer studies.

MATERIALS AND METHODS

Sample collection

The rhizosphere soil samples were collected from three different locations *viz*., BTM lake (12.9060° N, 77.6162° E), Butterfly park, Bannerghatta biological park (12.7961° N, 77.5764° E) and mulberry field, Seri Biotech (12.8820° N, 77.7102° E) in Bangalore Karnataka (India).

Screening for L-methioninase producing strains

Serially diluted soil samples (1 g 100 mL^{-1} water) were spread over the plates containing nutrient agar (NA), Czapek Dox agar (CDA) and actinomycetes isolation agar (ISP-2) media (Shirling and Gottlieb, 1966; Aneja, 2003) aseptically to isolate bacteria, fungi and actinomycetes, respectively. The plates were observed at frequent intervals for the development of colonies. Pigmented colonies were sub-cultured and subjected to rapid screening using M9 medium to identify L-methioninase producing isolates. A customized M9 medium was employed to swiftly screen for microbes capable of producing L-methioninase. This adapted M9 medium had ingredient components (mL^{-1}): Na_2HPO_4 6.0 g, L-methionine 5.0 g, KH_2PO_4 3.0 g, NaCl 0.5 g, $MgSO_4$ 0.24 g, $CaCl_2$ 0.011 g, D-glucose 2.0 g, and phenol red as an indicator, and 2% agar, all adjusted to a pH 7.0. The plates were then incubated for 3 days. The colonies displaying a pink colouration were considered positive for L-methioninase, and further purified as pure cultures.

Extraction of L-methioninase enzyme

The microbial isolates, bacteria, fungi and actinomycetes, were inoculated into NA, CDA and ISP-2 media, respectively (Aneja, 2003) so as to check the production of L-methioninase. The isolates were incubated for 7 days with bacteria at 37$\pm$1°C, and fungi and actinomycetes at 24$\pm$2°C. The culture media was then centrifuged at 8000 rpm to collect clear supernatant containing the enzyme and was used for subsequent assays.

L-methioninase assay

L-methionine activity was assayed by measuring the methanethiol (MTL) produced from L-methionine (Laakso and Nurmikko, 1976; Arfi, 2003). The reaction mixture consisted of 0.25 mM 5,5 dithio-bis-2-nitrobenzoicacid (DTNB), 0.01 mM pyridoxal phosphate (PLP) and 20 mM methionine in a final volume of 1 mL. The reaction mixture was diluted with 0.1M phosphate buffer to make a final volume of 3 mL. The MTL liberated from the substrate formed thionitrobenzoic acid after reacting with DTNB. The amount of MLT was measured at 412 nm by using UV-Vis spectrophotometer (Shimadzu UV-1800) and extrapolated by using a standard of sodium methane thiolate. The protein content of crude enzyme was determined by measuring the absorbance at 280 nm against a standard BSA. Finally, the specific activity was calculated and expressed in U mg^{-1} protein.

Characterization of isolates

Morphological characterization of the selected isolates was carried out by observing the colony shape, colour, uniformity, transparency, and position of the pure cultures. Gram staining was performed and the isolates were observed under 100X magnification of a binocular microscope (Labomed, Model Lx-300). The isolate with the highest specific activity for L-methioninase was selected for 16sRNA sequencing (Mignard and Flandrois, 2006), which was performed at Biokart in Bengaluru, India utilizing the Sequence Scanner software version 2.0. The resulting 16s rRNA nucleotide sequence was subsequently uploaded to the NCBI database, and the similarity of the sequence was assessed through BLAST analysis tool. Based on BLAST analysis results, a phylogenetic tree was created using Mega X software (Kumar *et al*., 2018), specifically version 10.1.7.

Purification of L-methioninase

Acetone precipitation: The method given by Nejadi *et al*. (2014) was followed. The clear supernatant of selected isolate was used as crude enzyme for acetone precipitation (Thermo Scientific protocol). Cold acetone was added to the supernatant in 1:4 ratio of sample and acetone. The solution was vortexed for mixing, incubated for 60 min at 20°C and then centrifuged at 10,000 rpm for 10 min. The pellet was air dried, dissolved in 0.1 M phosphate buffer and stored at -20°C until further use.

Ion exchange chromatography: The acetone purified sample was applied on top of a DEAE-cellulose column (15 cm × 2.5 cm) equilibrated with acetate buffer of pH 5.0 (0.1 M). The fractions from the sample were eluted using a linear gradient of sodium chloride (200 mM) in the same buffer at a flow rate of 0.3 mL min^{-1}. The fractions were collected separately and their activity measured spectrophotometrically at 280 nm (Selim *et al*., 2015b). The amount of L-methioninase was also determined quantitatively. The active fractions with the highest absorbance (protein fractions) were selected for determination of L-methioninase activity and those with similar specific activity were combined for further cytotoxicity analysis.

In vitro anticancer activity assessment

To analyze the potency of L-methioninase against cancer, the cancer cells were treated with different concentrations of the isolated enzyme for 48 h and later their viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). The cancer cell line (HeLa) obtained from National Center for Cell Sciences (NCCS), Pune, in India, was seeded in a 96 well plate for 24 h prior to the addition of pure enzyme. The mixture was incubated for 48 h along with a control for reference, followed by the addition of MTT and DMSO. After incubation in dark for 4 h, the optical density was checked at 540 nm, using an ELISA reader (LisaPlus, Aspen Diagnostics). The cytotoxicity of enzyme was determined. The cancer cell viability was calculated as a percentage viability of control (untreated cancer) cells.

Morphology observation of HeLa cells

HeLa cells treated with L-methioninase from B12 isolate were observed under an inverted microscope (Labomed) for the changes in cell morphology and were photographed.

Statistical analysis

Statistical analysis was done by using Graph Pad Prism version 6 software (Gra[phPad Software, San Diago, USA) to assess the significance of each test, which were performed in triplicates, with significance determined by a p-value < 0.05 (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

A total of 36 microbes were isolated from the soil samples which comprised of 8 actinomycetes, 9 fungi and 19 bacteria. Among these, 20 isolates were selected based on their pigmented colonies, and were sub-cultured to maintain their pure cultures.

L-methioninase enzyme activity

Of the selected 20 isolates, grown on modified M9 medium with phenol red as an indicator, 4 isolates displayed potential L-methioninase activity as these colonies turned pink in colour. This colour change in colonies occurs due to pH change caused by ammonia production by L-methioninase acting on L-methionine. The rate of pink colour intensity produced by the colonies depends on cultivation period and the formation of enzyme (Selim *et al.*, 2015b). The colonies that developed pink colour were selected for enzyme activity and total protein content assay.

The standard calibration curve for enzyme activity was prepared by using sodium methane thiolate while bovine serum albumin was used as a standard to ascertain total protein content at 280 nm. The supernatant from broth culture was used for estimating the enzyme activity, and specific activity was calculated after estimating the protein content of the samples (Table 1). The results indicated that isolate B12 had highest specific activity (0.141 U mg^{-1} protein) at 1:25 dilution as compared to the other isolates with 0.110 U mg^{-1} for B18, 0.097 U mg^{-1} for B11-2, and therefore was selected as the best isolate for further studies.

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Table 1: Quantitative analysis of crude enzyme activity of selected bacterial isolates, evaluated at different dilutions

Isolates	Dilution	Absorbance		Enzyme activity	Protein content	Specific activity
		412 nm	280 nm	(µmoles min^{-1} mL^{-1})	(mg mL^{-1})	(U mg^{-1} protein)
A6A	1:1	0.156	1.992	0.025	1.769	0.014
	1:10	0.340	2.500	0.054	2.221	0.024
	1:25	1.725	2.925	0.273	2.598	0.105
B 11-2	1:1	0.648	4.122	0.103	3.662	0.028
	1:10	1.750	4.730	0.277	4.202	0.066
	1:25	3.125	5.775	0.495	5.130	0.097
B18	1:1	0.284	3.030	0.045	2.692	0.017
	1:10	1.140	4.560	0.181	4.051	0.045
	1:25	2.825	4.600	0.448	4.086	0.110
B12	1:1	0.290	1.882	0.046	1.672	0.027
	1:10	1.080	2.310	0.171	2.052	0.083
	1:25	2.525	3.200	0.400	2.843	0.141

Characterization of the best isolate

The morphological characterization of bacterial isolate B12 (Fig. 1a) revealed that the bacterium was irregular in shape, opaque, creamy-white colonies with concentric rings and minimal elevation. Gram's staining revealed purple colored, rod shaped organism indicating that it was a Gram-positive bacterium (Fig. 1b).

Identification of isolate (16S rRNA sequencing)

The molecular identification of isolate B12 was done by 16S rRNA sequencing (McDonald *et al*., 2012).

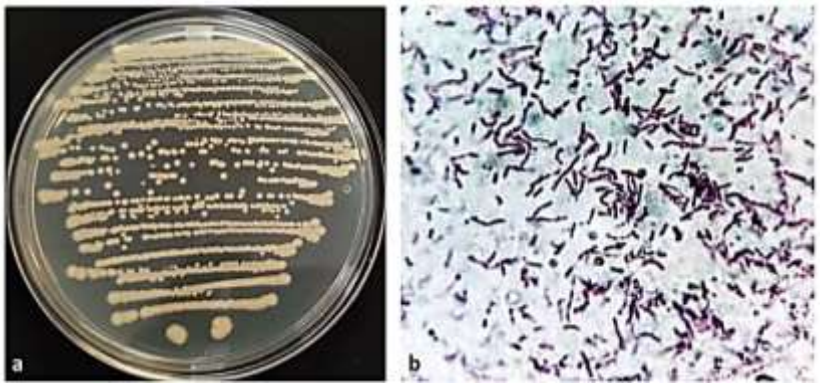

Fig. 1: a) Pure culture of isolate B12; b) Gram's staining of isolate B12

The phylogenetic analysis of B12 isolate revealed 98.87% similarity with *Bacillus cucumis* and the bacterium was identified as *Bacillus cucumis* LAKHS12. The rRNA sequence was deposited in GenBank under accession No. MT775456. The phylogenetic tree developed is shown in Fig. 2.

Fig. 2: Phylogenetic analysis of *Bacillus cucumis* LAKHS12

Purification of crude enzyme in culture supernatant

The 80 mL supernatant having crude enzyme was subjected to acetone precipitation which yielded 147 mg pellet upon centrifugation from *Bacillus cucumis* LAKHS12. The pellet was dissolved in

Table 2: Quantitative analysis of the activity of acetone purified L-methioninase enzyme derived from *B. cucumis* LAKHS 12

Dilution	Absorbance		Enzyme activity (μmoles min^{-1} mL^{-1})	Protein content (mg mL^{-1})	Specific activity (U mg^{-1} protein)
	412 nm	280 nm			
1:10	0.313	0.833	0.495	7.843	0.063
1:25	0.387	0.341	1.532	7.573	0.202
1:50	0.400	0.131	3.168	5.817	0.545

phosphate buffer to a final concentration of 5 mg mL^{-1} which was further purified through ion-exchange chromatography by using the DEAE cellulose column. The fractions collected separately were checked for protein content by taking the OD at 280 nm in a spectrophotometer. Active fractions having similar enzyme activity were pooled together. Fractions 3-16 were clubbed to form 4 major fractions having similar absorbance values.

Purified fractions were analyzed for their enzyme activity and total protein content, to calculate the specific activity (Table 2). The acetone purified samples exhibited highest specific activity of 0.545 U mg^{-1} protein. After acetone purification, *B. cucumis* exhibited higher specific activity which is a 3.86-fold increase in specific activity when compared to the crude enzyme. Further purification was performed by ion-exchange chromatography using DEAE-cellulose as the solid matrix. Specific activity of enzyme after column purification was estimated as 0.67 U mg^{-1} protein for the 4th fraction, which is 4.8-folds increase than that of the crude enzyme (Table 3). From *Pseudomonas putida*, the DEAE-cellulose purified L-methioninase had a specific activity of 0.161 U mg^{-1} protein (Ito *et al.*, 1975). The specific activity of L-methioninase purified from *Pseudomonas ovalis* using DEAE-cellulose was observed to be 0.220 U mg^{-1} (Tanaka *et al*., 1976). The values in this study are approximately 2.5 times greater than these earlier reports. Previously, it was reported that under ideal circumstances, *Bacillus subtilis* demonstrated a specific activity of 19.6 U mg^{-1} of protein as per Bhawana and Priyanka (2018). In comparison, *B. haynesii* exhibited a specific activity of 9.22 U mg^{-1} protein. These values surpass the specific activity of the current L-methioninase from *B. cucumis*, which was 0.673 U mg^{-1} protein. But this has a marked increase in the specific activity compared to specific activity of the crude sample reported earlier from *Pseudomonas putida* (Ito *et al.,* 1975). In present study the acetone purified and column purified enzymes showed 3.86- and 4.77-fold increase over crude enzyme (Table 4).

Table 3: Quantitative analysis of the activity of column purified L-methioninase enzyme isolated from *B. cucumis* LAKHS 12

Fraction No.	Protein content (mg mL^{-1})	Enzyme activity (μmoles min^{-1} mL^{-1})	Specific activity (U mg^{-1} protein)
1	0.263	0.012	0.044
2	0.304	0.010	0.033
3	0.140	0.018	0.127
4	0.027	0.018	0.673

Table 4: Purification folds and activity of L-methioninase enzyme isolated from *Bacillus cucumis* LAKHS12

Enzymes	Enzyme activity (μmoles^{-1} min^{-1} mL^{-1})	Total protein (mg mL^{-1})	Specific activity (U mg^{-1} protein)	Fold increase in specific activity over crude enzyme
Crude	0.400	2.843	0.141	1
Acetone purified	3.168	5.817	0.545	3.86
Coloumn purified	0.018	0.027	0.673	4.77

Cytotoxicity of L-methioninase on HeLa cells

The cytotoxicity of L-methioninase from *B. cucumis* was checked by performing MTT assay on HeLa cells (48 h) [Fig. 3]. Out of the 4 dilutions tested, 1:10 dilution exhibited least cell viability at 35.7%, followed by 1:1 dilution which exhibited 57% cell viability as compared to the control. The L-methioninase from *B. Cucumis* demonstrated higher cytotoxicity of 65%, with a percentage cell viability of 35%, which is higher than the cytotoxicity of column purified or acetone purified L-methioninase from *B. haynesii* with 30% and 25.1% respectively on MCF-7 cells with 70.0 and 74.9% viabilities (Bopaiah *et al*., 2020). The anti-cancer potential of a bioactive fraction B5, from *Bacillus endophyticus* JUPR15 was reported on HeLa, MCF-7, HepG2 cancer cells (Venkatachalam and Nadumane, 2018), but L-methioninase is not being reported from this species. Though L-methioninase from *B. subtilis* UBTn7 was reported, its anticancer activity was not studied (Prinhanto, 2018). Hence it is evident that, this study is the first to report about L-methioninase from *B. cucumis* to the best of our knowledge, and its anti-cancer activity against HeLa cells which exhibited such low cell viability. In a study by Selim *et al*. (2015b), L-methioninase was reported as a powerful anti-cancer substance, demonstrating IC_{50} values comparable to the standard drug doxorubicin in different cancer cell types, with an IC_{50} value of 0.127 U mL^{-1}. The *in vitro* assessment of *Candida tropicalis* L-methioninase's anti-tumor effectiveness on three human tumor cell lines (HEPG2 for liver, MCF7 for breast, and HCT 116 for colon) revealed that this enzyme exhibits potent anti-tumor properties against liver and breast cancer cell lines, and this effect is influenced by the concentration of the enzyme. Furthermore, the sensitivity of cancer cells to methionine depletion via enzymatic action varied among the cell lines, with the breast cancer cell line demonstrating greater sensitivity (IC_{50} of 0.13 U mL^{-1}) compared to the liver cancer cell line (IC_{50} of 0.2 U mL^{-1}). Multiple authors have reported the efficacy of L-methioninase against various cell lines. Additionally, in a separate study, the sensitivity of cancer cell lines to L-methioninase produced by *Pseudomonas putida* was assessed and found that leukemia cell lines exhibited higher sensitivity to L-methioninase compared to solid tumor cell lines, with an IC_{50} in leukemia cell lines being less than 0.5 U mL^{-1} (Hori *et al*., 1996). Our present study reaffirmed this finding, where we got 57.7% viability for 1:1 diluted sample (0.046 U mL^{-1}) and 35.7% viability for 1:10 diluted sample of L-methioninase (0.171 U mL^{-1}), which are much lower than that reported for *C. albicans* or *P. putida*. Thus, the strong cytotoxic effect of the L-methioninase from *B. cucumis* in the current study confirms the methionine dependency of the cancer cells.

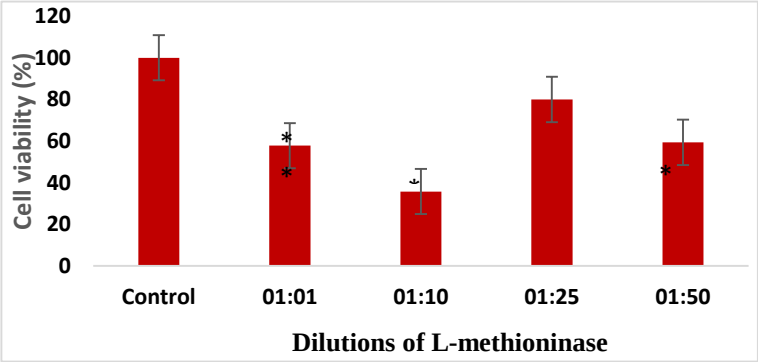

Fig. 3: Cytotoxicity of L-methioninase enzyme from *B. cucumis* LAKHS 12 on HeLa cells (48 h) (*p<0.05; **p<0.01)

HeLa cell morphology

The cervical cancer (HeLa) cells treated with the enzyme (for 48 h) when observed under the microscope revealed reduced cell concentration and cells rounding up and undergoing apoptosis as shown by the presence of apoptotic bodies, thus giving further evidence for the anticancer application potential of L-methioninase from *B. cucumis* (Fig. 4). Most of the human malignant cells have high requirement of amino acid methionine due to their requirement in transcription and protein synthesis (Swisher *et al*., 2009). This metabolic defect resulting in methionine dependency for growth is observed only in cancer cells, and these cells are unable to grow in methionine-free medium. An enzyme capable of cleaving methionine would be useful in cancer, to lower methionine levels and hence, has better effects therapeutically. Therefore, L-methioninase is commonly used by researchers

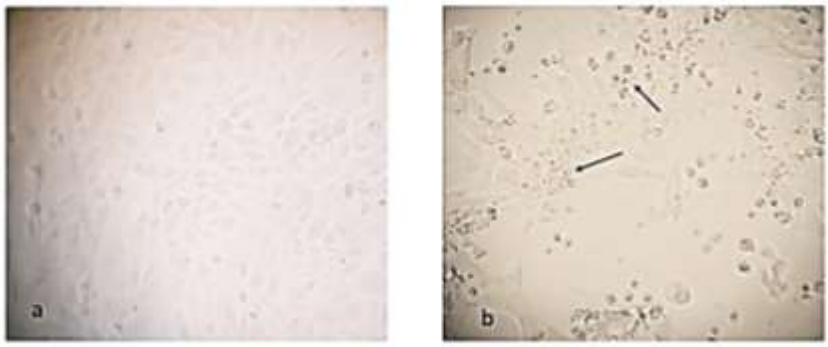

Fig. 4: Morphology of HeLa cells (a) Control HeLa (b) HeLa treated with L-methioninase. Arrows indicate apoptotic bodies

as anticancer agent for diverse types of cancers.

Conclusion: The bacterial isolate, *B. cucumis* LAKHS12, demonstrated promising L-methioninase activity along with *in vitro* cytotoxicity to cervical cancer cell line. Regarding L-methioninase production, the current study is the first report of L-methioninase production from *B. cucumis* and due to its high cytotoxicity, this enzyme holds promise towards future purification using gel filtration chromatography, and high performance liquid chromatography and further validation of anticancer activity on *in vivo* mice models.

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Author contribution: All the authors contributed to the study conception and design. Main experiment, data collection and analysis was done by Akhila Warrier (AW), Hara Gopal (HG), Kadambari Anand (KA), Legha (LE) and Soumitri (SNS). The first draft of manuscript was written by AW, HG, KA, LE and SNS. The paper was manuscript was finalized by Ashrini and Varalakshmi.

Conflicts of Interest: The authors further declare that they have no conflict of interest.

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