BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, June 2025. Vol. **22**(2), p. 699-710

Assessment of Antimicrobial and Cytotoxicity Effect of GABA extracted from *Lactiplantibacillus plantarum*

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http://dx.doi.org/10.13005/bbra/3395

(Received: 06 November 2024; accepted: 18 April 2025)

This study aimed to extract ā-aminobutyric acid (GABA) from *Lactiplantibacillus plantarum* isolated from milk. The microorganism was identified through standard microbiological techniques, DNA isolation, polymerase chain reaction (PCR), and gene sequencing analysis, followed by database submission to NCBI, obtaining the accession number PP391551. GABA extraction was performed using ethyl acetate in MRS broth, and its presence was confirmed through Fourier-transform infrared spectroscopy (FTIR), thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC) and obtained yield of 1.8 g/L. The extracted GABA exhibited antibacterial activity against *Shigella dysenteriae* (21±0.044 mm), *Salmonella typhi* (22±0.08 mm), *Escherichia coli* (11±0.12 mm), and *Klebsiella pneumoniae* (13±0.48 mm), as well as antifungal activity against *Aspergillus niger* (14±0.072 mm) and *Aspergillus flavus* (11±0.061 mm). Additionally, cytotoxicity analysis against the HCT 116 cell line revealed an IC… € value of 103.48 μg/ml, highlighting its potential biomedical applications.

Keywords: *Lactiplantibacillus plantarum*; GABA; FTIR; TLC; HPLC; Antimicrobial; Cytotoxicity.

GABA, also known as gamma-aminobutyric acid (GABA), is a non-protein amino acid produced through the glutamate decarboxylation pathway. GABA serves as an antidepressant, antihypertensive, antidiabetic, antimicrobial, anticancerous and immune system booster while also benefiting neural health. It is present in various foods, including grains, vegetables, fruits, [1] and animal products, as well as in different organisms such as bacteria, cyanobacteria, fungi, algae, and plants. [2]

Over the years few researches has been undergone to find the most suitable strategies to increase the amount of GABA in food with plant enrichment, chemical synthesis and microbial fermentation. Microbiological production of GABA is safer and more environmentally friendly, high specificity, and cost effectiveness than chemical methods. Additionally, using microorganisms for production allows for better control of conditions compared to extraction from plants and animals. Given its pharmaceutical properties, it is essential to establish optimal conditions for GABA production. [3,4,5] GABA production by beneficial microorganism has potential to increase the functional effect of some foods and beverages.[4] Lactic acid bacteria

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are often naturally present in some traditional food fermentations and which is used as starters in few industrial food fermentations for the pro technological properties.[6] For the fortification of food like GABA-fortified foods, high amount of GABA producing lactic acid bacteria are being used recently. [7] Lactic acid bacteria have the intrinsic property and numerous strains are proposed as human and animal probiotics. [8]

GABA producing lactic acid bacteria strains belonging to the genera of *Pediococcus, Lactobacillus, Enterococcus, Lactococcus, Streptococcus, Weisella, Lacticaseibacillus, Secundilactobacillus, Leuconostoc* etc. [9,10] Among this *Lactobacillus planatrum* is facultative hetero fermentative species with high adaptability to many conditions, which already isolated from cereals, vegetables, bee bread, meat, milk, fruits and fermented foods.[11] *L.plantarum* is a normal inhabitant of the gastro-intestinal tract insects, mammals, fish and which includes in qualified presumption of safety and in generally recognized as safe lists.[12]

In recent years, researchers have investigated *Lactobacillus* spp., particularly for its ability to synthesize GABA. However, comprehensive bioactivity evaluations remain limited. The present study aimed to isolate a unique strain of *Lactiplantibacillus plantarum* from milk, extract and characterize GABA, and conduct a detailed bioactivity evaluation. This included an antimicrobial activity study against foodborne pathogens and an anticancer study against the HCT 116 cell line.

MATERIALS AND METHODS

Collection of sample and isolation of *Lactobacillus*

The milk sample was collected from the cow farm of Eachanari, Coimbatore, TamilNadu, India in a sterile container and processed. MRS broth (Himedia, Mumbai, India) was prepared by dissolving 55.15g in 1000ml of distilled water and sterilsed under autoclave at 121^{0}C for 15minutes with 15lbs. The sterilized media was cooled to room temperature and the collected milk sample was added (10ml broth and 0.5ml milk) and incubated at 37^{0}C for 24 to 48 hours for the enumeration and isolation of the lactic acid bacteria.

Isolation of pure culture from the broth one loop of the bacteria was used to streak on MRS plate and incubated at 37^{0}C upto 24 hours. After incubation single colony was re streaked on to MRS agar and incubated which was repeated for 3 to 5 time to get the pure *Lactobacillus* spp. and was used for further study. [10]

Molecular confirmation of *L.plantarum*

Isolated bacterial DNA was extracted with phenol chloroform method [13] and 16s ribosomal RNA sequence was amplified using the universal forward primer 27 F (52 -AGA GTT TGA TCC TGG CTC AG-32) and reverse primer 1492R (52 -CTA CGG CTA CCT TGT TAC GA-32) by AB Applied Biosystem PCR. The PCR cycling condition were ; initial denaturation at 95^{0}C/5 min, 30 cycles of denaturation at 95^{0}C/30 sec, annealing at 55 ^{0}C/20 sec, and extension at 72^0 C/5 min, and a final extension at 72o C/5 min. The amplified gene product was subjected to sequencing analysis and the obtained data was compared using the Basic Local Alignment Search Tool (BLAST) available at the NCBI (National Center for Biotechnology Information, USA) followed by the phylogenetic tree was constructed.[14]

Production and Characterization of GABA – TLC, FTIR and HPLC

MRS broth was used as the production medium. After inoculation, the medium was incubated at 37°C for 5 to 7 days, followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was then precipitated using a 60% ethyl acetate solution. Intermediate mixing was carried out for 3 hours, and the mixture was incubated under ice-cold conditions (4°C) for 24 hours. The aqueous layer was then separated and stored at 4^{0}C in a sealed amber vial and used for analysis. [15]

Initially TLC study was carried out with standard GABA using R_f value to confirm the presence of the compound in the sample. About 10 µL of both standard GABA and the extracted GABA were loaded onto a TLC sheet coated with silica, then placed in a TLC chamber containing an N-butanol, acetic acid, and water mixture in a 5:3:2 ratio. Once the solvent reached approximately 1 cm from the top of the sheet, it was removed and allowed to dry. 0.2% of the ninhydrin solution was sprayed on the sheet and dried to get the visibility of the compounds and the R_f value was calculated.[16] FTIR study was carried out for the extracted and standard GABA using Shimadzu

infrared spectrophotometer from 4000 to 400 cm-1 to identify the functional group in the GABA.[15] A High-Performance Liquid Chromatography (HPLC) study was conducted for both standard and extracted GABA following the specified protocol of [17,18]. The samples were injected in the HPLC system equipped with a C18 column (250 X 4.6mm, Shimadzu) and photo diode Array detector. The mobile phase used was methanol and trifluoro acetic acid in the ratio of 60:0.1 with the flow rate of 2 ml/min with 254nm. The presence of GABA was confirmed with standard curve obtained in different concentration of standard GABA.

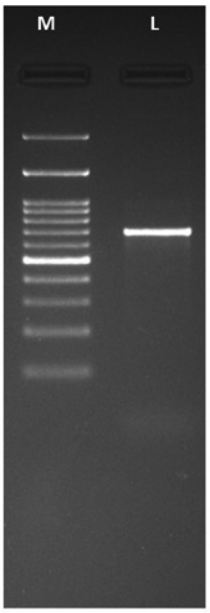

Fig. 1. PCR amplification of the isolated bacteria Lane 1 (M) - 100bp marker; Lane 2 (L) - Amplified product of *L.plantarum*

Antimicrobial study

For the antibacterial study, nutrient agar plate (28g in 1000ml of distilled water followed by sterilized) was used. 24hours old culture of *Escherichia coli*, *Salmonella typhi*, *Klebseilla pneumoniae* and *Shigella dysentriae* were swabbed (70µl) aseptically swabbed on to the plates. Wells were created with cork borer, and samples were added along with positive control (chloramphenicol-C30mcg) and negative control (DMSO). The plates were then incubated at 37^{0}C for 24hrs. After incubation the zone of inhibition (in mm) was measured using antibiotic zone scale (Himedia) in triplicates.

Antifungal study – malt agar (in 1000ml of distilled water followed by sterilized) was seeded with 70µl of *Aspergillus niger* and *Aspergillus flavus*. The test samples were added to the wells following previously mentioned method. Fluconazole (1mg/ml) was used as the positive

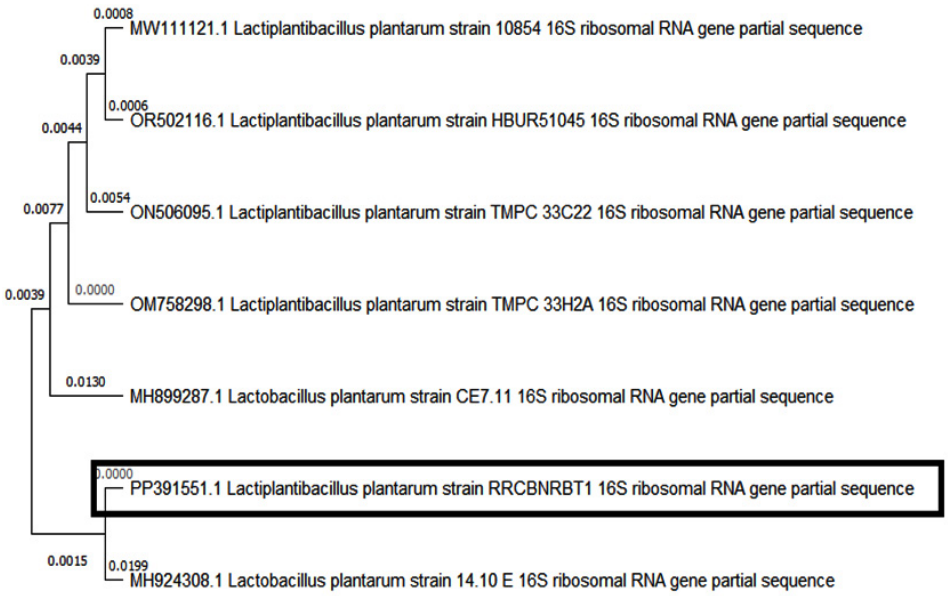

Fig. 2. Phylogram

control. The plates were then incubated at 30°C for 5 days, and the zone of inhibition was measured.[19]

Cytotoxicity study against HCT 116 Cell Line

Human colon cancer cell line (HCT 116 Cell Line) was purchased from NCCS, Pune, India and sub cultured in DMEM medium (Himedia, India) with the addition of 10% FBS, 1 mM L-glutamine and 1% penicillin to avoid the contamination. Cells were cultured in a humidified incubator at 37^{0}C with 5% CO_2 saturation for 72hours. Viability of the cells were confirmed with MTT assay by adding 100µl of cell line in 96 well plate with different concentration (12.5, 25, 50, 100, 200µg) of sample along with control (untreated cell line) and standard (doxorubicin-1uM/ml) for 24 hours. After incubation the cells were washed with trypsin and DMSO (50µl each) followed by adding MTT dye and incubated for 4 hours. Viability of the cells was measured using ELISA reader at 570nm and the percentage of viability was calculated in triplicates.[20]

Data analysis

The experimental data were analyzed in triplicates and expressed as mean ± standard deviation (SD). The results were visually represented using graphs for better clarity and comprehension

Fig. 3. TLC profile of GABA
(1- Extracted GABA; 2- Standard GABA)

RESULTS

Molecular confirmation of the bacteria

Molecular identification of the isolated bacteria was performed with Polymerase Chain Reaction (Figure 1) to amplify the 16SrDNA. Alignment of the amplified product

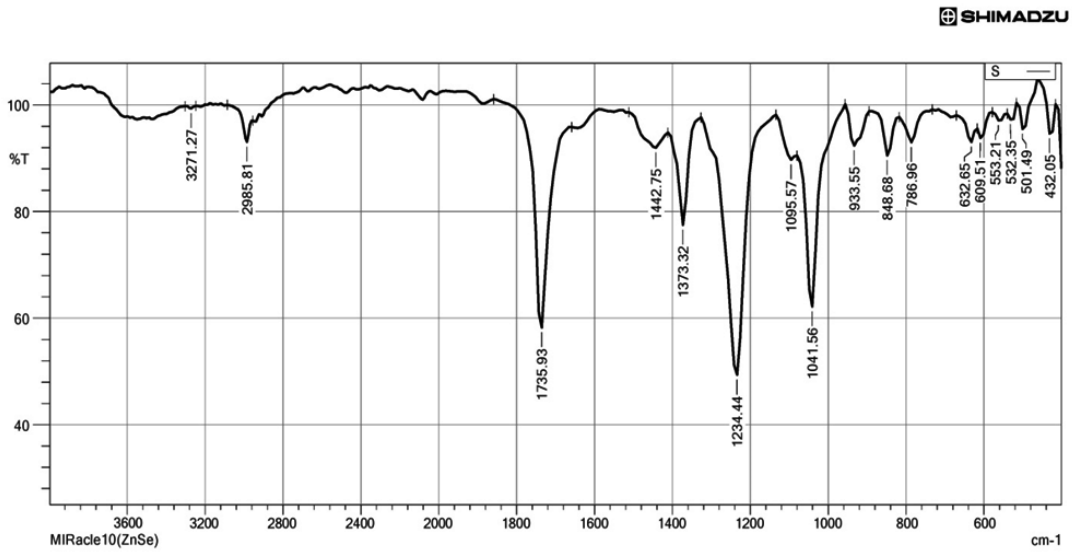

Fig. 4. FT-IR spectrum of the standard GABA

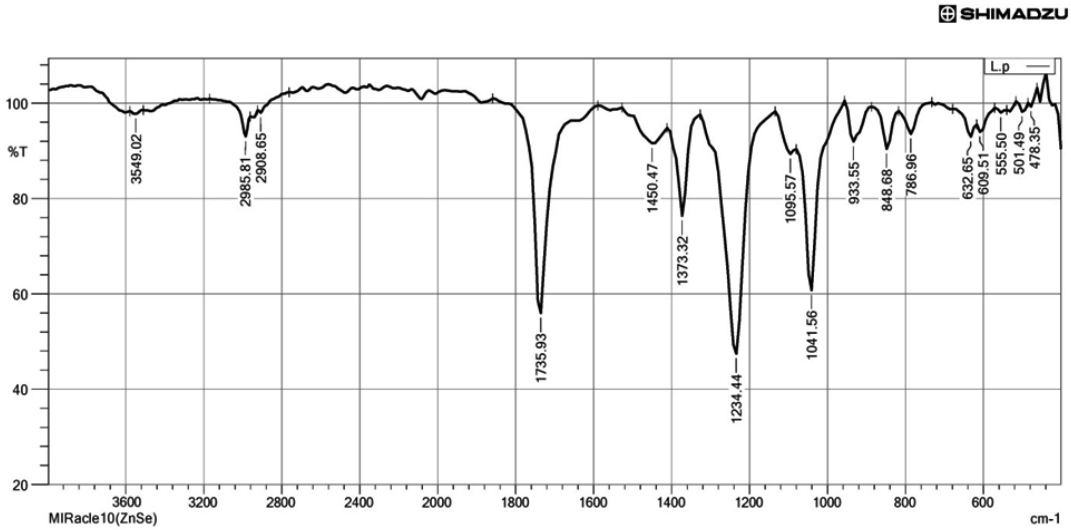

Fig. 5. FT-IR spectrum of the extracted GABA

of 16SrDNA gene from Gen bank database resulted in the identification of *Lactobacillus* spp as *Lactiplantibacillus plantarum* with 100 % homology and the accession number was PP391551 (Figure 2).

Production and Characterization of GABA – TLC, FTIR and HPLC

The presence of GABA was identified using a TLC study, the R_f value was recorded as 0.63, which was similar to that of standard GABA (Figure 3).

The FTIR spectrum of the extracted GABA was compared with standard GABA and 12 functional groups were observed as similar in both sample, which are Peaks at 2985.81 denoted the presence of CH weak, 1735.93 is the presence of C=O ester strong, 1373.32 – CH3 bend, 1234.44 C-O-C, 1095.57 and 1041.56 denotes the presence of C-OH stretch, 848.68 correspond to C-F bond, 786.96 - C-Cl strong, 632.65, 609.51 is corresponding to strong C-Br, 501.49 is denoting the presence of C-I strong bond respectively. FTIR results were presented in Figure 4 and 5.

Further, the presence of extracted GABA was confirmed and quantified using HPLC study (Fig. 6), which revealed that *L.planatarum* can produce 1.8 g/L of GABA. Similarly other researchers also quantified the GABA with HPLC study and the amount may be varying based upon the organism and medium condition.

Antimicrobial study

In the agar diffusion assay, the antibacterial activity of *L.plantarum* was evaluated using the cell free supernatant (CFS) and purified compound (GABA) against gram-negative bacteria such as *Escherichia coli, Klebsiella pneumoniae, Salmonella typhi,* and *Shigella dysentriae*. The zone of inhibition was examined around the well after 24 hours of incubation at 37^0 C. For GABA extract from *L.plantarum,* the highest zone of inhibition was observed against *S.dysentriae* (21±0.044), and *S.typhi* (22±0.08) and the lowest zone of bacterial inhibition was observed against *E. coli* (11±0.12) and *K.pneumoniae* (13±0.48). Similarly in case of CFS the highest zone of inhibition was observed against *K.pneumoniae* (9±0.02), and *S. typhi* (15±0.01) respectively. No visible inhibition was examined for CFS sample against *E. colli* and *S.dysentriae*. Reference antibiotic (chloramphenicol-C30mcg) showed marked zone of inhibition against test organism. There is no inhibitory zone for DMSO which serves as negative control (Table 1).

Gamma-aminobutyric acid (GABA) produced by *Lactobacillus* strains has shown promising antifungal activity against *Aspergillus*

niger and *Aspergillus flavus*. Studies suggest that GABA can disrupt fungal growth by altering membrane integrity, inhibiting spore germination, and interfering with metabolic pathways essential for fungal survival. This bioactive compound offers a natural and potentially safe alternative for controlling fungal contamination in food and agricultural products (Table 2).

Cytotoxicity study against HCT 116 Cells

The cytotoxic effect of GABA from *L.plantarum* on the HCT 116 cell line was evaluated using MTT assay. After incubation, cell viability of the sample was observed to be 96.03, 75.73, 53.74, 37.85 and 28.04 % at concentrations of 12.5, 25, 50, 100, 200µg respectively. In comparison, the standard drug doxorubicin exhibited a cell viability of 38.05%. The IC_{50} value for 24 hours in HCT116 cells was calculated to be 103.48ìg/ml, which suggests that *Lactobacillus*-derived GABA has moderate anticancer activity. The image acquired with the inverted microscope (Fig. 8) showed that the treated cells with an IC_{50} value of

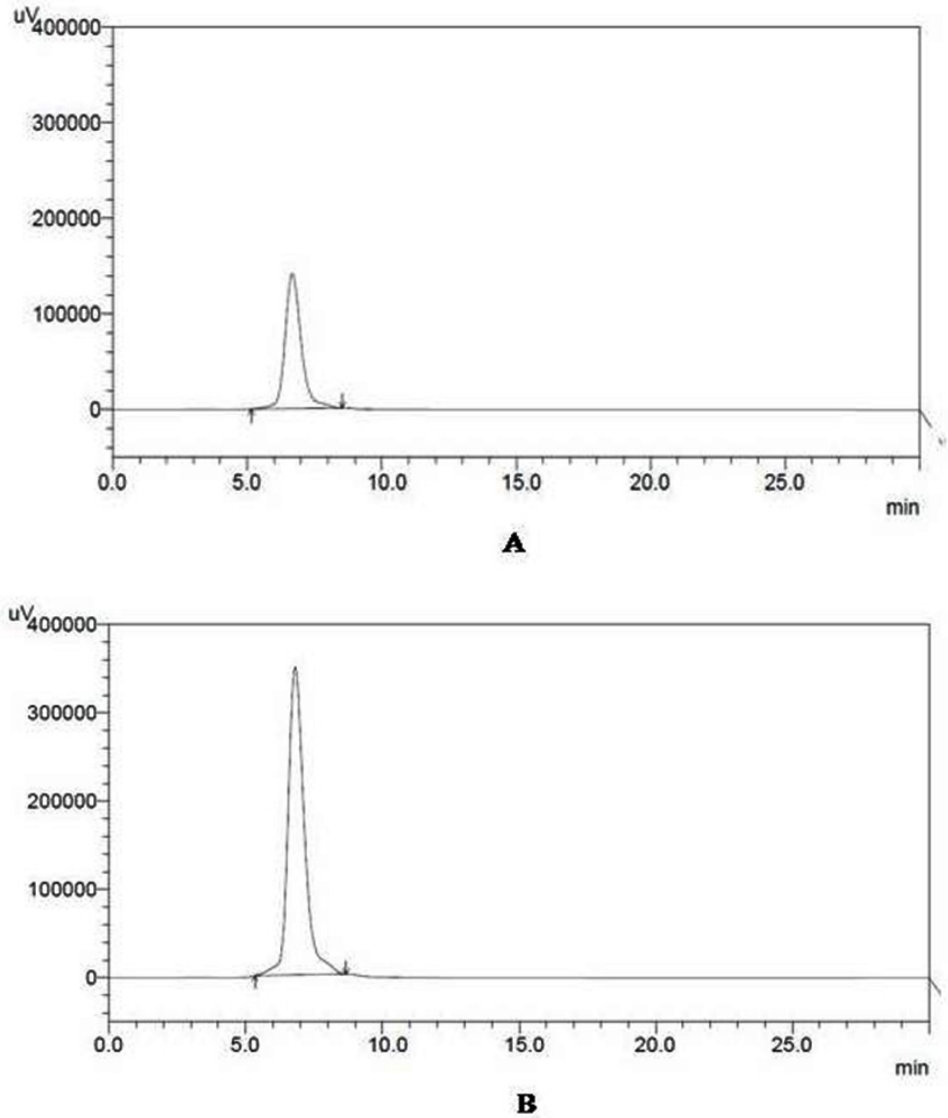

Fig. 6. HPLC chromatogram
A- Chromatogram of standard GABA ; B - Chromatogram of GABA extracted from *L.planatrum*

GABA extract were wrinkled, and as time passed, cell rupture and fragmentation were detected. The MTT assay results revealed that the cytotoxic effect of the investigated GABA was dose- and time-dependent, with a decrease in cell survival as the parameters were increased. A comparison of the 24-hour IC_{50} value for HCT116 cells to the results of other investigations demonstrates that the extracted GABA compound could have considerable cytotoxic effects (Fig 7).

DISCUSSION

This study focuses on the isolation of *Lactiplantibacillus plantarum* from milk, its molecular identification, and the subsequent extraction of gamma-aminobutyric acid (GABA), highlighting the potential application as antimicrobial and anticancer activity.

Initially, *Lactiplantibacillus plantarum* was successfully isolated, molecularly confirmed,

Table 1. Antibacterial activity of the extracted GABA from *L.planatrum* against different bacteria

S.No	Name of the organism	Zone of Inhibition (mm ± mean SD)			
		CFS	GABA	DMSO	Disc chloramphenicol
1	*E. coli*	Nil	11±0.12	Nil	6±0.06
2	*S. typhi*	15±0.01	22±0.08	Nil	16±0.02
3	*K. pneumoniae*	9±0.02	13±0.48	Nil	17±0.07
4	*S.dysentriae*	Nil	21±0.044	Nil	13±0.01

Table 2. Antifungal activity of GABA from *L.planatrum*

S.No	Name of the organism	Zone of Inhibition (mm ± mean SD)			
		Crude	GABA	DMSO	F-fluconazole
1	*A.niger*	8±0.022	14±0.072	Nil	Nil
2	*A. flavus*	8±0.043	11±0.061	Nil	Nil

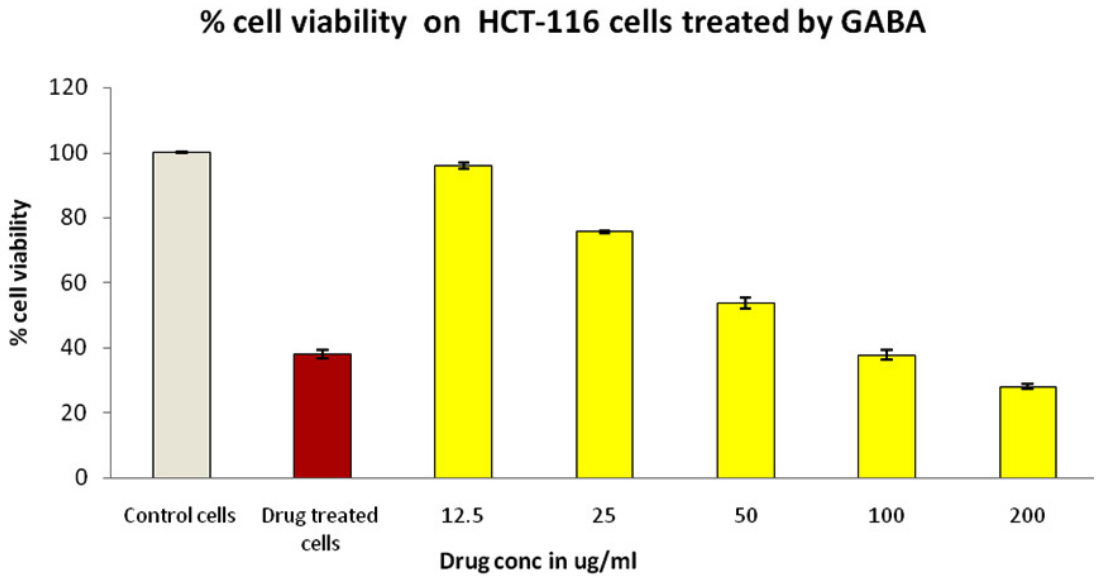

Fig. 7. Viability percentage of GABA treated on HCT-116 cells

and submitted to the NCBI GenBank, in reference to the work published by Duyen *et al*, [21] the selected *Lactiplantibacillus plantarum* possesses 95.70% similarities in the 16SrDNA study and the submission to NCBI Gene Bank. Fifty lactic acid bacteria from the Iranian dairy products were screened for the GABA production and the isolates shows >94 % of similarities and which were considered as belongs to same strains. GABA production may significantly vary between isolates with high similarity and low similarity.[22]

Phong *et al,* [23] isolated 12 *Lactobacillus* strains from Nem Chua samples and fermented them in MRS broth with 1% MSG. The results are consistent with,[24] who isolated *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* from the fermented bamboo shoot and in the TLC similar spots was observed as standard GABA.

Many researchers studied the compound characterization using FTIR and the peaks are varying based upon the nature of extraction and origin of the extraction. Similarly Lee *et al,* [25] isolated *Lactobacillus* sp from Korean salted and fermented sea foods for effective fermentation of strawberry leaf extract to enhance its anti-inflammatory activity and the raw and fermented extracts were subjected to FTIR analysis for the detection of functional groups. In contrast to the extract, different peaks were detected such as peaks at 3218 and 1358 cm”1 are indicators of OH, peaks at 2958 and 2924 cm”1 corresponds to –CH group. 1595 and 1043 cm”1 could be attributed to the stretching of C=C and C–O groups, respectively. [26]

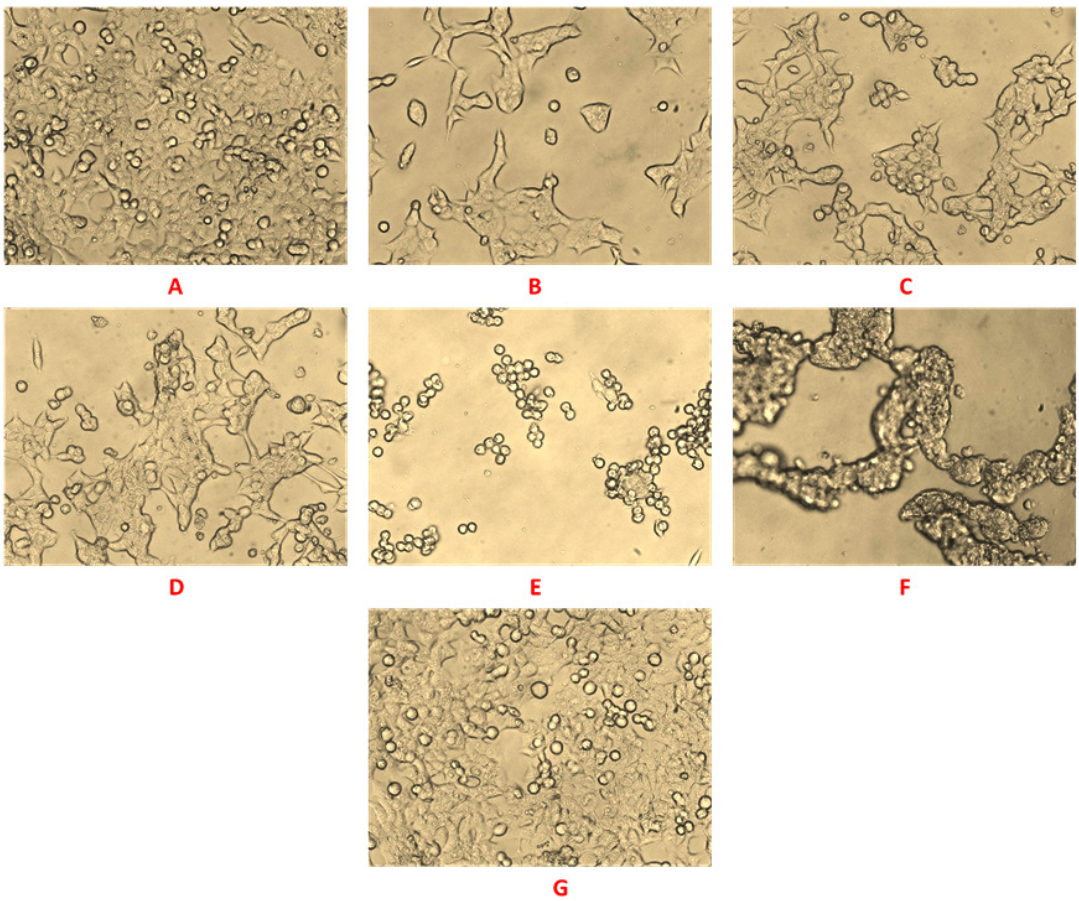

Fig. 8. Effect of GABA against HCT 116 Cells
Cell invasion image of the HCT 116 Cells A-12.5µg; B-25 µg; C-50 µg; D-100 µg; E-200 µg; F- standard and G-untreated cells

An antimicrobial compound was isolated from *Lactobacillus delbrueckii* subsp. lactis and characterized using FTIR, which revealed peaks ranging from 3227.4 to 1260 cm{ [1], corresponding to different functional groups such as C=N, NH, and OH.[27] GABA from the mulberry leaf shows peaks at around 3379, 2925, 2851, 1738, 1650, 1550, 1247, 1084, and 768 cm-1 and no new chemical group was produced, the particle size were similar. Peak at 3379cm-1 corresponds to intermolecular hydrogen bond OH. [28]

Zhang *et al*,[29] reported that *L. planatrum* was produced 1.52±0.07g/L. Valenzuela *et al* [30] stated *L.brevis* has the ability to produce GABA and which consistent with other reported level of *L. brevis* LMG9606 (0.29g/L). Hwang *et al,* [31] examined GABA production with MRS broth with *L.lactic* strain and found that 1.37g/L of the GABA after 40h of incubation.

Iman *et al,* [32] found that, *E.coli, P.aeruginosa, S. typhi, S.aureus,* and *E.faecalis* exhibited zone of inhibition values of 16mm, 14mm, 19mm, 18mm, and 11mm respectively when tested against *Lactobacillus* using the agar well diffusion method. The supernatant showed the highest inhibitory zone against *S.typhi* growth at 19mm, while the least inhibitory zone was observed against *E. faecalis* at 11mm.

Tanim *et al,* [33] stated that the antimicrobial activity of the two lactic acid bacteria was primarily assessed using the agar diffusion assay. The cell-free supernatant (CFS) was used to test the inhibitory effects against seven Gram-negative bacteria (*A. baumannii, E. coli, K.pneumoniae, P.aeruginosa, S.abony, S.typhi, S.flexneri*) and three Gram-positive bacteria *(B. cereus, B. subtilis, S. aureus*). The CFS of both isolates exhibited a broad antimicrobial spectrum, indicating the presence of inhibitory activity. The *L. fermentum* strain demonstrated the ability to inhibit all test strains, with a minimum inhibition zone of 9 mm observed against most pathogens. On the other hand, the *L. brevis* strain displayed a similar antimicrobial spectrum, albeit with relatively smaller inhibition zones (<6 mm) and a minor zone against *A. baumannii*. Notably, both isolates exhibited the highest effectiveness against *P. aeruginosa* in the diffusion assay.

Isolation of *L. plantarum* MYSN7 from a traditional fermented food, haria against fungi shows good antifungal effect against *T.tonsurans.* [34] The antifungal activity of the GABA against the postharvest pathogen *A.alternata* as a controlling post harvest disease in fruits and vegetables, these findings confirmed the information about the antifungal activity of the GABA. [35] In another study, the best antifungal agent was screened from 60 isolated *Lactobacillus* species, revealing that many *Lactobacillus* strains exhibit antifungal activity against molds and yeasts, including *Aspergillus* sp., *Alternaria* sp., *Geotrichum* sp., *Mucor*, and *Fusarium* sp. However, the level of antifungal activity varied, ranging from moderate to poor, depending on the specific compounds extracted. [36]

The GABA compound can elicit morphological changes that indicate programmed cell death. Probiotics, particularly *lactobacilli*, have been studied for their anticancer effects on colorectal cancer cells. *L. acidophilus* extract and supernatant reduced colorectal cancer cell proliferation and increased apoptosis and necrosis. [37] Another study found that *L. acidophilus* causes apoptosis in cervical, gastric, breast, and colorectal cancer cells. [38]

Combining *L. acidophilus* and *L. casei* extracts can cause apoptosis in colorectal cancer cells. [39] A different study on *Lactobacillus* revealed that external polysaccharides reduce BCL-2 and survivin gene expression while increasing Caspase-3 (Cas3), Caspase-9 (Cas9), and BAX gene expression in colorectal cancer cells, ultimately inducing cell death. [40]

The anticancer activity of CaCo-2 and HT 29 cells using *Lactobacillus* culture free supernatant showed increasing cytotoxicity at the concentration of 800 µL/mL. Similarly, the growth of HT-29 cells was inhibited in a dose-dependent manner following treatment with CFSs. The inhibitory rates were comparable to those of 2.5 ìM 5-fluorouracil (5-FU), a positive control known to inhibit approximately 50% of HT-29 cells. [41] These discussions suggests that, *Lactobacillus plantarum* components in the GABA compound may promote colorectal cancer cell death by regulating apoptosis-related gene expression. This preliminary study focused on the anticancer effects of GABA from *Lactobacillus plantarum* on HCT 116 cells. While normal cell comparisons were not included, *Lactobacillus plantarum* is a probiotic

known for its non-toxic nature, suggesting no impact on healthy cells.

CONCLUSION

This study demonstrated the successful isolation of *Lactiplantibacillus plantarum* from milk using MRS broth and the production of ã-aminobutyric acid (GABA), which was precipitated using an ethyl acetate solution. The extracted GABA was characterized using Fourier Transform Infrared Spectroscopy (FTIR) and confirmed through Thin-Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC), yielding 1.8 g/L. Antimicrobial studies revealed significant antibacterial activity against *Escherichia coli, Salmonella typhi, Klebsiella pneumoniae*, and *Shigella dysenteriae*, as well as antifungal activity against *Aspergillus niger* and *Aspergillus flavus*. Additionally, the cytotoxicity study demonstrated effective toxicity against the HCT 116 cell line, with an IC_{50} value of 103.48 µg/mL. This study presents a novel approach for extracting and confirming GABA from *L. plantarum*, highlighting its potential as a natural antimicrobial and anticancer agent.

ACKNOWLEDGEMENT

The authors express heartfelt thanks to the management and staffs of Centre for Bioscience and Nanoscience Research, Eachanari, Coimbatore-21, Tamilnadu, India, for giving the facility to complete the research work.

Funding Sources

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Conflict of interest

The authors do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials**.**

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Not Applicable.

Author's Contribution

Gopalakrishnan. Rajesh – Research work and Writing, Ramachandran Ragunathan- Conceptualization and Reviewing, Jesteena Johney- Supervision, Reviewing and Editing

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