

IN VIVO ACUTE TOXICITY, ANTIBACTERIAL, ANTI-AQUATIC FUNGAL, ANTHELMINTIC ACTIVITY OF *LACTOBACILLUS PLANTARUM* KP894100 AND *LACTOBACILLUS ACIDOPHILUS* KP942831

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ABSTRACT

Objective: This study observed the antibacterial, antifungal (aquatic and pathogenic fungi), and anthelmintic properties of *Lactobacillus plantarum* (LP) KP894100 and *Lactobacillus acidophilus* (LC) KP942831, isolated from a dairy product.

Methods: Lactic acid bacteria traditionally used to improve human health. For *in vitro* antibacterial, antifungal, and anthelmintic studies of intracellular cell-free extract (ICFE) from LP and LC were produced separately by using filtration methods. ICFE was freeze-dried then resuspended in citrate phosphate buffer. This ICFE is further used for the antimicrobial and anthelmintic assay. In the antimicrobial assay, ICFE were tested against pathogenic bacteria, i.e., *Bacillus subtilis*, *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, and aquatic fungal species including: *Aspergillus clavatus*, *Pythium aphanidermatum*, *Saprolegnia parasitica*, *Candida albicans*, *Aspergillus fumigatus*, *Fusarium oxysporum*, *Alternaria alternata*, *Curvularia* sp., *Mucor* sp., *Rhizopus stolonifer*, *Aspergillus niger*, and *Penicillium chrysogenum*. In the anthelmintic assay, young stages of *Pheretima posthuma* (Indian earthworms) were used. For the analysis of acute toxicity assay different graded doses (100, 250, 500, 1000 mg/kg bw) of ICFE of LP and LC was administered in Wistar albino rats, respectively, and the control group received distilled water.

Result: The ICFE of both *Lactobacillus* strains showing strong antibacterial and weak antifungal activity against aquatic fungi except, *S. parasitica*, and *C. albicans*. The ICFE shows 100% paralysis and killing efficacy against *P. posthuma* in 48-72 hrs. In the acute toxicity test, LP and LC did not produce any toxic signs or death at the maximum concentration 1000 mg/kg bw.

Conclusion: ICFE of LP and LC possess anthelmintic activity against *P. posthuma* with only strong antibacterial activity. Both the *lactobacillus* strains have strong antibacterial activity and have potential activity against *P. posthuma* helminthes.

Keywords: Antimicrobial, Antifungal, Aquatic fungi, Anthelmintic activity, *Pheretima posthuma*.

INTRODUCTION

Contagious and parasitic infections continue to be a considerable burden in developing countries. Helminth infections are the most common infections in humans, distressing an enormous population of the world. Helminthiasis is the most important animal diseases inflicting heavy production losses. Chemical control of helminth infection coupled with improved management has been the important worm control strategy throughout the world [1]. However, the majority of parasitic infections is mostly limited to tropical regions and causes a huge threat to human health and contributes to the occurrence of malnutrition, anemia, eosinophilia, and pneumonia [2]. Parasitic diseases cause ruthless morbidity affecting the principal population in endemic areas [3]. The gastrointestinal (GI) helminths become resistant to currently available anthelmintic drugs; therefore, there is a foremost problem in the treatment of helminth diseases [4]. However, increasing problems of development of resistance in helminths against drug have led to the proposal of screening labor, natural products for their anthelmintic activity which are safe for human as well as animal.

The validation of the antibiotic properties of lactic acid bacteria (LAB) is greatly needed because the development of new drugs from these bacteria will complement the decreasing arsenal of potent antibiotic and antiparasitic agents currently available. Nowadays, probiotic bacteria are most commonly used as an alternative to antibiotics [5]. Using of probiotic bacteria, the chance of drug resistance may decrease. In addition, LAB with health promoting activity is willingly accessible for human use and once their scientific validation and efficacy are

known the value of these bacteria need no longer be based on folkloric recommendations. Therefore, the objective of this work was to explore the anthelmintic and antimicrobial properties of LAB. Previously, no paper reported the effect of LAB on aquatic fungi and helminths *Pheretima posthuma*.

The LAB are conventionally used to improve immune system also used in pharmaceutical as an alternative of antibiotic [6], antimicrobial [7], anticancer [8], antidiabetic [9], Immunomodulatory [10], lactose intolerance, as well as bio preservatives in food [11,12], and improvement of gut microflora or to manage gut related problems [13]. Certainly, in a recent study, the intracellular cell-free extract (ICFE) from LAB showed *in vivo* antiproliferation of cancer, antimutagenic and anticarcinogenic activity [14]. Since LAB was safe recognized by WHO and FDA in 1990 [15], at the present increasing use of this bacteria as living medicine [16-18]. Since few studies so far exist on the biological activity of these bacteria to the best of our knowledge. The study reported here focuses on the *in vivo* acute toxicity of ICFE of LAB as well as its efficacy as an antimicrobial and anthelmintic agent.

METHODS

Bacterial culture

220 *Lactobacillus* strains have been isolated from milk and different dairy products commercially available in the local markets such as cheese, yogurt, and curd. The isolation was performed by the routine microbiological procedure and inoculation on MRS broth [19]. The isolated colonies will be screened for antimicrobial potential against

Escherichia coli; those bacterial colonies give the highest inhibition zone will be further identified by 16S rRNA sequence analysis. The sequence of the organisms was deposited in the GenBank nucleotide sequence databases under accession number KP894100, KP942831 for *Lactobacillus plantarum* (LP) and *Lactobacillus acidophilus* (LC), respectively.

Preparation of ICFE of LP and LC

For antimicrobial activity, preparations of ICFE are done according to the method is given by Chiu *et al.* (2013) with slight modification [20]. 800 ml of MRS broth contains cultures of LP and LC was harvested by centrifugation (10,000 g, 30 minutes, and 4°C) and washed twice with phosphate buffered saline, total cell numbers were adjusted to 10⁸ CFU/ml, respectively. Pressure Cells Press (Thermo Electron) was used for cell disruption, then centrifuged at 10,000 g for 15 minutes, and the supernatant was filtered through syringe filters (0.45 mm pore size; Millipore). The sterile cell-free supernatant (CFS) was freeze-dried and resuspended (to a 15-fold concentration) in 20 mM citrate phosphate buffer (pH 3.4). This ICFE is used for further different assays.

Acute toxicity

For acute toxicity testing in animals, preparation was carried out according to the standard method is given by Zhou *et al.* [21] and Wolf *et al.* [22]. Wistar rats of either sex (weighing 125-150 g) were purchased from the Department of Research and Defence Establishment, Gwalior. The animals were maintained in a well-ventilated room, fed on commercial balanced pellet feed, and water ad libitum. Prior to acute toxicity evaluation, the animals were acclimatized to standard conditions of temperature, moisture, aeration, and nutrition in the animal house of the Department of Pharmaceutical Sciences, Dr. Hari Singh Gour University. All studies on animals were approved by the Institutional Animal Ethics Committee (IAEC, DHSGU Sagar) with sanction number CPSEAE-48/13.

The Wistar rats were divided into 5 groups; each group contains 6 rats. Group 1 serves as a control; the rest is treated by oral administration of graded doses 100, 250, 500, 1000 mg/kg bw of ICFE for 7 days.

Antimicrobial activity

Pathogenic bacterial and aquatic fungal strain; culture media

The bacterial strains used in this study included as representative of Gram-positive bacteria *Lactobacillus viridescens* NCIM 2167, *Lactobacillus jugurti* NCIM 2367, *Lactobacillus pentosus* NCIM 2669, *Bacillus subtilis*, MTCC 1143, *Enterococcus faecalis* MTCC 439, *Staphylococcus aureus* MTCC 9886 and *Pseudomonas stutzeri* MTCC 8350, *Pseudomonas aeruginosa* MTCC 6642, *E. coli* MTCC 433, *Proteus vulgaris*, *Staphylococcus epidermidis* MTCC 7919, *Salmonella* Typhimurium, *Vibrio cholera* MTCC 3906, *Klebsiella pneumonia* was used as representative of Gram-negative strains.

The pathogenic aquatic fungus culture is *Aspergillus clavatus* MTCC1323, *Candida albicans* MTCC 7315, *Pythium aphanidermatum* MTCC 10247, *Saprolegnia parasitica* MTCC718, were procured from microbial culture collection center (MTCC) Chandigarh, India. *Alternaria alternate*, *Aspergillus niger*, *Aspergillus fumigatus*, *Curvularia* sp., *Fusarium oxysporum*, *Mucor* sp., *Penicillium chrysogenum*, *Rhizopus stolonifer*, is represented as pathogenic fungi collected from the culture collection center of the department of botany, Dr. H.S. Gour University, Sagar, India. The preparation of bacterial strain and fungal spore for antibacterial activity was done according to the method given by Hladikova *et al.* [23].

Antimicrobial assay

The disk diffusion method was used for antimicrobial assay according to the method given by Oliveira *et al.* [24]. Briefly, indicator bacteria were grown in Muller-Hinton broth (HiMedia, India) overnight and spread onto the soft agar (1%, w/v) plate of MH Agar after diluting to 10⁷ CFU/ml. Subsequently, 50 µl samples prepared from filtrate was absorbed by sterile disc these discs transferred to MHA plates then

incubated at 37°C for 24 hrs, the inhibitory activity was determined by measuring the radius of inhibition zone around the disc in mm. MRS broth adjusted at pH 6.5 served as control. All the assays were carried out in triplicate. Gentamycin and streptomycin were used as positive control.

Antifungal assay

The LAB was screened for antifungal activity by disk diffusion assay as described by Magnusson and Schnurer, 2006 with slight modification [25]. Briefly, the MRS agar plates containing 10⁴ spores/ml agar were prepared. Sterile disk with 50 µl of ICFE was put on the MRS agar plate allowed to diffuse into the agar during a 5 hrs pre-incubation period at room temperature, followed by aerobic incubation at 30°C for 48 hrs. The antifungal effects recorded were as clear zones around the disk; Standard drug Nystatin, griseofulvin is used as positive control.

Anthelmintic assay

Anthelmintic activity was carried out according to the method suggested by Ahirwal *et al.* (2010) with slight modifications [26]. Indian young earthworms (*P. Posthuma*) collected from the moist soil of the vermin farm Sagar (MP), India and washed with normal saline and used for the study. The ICFE of both bacteria were dissolved in 20 mM citrate phosphate buffer and prepared desired concentrations (100, 250, 500, and 1000 mg/ml). Piperazine citrate (20 mg/ml) was used as the standard drug. All the samples, standard drug and 4 different concentrations of the ICFE were freshly prepared before starting the experiment. 14 groups containing 6 earthworms in each group were placed into 15 ml of ICFE concentration. Group 1 served as control being treated with 20 mM citrate phosphate buffer (control group) while Group II was treated with the standard drug (Piperazine citrate) 20 mg/ml and 2 sets of 5 different groups were treated with extracts of respective concentrations. Observations were made for the time taken for paralysis and death of individual worm. The paralysis was said to occur when the worms were not able to move even in normal saline while the death was concluded when the worms lost their motility followed with fading away their body colors [27]. Assays were conducted in duplicate.

Statistical analysis

Data were expressed as the mean, standard deviation of the means and statistical analysis were carried out employing one-way ANOVA. Differences between the data were considered significant at p<0.05. For the anthelmintic assay, each experiment was run in duplicate and the mean calculated using Excel software.

RESULTS

Acute toxicity studies

There was no mortality in the treatment and control groups after 24 hrs of ICFE administration. None of the rats died during the 7 days of observation. No adverse effects were noticed in the behavior of the rats in any of the treatment or control groups. There was no drop in body weight in any of the groups as well. A physical examination prior to euthanasia on the 7th day showed that the rats looked healthy with no ruffled hair or any abnormality in the body. Organ examination did not reveal any abnormalities or gross lesions. Acute toxicity studies of ICFE of LAB have been extensively carried out and found non-toxic at concentrations as high as 1000 mg/kg body weight of animals.

Antibacterial spectrum

To evaluate the antibacterial activity of the ICFE of LP and LC, the pathogenic bacterial strains were exposed to the 50 µl of ICFE. Both the tested ICFE showed different bioactivities at 50 µl of concentration. Remarkably, the ICFE of LP showed the highest degree of activity against all microbial strains (Figs. 1 and 2). Furthermore, both ICFE showed the highest antibacterial activity against *S. Typhimurium*.

Antifungal assay

The disk diffusion method was used to detect the antifungal spectrum of ICFE of LP KP894100 and LC KP942831 against toxin producing

fungi (*Aspergillus* spp., *Penicillium* spp., *Fusarium* spp.), human disease-causing aquatic fungi (*C. albicans*) and food spoilage fungi (*Penicillium* spp., *Fusarium* spp.) were determined by disk diffusion assay. ICFE of LP had a broad antifungal inhibitory spectrum, against 2 aquatic fungi, i.e., *S. parasitica* and *C. albicans*. The inhibitory effects of ICFE on the growth of different fungal strains are presented in Figs. 3 and 4. Both *Lactobacillus* strains restricted the germination of fungal spore of most of the fungal strains. However, great variation in growth inhibition was observed between different fungi, some fungi being more resistant to the antimicrobial substances produced by these lactobacilli. However, the fast-growing Ascomycetes *A. niger* was only marginally affected.

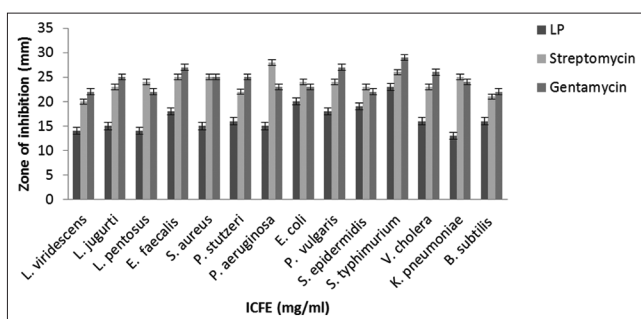


Fig. 1: Growth inhibition (zone of inhibition) of pathogenic bacteria by intracellular cell-free extracts of *Lactobacillus plantarum* as measured by disk diffusion methods. Streptomycin and Gentamycin are used as standard drugs. The results are means±standard deviation of triplicates, p≤0.05

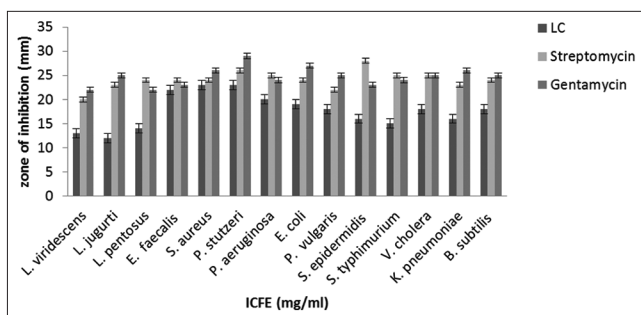


Fig. 2: Growth inhibition (Zone of inhibition) of pathogenic bacteria by intracellular cell-free extracts of *Lactobacillus acidophilus* as measured by disk diffusion methods. Streptomycin and gentamycin are used as standard drugs. The results are means±standard deviation of triplicates, p≤0.05

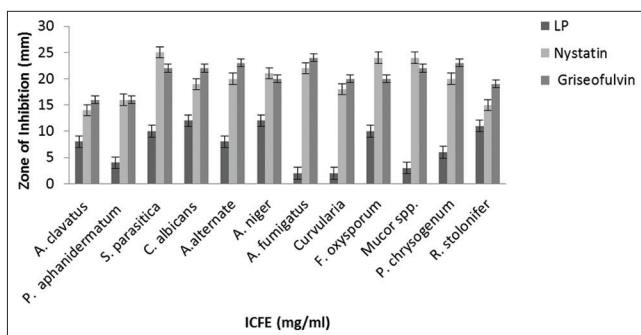


Fig. 3: Growth inhibition (zone of inhibition) of fungi by intracellular cell-free extracts of *Lactobacillus plantarum* as measured by disk diffusion methods. Nystatin and Griseofulvin are used as standard drugs. The results are means±standard deviation of triplicates, p≤0.05

The antifungal activity differed only slightly between incubation temperature at 25 or 30°C. In particular, the *C. albicans*, *S. parasitica*, and *A. niger* were insensitive to ICFE. The LAB strains were also effective against *Rhizopus*, *Mucor*, *Curvularia* strains. Although the strains of lactobacillus are isolated from different sources had surprisingly similar antifungal spectra.

Anthelmintic assay

The anthelmintic activity of ICFE was assessed on the soil nematode *P. posthuma*. Anthelmintic activity was observed against the young stage of *P. Posthuma* mostly during 48-72 hrs post-treatment (Fig. 5), while both the ICFE from LAB demonstrated potent anthelmintic activity, killing 100% of the young worms at 100 mg/ml in 48-72 hrs (Fig. 6). From the observations made, a higher concentration of ICFE produced a paralytic effect much earlier and the time of death was shorter for all worms. ICFE showed anthelmintic activity in a dose-dependent manner, giving the shortest time of paralysis (P) and death (D) with 500 mg/ml concentration, for all young worms. ICFE exhibited more potent activity at higher concentration (1000 mg/ml). Evaluation of anthelmintic activity was compared with reference standard Piperazine citrate (Fig. 5).

DISCUSSION

The selection of an appropriate method for the ICFE production is fundamental to initial successful prediction of the biological activity of LAB. Based on the activity observed, the efficacy of the general preparations of ICFE might be enhanced by the use of different purification techniques such as purified by reversed phase-high-performance liquid chromatography. The LAB are traditionally used without extraction of ICFE and have the possibility of giving different results compared to ICFE. Unfortunately, the effect of the LAB when

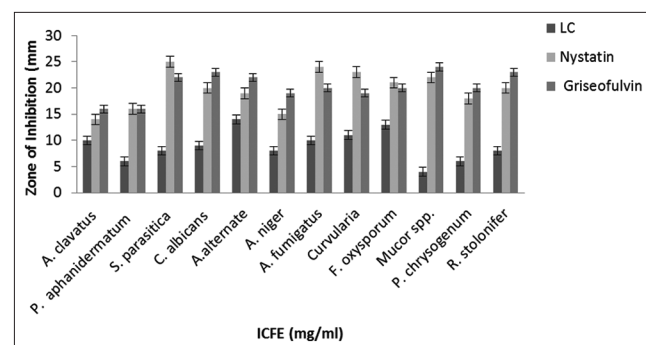


Fig. 4: Growth inhibition (zone of inhibition) of fungi by intracellular cell-free extracts of *Lactobacillus acidophilus* as measured by disk diffusion methods. Nystatin and Griseofulvin are used as standard drugs. The results are means±standard deviation of triplicates, p≤0.05

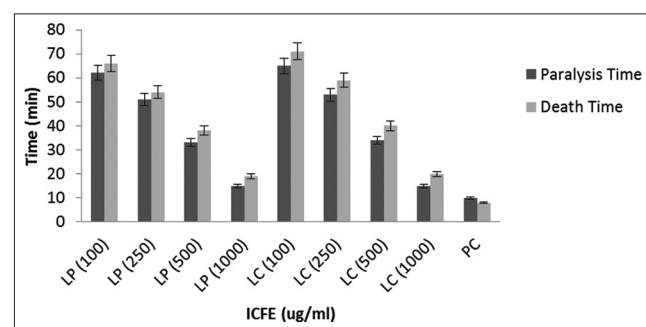


Fig. 5: Anthelmintic activity of intracellular cell-free extract of *Lactobacillus plantarum* and *Lactobacillus acidophilus*, Piperazine citrate used as standard drug. The results are means±standard deviation of triplicates, p≤0.05

consumed directly can only be measured properly in an *in vivo* model. Nevertheless, *Lactobacillus* sp. Least reported in the literature against different disorders prevalent in the human population; hence, assessment of toxic effects of ICFE from both the bacteria were essential in our study too. Finally, 4 doses of each ICFE were chosen on the basis of acute toxicity studies for anthelmintic activity.

The antibacterial activity indicates that ICFE of LP possesses strong activity against the microorganisms as compared to ICFE of LC (Figs. 1 and 2); However, the ICFE of LC showed moderate activity against the fungus *S. parasitica* (10.35±0.21) and *C. albicans* (12.32±0.44) (Fig. 4). The antibacterial activity of LC observed against *S. aureus* (Fig. 2). Compared to the observed by Oh *et al.* (2000), the CFS of LC 30SC have the potential to inhibit some gram-positive pathogenic bacteria including *Bacillus cereus* ATCC 11778, *Listeria ivanovii*, and *S. aureus* [28]. The ICFE may have potential use as a food additive in the food industry, particularly processed food, in which some *Bacillus* species are potential spoilage microorganisms.

The young earthworms (2-4 cm in length and 0.1-0.2 cm in width) were used for all the experimental protocols due to their anatomical and physiological resemblance with the intestinal roundworm parasites of human beings. More than one billion people are infected worldwide annually by soil-transmitted helminths, which are parasitic intestinal nematodes [29-31]. The validation of anthelmintic activity of LAB is a great necessity as this could lead to recommendations for the improved use of LAB as well as provide opportunities for the discovery of new anthelmintic drugs. In this study, there was good antiparasitic activity was recorded against the young stage of the nematode *P. posthuma*. The ICFE of LP and LC demonstrated the best activity killing 100% of the young worms within 72 hrs at 1000 mg/ml (Fig. 5). Further studies to identify the active antiparasitic compound(s) in ICFE, as well as activity against other helminths is recommended. There is no study reported on the anthelmintic effect of LAB while traditionally many plant parts are used as anthelmintic drugs [32-34].

The LAB did not produce mortality in rats at the highest CFU tested. Attempts to escalate the dose failed due to the fact that 10⁸ cfu/ml was the maximum amount that was able to maintain healthy gut intestinal health. Despite the fact that no adverse effects were observed, it is recommended that a chronic toxicity study be done in the future to confirm the safety of this product for long term usage. Even though LAB is used as probiotic drugs for the treatment of many disorders in some developing countries where access to modern health facilities is limited, generally very little is known about their toxicity and this applies also to LAB. Despite the fact that efficacy is one of the overriding criteria in the selection of LAB for use in healthcare systems, safety should never be overlooked, and formal toxicological evaluation of LAB should be done as part of the validation process.

From the above results, it is concluded that the presence of LAB in dairy products, traditionally used by humans to improve intestinal health, showed significant anthelmintic activity. The experimental evidence obtained in the laboratory model could provide a rationale for the traditional use of this LAB as anthelmintic. The ICFE may be further explored for its biochemical profile to recognize the active constituent accountable for anthelmintic activity.

CONCLUSION

This study exhibited an anthelmintic as well as a strong antibacterial and weak antifungal activity of 2 lactobacillus species, i.e., LP and LC. There is no-observed adverse-effect of the bacteria in the acute toxicity study in rats at the highest concentration of ICFE. Both the bacteria are safe for long-term treatments considering the live bacteria at which no toxicity is detected, the anthelmintic dose may, in fact, be reached in the GI-tract and thus give support to the traditional use of LAB as living medicine to control worms. On the other hand, the strong antibacterial activity founds in ICFE confirm that its use in the treatment of infectious diseases is causing by pathogenic bacteria.

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