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THERAPEUTIC ACTIVITY OF CONJUGATED LINOLEIC ACIDS SYNTHESIZED BY *LACTOBACILLUS PLANTARUM*

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ABSTRACT

Conjugated linoleic acids are the most biologically active fatty acids, synthesized by biohydrogenation of linoleic acid (C18:2).In the present study, *Lactobacillus plantarum* was used as a probiotic model for the biotransformation of linoleic acid to conjugated linoleic acids. The therapeutic applications of conjugated linoleic acids were validated through in-vitro and in-vivo studies. The anti-inflammatory activity was evaluated with cotton pellet induced granuloma and hydroxyl proline content in wistar rats. Oral administration of conjugated linoleic acid and linoleic acid showed dose-dependent anti-inflammatory activity. Antioxidant propertiesof conjugated linoleic acid analyzed by catalase, superoxide and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay showed significant antioxidant activity. The anti-proliferative activity assessed through morphological findings and neutral red assay exhibited conjugated linoleic acid as an effective cytotoxic agent against Estrogen Receptor-negative breast cancer cell line. The results clearly endorse the traditional use of probiotics by confirming the therapeutic potential of conjugated linoleic acids.

KEY WORDS: **Conjugated linoleic acids, Lactobacillus plantarum, linoleic acid, Neutral red assay**

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INTRODUCTION

Probiotics are considered as one of the most important component of functional foods and have varied beneficial effects on human health. Probiotics are defined as live microorganisms which when administered in adequate quantities; they confer health benefits on the host. It is well documented that probiotics use specific cellular and molecular mechanisms with diverse functions like blocking pathogenic bacteria effects, regulation of immune responses, alteration of intestinal epithelial homeostasis by promoting cell proliferation.[1]Lactobacillus, Bifidobacteriumand *Saccharomyces cerevisiae* are the commonly used strains, as probiotics.[2,3] Both Bifidobacterium and Lactobacillus are Gram +ve bacteria and can grow in an environment with a pH range from 4.5 to 8.5. The consumption of a large quantity of dairy products and fermented milk with Lactobacillus or Bifidobacterium lessened the occurrence of colon cancer.[4]Research reports on probiotics offer evidencesfor the synthesis ofconjugated fatty acid isomersfrom essentialunsaturated fatty acids.Conjugated linoleic acid (CLA) is a refers to a group of 28 distinct positional and geometric isomers of linoleic acid (LA,C 18:2) in which the double bonds are conjugated instead of the methylene interrupted configuration of LA. Out of these isomers, cis-9, trans-11 and Trans 10, cis12-octadecadienoicacids have been reported as the most biologically active fatty acids. CLA isomers are found mostly abundantly in milk, dairy products and meat of ruminant animals.The isomers of CLA are found naturally in some plants or formed in ruminant animals by the process of biohydrogenation, which is facilitated by microbial anaerobic fermentation. This process turns polyunsaturated fatty acids (PUFA) from the animal's food, in particular, plant matter into saturated fats, but any incomplete hydrogenation results in to positional and geometrical isomers of LA, a process which takes place in the rumen. CLA can also be produced commercially by partial hydrogenation of LA or by heating LA under alkaline conditions. The first step in the process of biohydrogenation is the transposition of the delta 12 double bonds of PUFA to form the cis-9,trans-11 isomer, often called rumenic acid (RA). RA can also be formed by the oxidation (introduction of a double bond) of trans-11vaccenic acid by a delta-9 desaturase. These two routes involved in the formation of RA probably contribute to its natural abundance. Further isomerization, and transposition of RA leads to the formation of other, less abundant isomers, such as the trans-10,cis-12 isomer, which exhibits anti-cancer effects.[5,6] (Markey et al 2014;). Probiotic bacteria such as *Lactobacilli* and *Bifidobacteria*species are capable of converting linoleic acid into conjugated linoleic acid (CLA). Currently, CLA has attracted much attention as a novel type of functional lipid due to its exciting implications in several biological activities.With this background, the present study has been designed to investigate the efficiency of *Lactobacillus plantarum* for the production of conjugated linoleic acid and also to demonstrate the therapeutic applications of conjugated linoleic acid derived from *Lactobacillus plantarum.*

MATERIALS AND METHODS

Lactobacillus plantarum (ATCC 8014); MRS broth (HiMedia Laboratories); Linoleic acid (99% purity, Sigma Aldrich); FBS (GIBCO Laboratories); DMEM, Trypsin, PBS (HyClone); Indomethacin, (Himedia Laboratories); LPS, Protease inhibitor cocktail for mammalian cell lysate.

Growth of Probiotics

Lactobacillus plantarum (ATCC 8014) is used as microbial model for the bioconversion of linoleic acid into conjugated linoleic acid. The cell-free extract of lactobacilli is employed for the extraction of lipids .[6] The CLA isomers were characterized based on the retention time after GC–MS analysis with authenticated standards.

Antibacterial activity of LP-CLA

The effectof LP-CLA on the growth of bacteria was evaluated by disc diffusion method .The extracted LP-CLA was dissolved in ethanol and 20µl of the resultant compound, in different concentrations ranging from 10 to 100µg/disc, were transferred onto the sterile 6mm discs and incubated at 37ºC for 24 h with Streptomycin (30µg/disc) as positive control. Negative control measurements were carried out with 1% ethanol. The growth inhibition was determined by measuring the diameter of the clear zone on the agar medium. All the experiments were carried out in triplicates and mean zones of inhibition were recorded.

DPPH radical scavenging assay

LP-CLA was tested for scavenging activity against DPPH (2, 2-Diphenyl-1-picrylhydrazyl).[7]Briefly, 1ml of 0.2mM DPPH solution in methanol was mixed with different concentrations of LP-CLA (25-200µg/ml). The reaction mixture was vortexed and kept in the dark for 15min at room temperature. The absorbance of the solutions was measured at 517ηm against a blank.The percent of radical scavenging activity was calculated based on the control value.

Effect of LP-CLA on the activity of Anti-oxidant enzymes

The antioxidant property of LP-CLA was determined using MDA-MB-231 cell line as *in vitro* model. The cells were treated with different concentrations of LP-CLA (20, 40 and 60µg/ml) for 24h and incubated at 37ºC in 5% CO_2 atmosphere. The treated and untreated cells were trypsinized and washed with PBS. The cells were lysed with 1% Triton X-100 with 1mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 10,000rpm for 15min. The supernatant was collected andfurther utilized for determining the activities of the antioxidant enzymes like Catalase (CAT), Superoxide dismutase (SOD) and Glutathione peroxidase (GPx). The protein content of the cellular extract was estimated according to BCA method.

Measurement of Catalase activity

Cellular catalase was determined as per the method of Claiborne.[8] Briefly, the assay mixture containing 2.4 ml of phosphate buffer (50mM, pH 7.0), 50µl of enzyme source, initiated with 10µl of 19mM hydrogen peroxide

(H_2O_2). The decrease in absorbance was measured immediately at 240ηm, against blank containing all the components except the enzyme source at the 15s intervals for 3min on a UV-Visible spectrophotometer (Shimadzu-1601). The catalase activity was determined using the molar extinction coefficient 43.6M/cm of H_2O_2 and the enzyme activity was expressed as μmoles of H_2O_2 consumed/min/mg protein.

Superoxide dismutase activity assay

The total cellular Superoxide dismutase activity (SOD) activity was measured by monitoring the auto-oxidation of epinephrine.[9] Briefly, 100μl of cellular extract was added to the mixture containing 880μl of 0.05M carbonate buffer containing 0.1mM EDTA (pH 10.4) and 20μl of 30mM epinephrine (in 0.05% acetic acid)and mixed well.The absorbance was measured at 480ηm at an interval of 30s for 5min and expressed as the SOD enzyme required for 50% inhibition of epinephrine /min/mg protein.

Glutathione peroxidase assay

Cellular GPx activity was measured with the reaction mixture (1ml) containing 50mM potassium phosphate (pH 7.0), 10mM reduced glutathione (GSH), 2.4 U/ml glutathione reductase (GR), 1.5mM NADPH and cell lysate and the activity was assayed using 2mM H_2O_2, and the rate of NADPH oxidation was monitored immediately at 340ηm against a blank.GPx activity is calculated and expressed as ηmole of NADPH oxidized/min/mg protein.[10-11]

Animals and Acute toxicity test

Wistar rats (120-150g) were fed with commercial diet, and water ad libitum and maintained at 25± 2ºC with the dark and light cycle of 14/10h. The animals were divided into three groups of six animals each. *Lactobacillus plantarum* (1x1012 CFU/ml) and LP-CLA (2000mg/kg) were administered orally as a single dose to rats as per OECD guidelines-423. After administration of Lactobacillus plantarumand LP-CLA, the rats were observed, periodically for symptoms of toxicity and death within first 24h and then every consequent p day for remaining next 14 days.[12] The serum was separated from the blood drawn from the control and the treated groups by centrifugation at 2500rpm for 10min and utilized for assessing liver function makers like Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline Phosphatase (ALP) and Bilirubin.

Cotton pellet-induced granuloma in rats

The chronic phase of inflammation was evaluated using cotton pellet method.[13] For the experiment, the male wistar rats were separated into seven groups with each group containing six animals (n=6). Group-(A) - served as control and received distilled water (10 ml/kg, p.o.); (B) - disease control group, (C) - animals were treated with Indomethacin (10mg/kg, p.o.); (D)- animals were treated with *Lactobacillus plantarum* (2x105 CFU/ml/kg, p.o.); (E) - animals were treated with *Lactobacillus plantarum* (2x107CFU/ml/kg, p.o.); (F) - animals were treated with CLA (5mg/kg, p.o.);(G) - animals were treated with CLA (10mg/kg, p.o.). Cotton pellets, weighing 5mg each, were sterilized and the pellets were implanted subcutaneously through a skin incision at the back of the animals. Every group was treated either with drug or vehicle through the oral catheter, and the treatment was continued for seven consecutive days from the day oneof cotton pellet implantation. At the end 7^{th} day, i.e., on the eighth day, the animals were anesthetized with ether and the granuloma was removed and dried for 24h at 60ºC and the dry weight of every granuloma was determined individually. The difference between the initial and final dry weight was considered as the weight of the granulomatous tissue produced and was expressed as percent Inhibition of granuloma.

Estimation of Hydroxyproline

In order to estimate the amount of hydroxyproline produced during chronic inflammation, the dried granuloma tissue was hydrolyzed with 6N HCl at 130ºC in sealed tubesfor 4h. The hydrolysate was neutralized to pH 7.0 and was subjected to chloramine-T oxidation for 20min. The reaction was terminated by addition of 0.4M perchloric acid. Ehrlich reagent was added and incubated at 60ºC to develop color and this color intensity was measured at 557ηm using UV-Visible spectrophotometer .[14]

Neutral red assay

The effect of LP-CLA on the proliferation of MDA-MB-231 cells was evaluated based on uptake of Neutral red by the cells.[15] The MDA-MB-231 cells were seeded with 1 X105 cells per well with a final volume of 0.2ml and incubated at 37ºC for 24h. The cultured MDA-MB-231 cells were treated with different concentrations of LP-CLA (20, 40, 60, 80 and 100μg/ml) and 1% ethanol as control and incubated for two different time periods, i.e. 24 and 48h. After the exposure to the LP-CLA, the media was removed, washed with 0.01M PBS and replaced with media containing 0.033% Neutral Red dye in warm PBS to stain unlysed cells. Later the cells were treated with 0.5ml of solubilization buffer for 20 min and the amount of neutral red dye released was measured in a microplate reader (Biotek-255907) at 570ηm. The percentage of cell survival per sample was calculated and accordingly, the IC50 value was determined.

RESULTS AND DISCUSSION

Characterization of LP-CLA isomers produced by Lactobacillus plantarum

The fatty acids extracted from the cell-free supernatant of *Lactobacillus plantarum* culture were analyzed by GC-MSanalysis. Fatty acids extracted from the *L. plantarum* showed two major peaks, with retention times of 21.42 and 21.50 (Figure-19C). The Peak-1 with 21.42 is confirmed as cis-9,trans-11 CLA and the peak-2 at 21.50 is confirmed as trans-11,cis-12 CLA based on the comparison with standard CLA and the verification with NIST database. From the results, it can be inferred that *L. plantarum* (ATCC:8014) is potential to convert linoleic acid into cis-9,trans-11 and trans-10,cis-12 CLA isomers and the relative abundance of these two isomers was found to be in the ratio of 60:40.

GC chromatogram of LP-CLA isomers

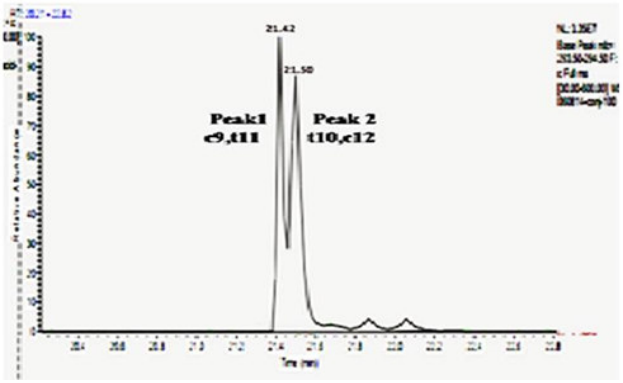

Figure 1
Partial GC chromatogram of LP-CLA isomers extracted from Lactobacillus plantarum

Anti-bacterial activity of LP-CLA

The antibacterial activity of LP-CLA at different concentrations was tested against human pathogens by disc diffusion method (Fig.2). Ethanol (1%) was used as vehicle control and streptomycin (30µg/disc) as standard antibiotic control.LP-CLA showed minimum inhibitory concentration of 40µg/disc with *Staphylococcus aureus* and 60µg/disc with *Bacillus cereus*, *Klebsiella pneumonia and Pseudomonas aeruginosa*.Gram-positive bacteria showed more distinct antibacterial activity comparedGram-negative bacteria.The influence of CLA on the growth of certain food borne and pathogenic bacteria were investigated and CLA-K inhibited the growth of food borne pathogens.[16]The results are in tune with the antibacterial activity of potassium salt of CLA.[16] However, CLA treatment did not show any activity against Escherichia coli.

Table 1
Evaluation of antibacterial activity of conjugated linoleic acid produced by Lactobacillus plantarum.

Bacterial species	* Zone of inhibition by LP-CLA in mm					*Streptomycin
	20µg/disc	40µg/disc	60µg/disc	80µg/disc	100µg/disc	30µg/disc
Bacillus cereus	-	-	4 ± 1	6 ± 2	10 ± 1	15 ± 2
Staphylococcus aureus	-	2 ± 2	6 ± 2	9 ± 2	13 ± 1	16 ± 1
Klebsiellapneumoniae	-	-	2 ± 2	5 ± 1	7 ± 2	14 ± 2
Pseudomonas aeroginosa	-	-	2 ± 1	7	11	17± 1

Represents mean ±SEM of three independent experiments.

Antibacterial activity of LP-CLA against Gram-positive and Gram-negative bacteria

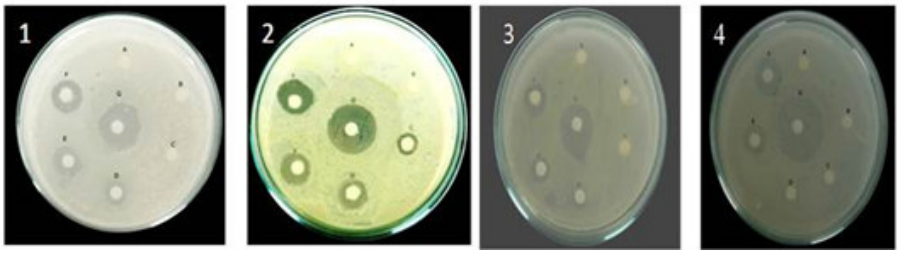

Figure 2
Antibacterial activity of LP-CLA against Gram-positive and Gram-negative bacteria.
(1)Bacillus cereus(2)Staphylococcus aureus(3) Klebsiellapneumoniaand (4)Pseudomonas aeruginosa. A:. 0.1% ethanol, B:. 20µg, C:. 40µg, D:. 60µg, E:. 80µg, F:.100µg and G: 30µg Streptomycin.

LP-CLA scavenges DPPH radical

The DPPH assay is a simple, fast and widely used method for measuring the free radical scavenging ability. The percent scavenging of DPPH radicals was measured at 517ηnm and the IC_{50} value was calculated by plotting the percent scavenging of DPPH at the steady state of reaction against the corresponding concentration of LP-CLA. The IC_{50} is the concentration of anti-oxidant needed to quench the DPPH by 50% under standard experimental conditions. The quenching of DPPH increased with increase in concentration of LP-CLA, 98% and 18% of inhibitions were noticed with 200 µg/ml and 25µg/ml of LP-CLA respectively (Fig 3). The IC_{50} for DPPH scavenging was found to be at 74.94 µg/ml of LP-CLA as per linear regression. This

reveals that LP-CLA has proton donating ability and acts as a free radical scavenger. DPPH is a stable, nitrogen-centered free radical which produces purple color in ethanol solution and fades to shades of yellow color in the presence of an antioxidant.[17]The free radical scavenging activity of CLA isomers have been reported.[18]From the present study, it is found that LP-CLA reduces the free radical to 2, 2′--diphenyl-1-picrylhydrazine when it reacts with hydrogen donors.Reactive oxygen species (ROS) or Reactive oxidative intermediates (ROIS) are partially reduced metabolites of oxygen that possess strong oxidation capabilities. The lower concentrations of ROS function as signaling molecules that regulate cell growth, and apoptosis.[19] Elevated levels of ROS can be observed in several chronic human diseases associated with inflammation.[20] The prolonged/chronic production of ROS is central reason to the progression of inflammatory diseases.The intracellular concentration of ROS depends upon the production/ or removal by the antioxidant system. Cells contain a large number of antioxidants to prevent or repair damage caused by ROS as well as to regulate redox-sensitive signaling pathways. Superoxide dismutase, Catalase and Glutathione peroxidase are the primary antioxidant enzymesinvolved in all oxygen metabolizing cells.

Induction of Catalase activity in LP-CLA treated MDA-MB-231 cells

Catalase is one of the antioxidant enzymes, which prevents the accumulation of hydrogen peroxide formed during oxygen transport. It is a tetrameric protein with heme group, measuring 250 kDa. The LP-CLA treated MDA-MB-231 cells showed significant catalase activity with increase in the concentration of LP- CLA and 1.63 folds increase in catalase activity was found at 60µg/ml of LP-CLA (Fig.-4). The data clearly indicates the functional role of LP-CLA in the induction of catalase activity.

Quenching of DPPH by LP-CLA

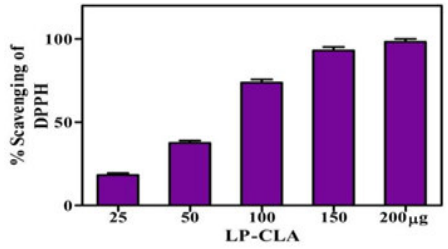

Figure 3
Concentration gradient quenching of DPPH by LP-CLA.

Concentrations ranging from 25-200µg/ml of LP-CLA were used to study the scavenging ability of free radicals generated by DPPH.

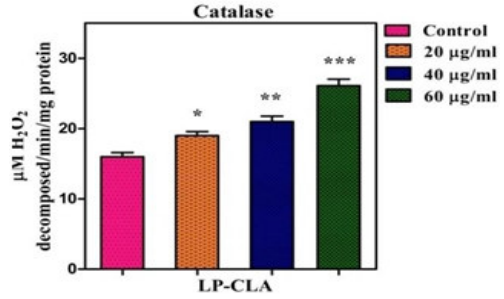

Figure 4
Effect of LP-CLA on Catalase activity.

MDA-MB-231 cells were treated with different concentrations of LP-CLA and incubated for 24h at 37ºC in CO_2 atmosphere. The cellular extracts were prepared and the catalase activity was measured by the amount of H_2O_2 consumed/min/mg protein at 240nm. Values are expressed as a mean ± SEM of three independent experiments. *Statistical significance *p<0.05, ** p<0.001 and ***p<0.0001* when compared with the control.

LP-CLA induces the activity of Superoxide dismutase

Superoxide dismutase enzyme catalyzes detoxification of superoxide anion either in the mitochondria or the cytoplasm of the cells. To detect the role of LP-CLA on superoxide dismutase, MDA-MB-231 cells were treated with 20, 40, and 60µg/ml of LP-CLA for 24h at 37ºC. The cytoplasmic extract was collected and analyzed for the activity of SOD in comparison with untreated control cells. The LP-CLA treated cells exhibited a slight increase in the activity of SOD (Fig 5) confirming the role of LP-CLA in boosting the primary antioxidant activity like SOD.

Increased Glutathione peroxidase activity by LP-CLA

Glutathione peroxidase protects the cells from oxidative damage particularly due to lipid peroxidation. This enzyme catalyzes the reduction of the lipid hydroperoxides to their corresponding alcohols and free hydrogen peroxide to water using reduced glutathione

as a source of reducing equivalent. To evaluate the role of LP-CLA on the activity of glutathione peroxidase enzyme, the MDA-MB-231 cells were treated with LP-CLA (20-60µg/ml) for 24h and the cellular extract was used to quantify the activity of glutathione peroxidase. The LP-CLA treated cells represented significant increase i.e. 1.59 folds in Gpxactivity in comparison with untreated cells at 60µg/ml (Fig–24). SOD converts the superoxide radical into hydrogen peroxide and molecular oxygen (O_2) while the catalase and peroxidase convert hydrogen peroxide into water. The net result is that two potentially harmful species superoxide and hydrogen peroxide are converted into water.[21]The activities of Superoxide dismutase, Catalase, and Glutathione peroxidase were induced in MCF-7 breast cancer and SW480 colon cancer cell lines exposed to CLA 20ppm over a period of 12 days. However, the greater extent was seen in MCF-7 cells than inSW480 cells.[22]The dietary CLA has anti-atherosclerotic and antioxidant activity by increasing oxidative stability in plasma and hepatic membrane in the vitamin E-deficient rats.[23]

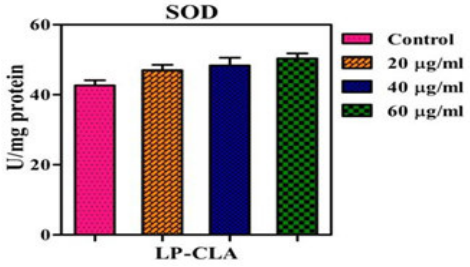

Figure 5
Activity of Superoxide dismutase induced by LP-CLA in MDA-MB-231 treated cells.

MDA-MB-231 cells were treated with 20, 40 and 60µg of LP- CLA and incubated for 24h at 37ºC. The cellular extract was analyzed for SOD activity and expressed as the SOD enzyme required for 50% inhibition of epinephrine /min/mg protein. Values are expressed as mean ± SEM of three independent experiments.

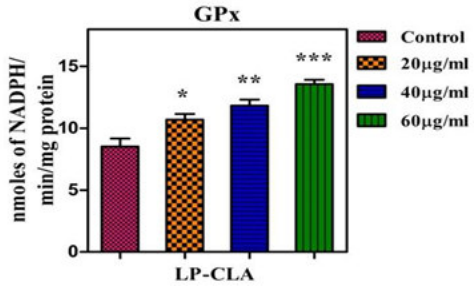

Figure 6
Change in the activity of Glutathione peroxidase by LP-CLA.

MDA-MB-231 cells were treated with 20, 40 and 60 µg/ml of LP-CLA and incubated for 24h at 37ºC in CO_2 atmosphere. The cellular extract was prepared and assayed for glutathione peroxidase with H_2O_2 as substrate. The activity described as the amount of protein required to oxidize 1η mole of NADPH /min. The results are the mean ± SEM of three independent experiments. *Statistical significance *p<0.05, ** p<0.001 and ***p<0.0001*when compared to control.

Non-toxic properties of LP-CLA in Wistar rats

The acute toxicity analysis of *Lactobacillus plantarum* ($1x10^{12}$ CFU/ml) and LP-CLA (2000mg/kg) were found to be non-toxic and there were no signs of mortality during the 14 day study period of administration. Physical observations showed no remarkable changes in the skin, eyes and mucous membrane including the behavioral pattern of rats. The body weights were not significantly different between the control, *Lactobacillus plantarum*and LP-CLA treated groups. During acute toxicity study, liver function tests were performed to know about the state/status of liver and its function after treatment with *Lactobacillus plantarum* and LP-CLA. In the present study (Table-1), the level of liver function enzymes like AST, ALT, ALP and liver proteins like bilirubin and albumin was analyzed after treatment with *L. plantarum* ($1x10^{12}$ CFU/ml/Kg) and LP-CLA (2000mg/kg). During the study, the protein levels (bilirubin and albumin) and the enzyme activities of AST, ALT and ALP were unaltered indicating the non-toxic nature of *Lactobacillus plantarum* ($1x10^{12}$ CFU/ml /Kg) and LP-CLA (2000mg/kg).

Table 2

Effect of Lactobacillus plantarum and LP-CLA on biochemical parameters of Wistar rats.

Male Rats	Aspartate aminotransferase (AST), U/L	Alanine aminotransferase (ALT), U/L	Serum alkaline phosphatase (ALP), U/L	Bilirubin, g/L	Albumin, g/L
Control	213.23 ± 5.23	58.40±5.54	187.87 ± 3	1.28 ± 0.3	24.7 ± 1
L.plantarum($1x10^{12}$FU/ml/kg)	208.00 ± 4.12	62.18 ±6.13	192.16± 3.48	1.32 ±0.4	26.12 ± 0.65
LP-CLA (2000mg/kg)	211.19 ± 2.89	61.15±7	184.83±1	1.35 ± 0.3	25.45± 1.2

mean ±SEM of three independent experiments.

Cotton pellet induced granuloma

The cotton pellet granuloma method is being widely used to assess thedifferent stages of sub-acute inflammation. The rats treated with normal saline, Indomethacin, *Lactobacillus plantarum* ($2x10^5$ and $2x10^7$ CFU/ml/kg) and LP-conjugated linoleic acid (5 and 10mg/kg) were anesthetized on the 8th day and the formation of granuloma around subcutaneously implanted pellets in the groin region of rats was observed. The cotton pellets were removed surgically, freed from extraneous tissues and dried for 24h at 60ºC. The dry weight of cotton pellet was taken as the measure of granuloma formation. Both *Lactobacillus plantarum*and LP-CLA has been found to reduce the weight of cotton pellet granuloma in a dose-dependent manner. The reduction in the dry weight of cotton pellet granuloma with different doses of *Lactobacillus plantarum* ($2x10^5$ and $2x10^7$ CFU/ml/kg)and LP-CLA (5 and 10mg/kg) was found to be 19.0, 46.9 percent with *L.plantarum* and 39.21 and 69.60% with LP-CLA respectively (Figure-7). The suppression of inflammation by LP-CLA (10mg/kg) was comparable to Indomethacin, which reduced the weight of cotton pellet granuloma by 66.5% (Table-5). The cotton pellet induced granuloma is an established animal model to evaluate the chronic inflammation.[24] The inflammatorycytokines, such as IL-1 and TNF-α, as well as growth factors influence proliferation of smooth muscle cells and fibroblasts and production of granuloma.[25]The anti-inflammatory drugs like NSAIDs reduce the size of granuloma, which results from the cellular reaction by suppressing granulocyte infiltration, forbidding generation of collagen fibers and inhibiting mucopolysaccharide.[26] Theprominent anti-inflammatory activity of LP-CLA compared to standard indomethacin sugges its activity in the transudative phase as well as the proliferative phase of inflammation.

Effect of Lactobacillus plantarum and LP-CLA on Hydroxyproline content

Wound healing is an intricate process where the skin or the body tissues repair itself after injury. During this process, an increase in synthesis of collagen occurs. The present work was carried out to assess the effect of *Lactobacillus plantarum* and LP-CLA on hydroxyproline content, which is an indicator for collagen synthesis and wound healing caused due to cotton pellet induced granuloma. The hydroxyproline content increased significantly at both doses of LP-CLA (5 and 10mg/kg) and found to be 10.77 and 19.52µg/mg tissue respectively whereas significant increase with *L. plantarum* was observed only at dose of $2x10^7$ CFU/ml/kgwith 13.1 g/mg tissue and indomethacin group showed 15.46µg/mg tissue. These results indicate that the treatment with LP-CLA effectively helped in healing the wound induced by cotton pellet on comparison with indomethacin (Figure-8).The granulation tissue of the wound is primarily composed of fibroblast, collagen edema and small new blood vessel.[27,28] The significant levels of hydroxyproline was observed with LP-CLA compared to L. plantarum and the higher dose of LP-CLA exhibited greater levels of hydroxyproline compared with standard NSAID Indomethacin.

Cytotoxicity of LP-CLA on MDA-MB-231 cells

The neutral red assay is one of the most widely used cytotoxicity assay with many biomedical and environmental applications. It provides a quantitative method for counting the number of viable cells. The viable cells take up the vital dye, neutral red (Basic Red 5, Toluylene Red) by active transport and incorporate the dye into lysosomes. As illustrated in the Figure-9A, the uptake of Neutral red dye by the cells was decreased steadily with morphological alterations with the increase in the concentration of LP-CLA. The percentage of cell proliferation of MDA-MD-231 cells was found to be 47.8% with 60µg and 10% with 100µg of LP-CLA after 48h (9B). The viability in the vehicle treated control (1% ethanol) was > 95.5% as determined by MTT and neutral red assay. The data obtained from both assays showed that LP-CLA has an IC50 value as 60µg. The IC50 concentration was defined as the concentration of LP-CLA inhibiting cell viability/cell survival by 50% compared with the vehicle-treated control (1% ethanol). Thus, LP-CLA displayed cytotoxic effect on MDA-MB-231 breast cancer cells.

Table3

Percent inhibition and mean weight of cotton pellet in treated groups.

Groups	Dry weight of cotton pellet (mg)	% inhibition of granuloma
Disease control	95.0± 1	0.00
Indomethacin (10 mg/kg)	33.45±1***	66.5
L. plantarum($2x10^5$ CFU/ml/kg)	80±1.2	19
L.plantarum($2x10^7$ CFU/ml/kg)	53.1±1**	46.9
LP-CLA (5mg/kg)	54.71±0.14**	39.21
LP-CLA (10mg/kg)	27.36±0.26***	69.60

Values are expressed as mean ± SEM (n=6), *p<0.05, ** p<0.001 and ***p<0.0001vs disease control group.

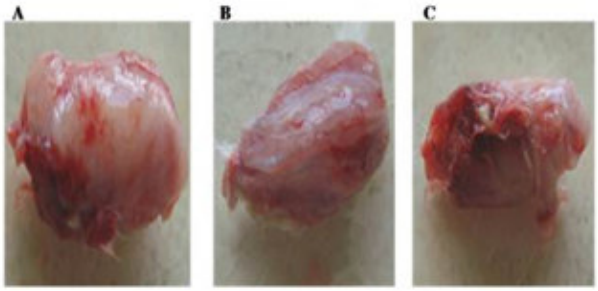

Figure 7
Changes in the weight of cotton pellet granuloma.
(A). Control; (B). Lactobacillus plantarum(2x10^7 CFU/ml/kg);
(C). LP-CLA (10mg/kg).

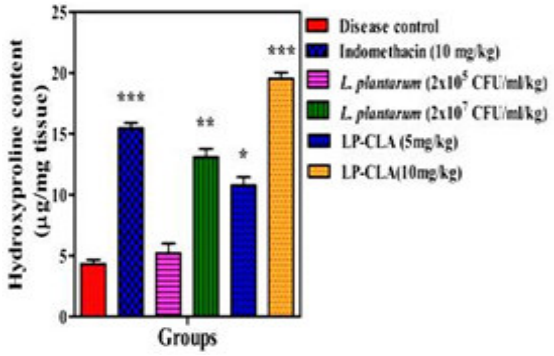

Figure 8
Changes in hydroxyproline levels of granuloma tissue treated with Lactobacillus plantarum and LP-CLA.
Values are expressed as mean ± SEM

Neutral red uptake assay

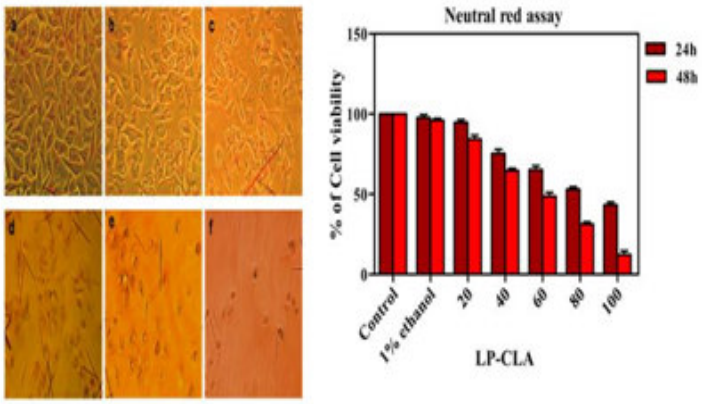

Figure 9(A)
Photomicrograph in optical inverse microscope shows the effect of LP-CLA on MDA-MB-231 cells survival by Neutral red uptake assay.

From 'a' to 'f' are 1% ethanol treated cells to 20, 50, 60 80 and 100µg of LP-CLA treated cells after 48h.(B)Effect of LP-CLA on the proliferation of MDA-MB-231 Cells. MDA-MB-231 cells were treated with 20, 40, 60, 80 and 100µg of LP-CLA and the cell survival was determined after 24 and 48h by Neutral red assay. Each bar represents the results of triplicate determinants and there is a significant difference on comparison with control($p < 0.01$).

CONCLUSION

The LP-CLA isolated from *Lactobacillus plantarum*on characterization by GC-MS analysis shown the presence of both cis-9,trans-11, and trans-10,cis-12 CLA isomers in 60:40 ratio.LP-CLA exhibited higher antibacterial activity against Gram-positive bacteria. The quenching of DPPH by LP-CLA was noticed as 98% at 200µg/ml reveals the free radical scavenging ability of LP-CLA.The functional role of LP-CLA on the induction of antioxidant enzymes was exhibited by the induced

activity of Catalase (CAT), Superoxide dismutase (SOD) and Glutathione peroxidase (GPx) in MDA-MB-231cell lines as in vitro model. The in vivoresults clearly indicates that the LP-CLA has anti-inflammatory activity by reducing the weight of cotton pellet granuloma in tune with NSAIDS. Thus LP-CLA may be considered as therapeutic agent based on its antimicrobial, antioxidant and anti-inflammatory activities.

CONFLICT OF INTEREST

Conflict of interest declared None.

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