RESEARCH ARTICLE

Probiotic Conjugated Linoleic Acid Mediated Apoptosis in Breast Cancer Cells by Downregulation of NF-κB

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Abstract

Conjugated linoleic acid, a functional lipid, produced from *Lactobacillus plantarum* (LP-CLA), has been demonstrated to possess apoptotic activity. The anti-proliferative and apoptotic potential of LP-CLA was here evaluated *in vitro* using the MDA-MB-231 human breast cancer cell line as a model system. Proliferation of MDA-MB-231 cells was inhibited with increasing concentrations of LP-CLA with altered morphological features like cell detachment, rounding of cells and oligonucleosomal fragmentation of DNA. Flow cytometry confirmed the apoptotic potential of LP-CLA by ANNEXIN V/PI double staining. Furthermore, outcome results indicated that the apoptosis was mediated by downregulation of the NF-κB pathway which in turn acted through proteasome degradation of IκBα, inhibition of p65 nuclear translocation, release of cytochrome-C from mitochondria and finally overexpression of Bax protein. Thus, conjugated linoleic acid, a natural product derived from probiotics, could therefore be a possible potential chemotherapeutic agent due to its apoptotic activity against estrogen receptor negative breast cancer cells.

Keywords: Conjugated linoleic acid - NF-κB - apoptosis - GSH - MDA-MB-231 cells - *Lactobacillus plantarum*

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Introduction

The nuclear factor kappa B (NF-κB), an eukaryotic redox sensitive transcription factor, plays an important role in the wide range of cellular functions starting from cell division, growth and differentiation to cell death by virtue of its ability to modulate expression of over 200 genes involved in immune, inflammatory and anti-apoptotic processes (Rothwarf and Karin, 1999; Karin, 2006). Till to date, only five members of mammalian NF-κB family were recognized, which are NF-κB1 (p105 and p50), NF-κB2 (p100 and p52), C-Rel, RelB and RelA(P65). The abundant form of NF-ϰB is p65/p50 heterodimer, which are generally retained in the cytoplasm in an inactive form associated with regulatory proteins called inhibitors of κB (IκB) (Hacker and Karin, 2006; Vunta et al., 2007). Under stimulation of the cells by a variety of agents such as LPS or TNF-α, the classical pathway of NF-κB is triggered with the phosphorylation of two regulatory serine residues (S32, S36) on IκB, which targets IκBα for poly-ubiquitination and proteolytic degradation allowing the translocation of NF-ϰB to the nucleus to bind with the promoter regions of its multiple target genes. Activation of NF-ϰB induces expression of anti- apoptotic genes which regulates the apoptosis by blocking the release of cytochrome C through polarization and membrane integrity of mitochondria (Karin, 2000; Kucharczak et al., 2003).

Literature reports that the oxidative stress, including the depletion of monomeric glutathione (GSH) or increase in dimeric glutathione disulfide (GSSG) levels, leads to rapid phosphorylation, poly ubiquitination and the degradation of IκB complex, thus activating NF-κB (Mercurio and Manning, 1999), Mihm SD, Galter, Droge (2006). In mammalian cells, Glutathione peroxidase (GPX) catalyzes the oxidation of GSH into GSSG and this oxidized GSSG is converted back to the monomeric GSH by the glutathione reductase. During oxidative stress, GPx is up-regulated by NF-κB and all the five NF-κB subunits bind to the promoter region of Glutathione peroxidase in response to LPS (Schreiber et al., 2006). The redox regulation of GSH is mainly linked to modulate kinases which are responsible for activation of NF-κB. Under cellular stress, Bcl-2 has also shown to promote sequestration of glutathione into nucleus and control apoptosis by regulating membrane integrity of mitochondria (Pardhasaradhi et al., 2003).

The usage of probiotics is currently gaining significance due to various health benefits such as improving immune system, prevention of colon cancer, reduction of cholesterol, lowering of blood pressure, and control of inflammation. Oral administration of lactic acid bacteria

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has proven to reduce DNA damage caused by chemical carcinogens, in gastric and colonic mucosa of rats. Additionally these probiotics are capable of producing conjugated linoleic acid (CLA) from linoleic acid (Ogawa et al., 2005). CLAs are heterogeneous geometric isomers derived from linoleic acid. CLA has attracted much attention as a novel type of functional lipid due to its exciting implications in anti-diabetic (Rungapamestry et al., 2012), anti-carcinogenic (Shen et al., 2014, Heinze and Actis, 2012) and anti-inflammatory activities (Yang et al., 2014). The present study reveals that the LP-CLA induces the apoptosis of MDA-MB231 cells through the down regulation of NF-κB.

Materials and Methods

Lactobacillus plantarum (ATCC 8014), MRS broth (HiMedia Laboratories); Linoleic acid (99% purity, Sigma Aldrich); cis-9, trans-11 CLA and trans-10, cis-12 CLA (≥98% purified Cayman chemicals); MDA-MB-231cells (National centre for cell sciences, Pune); FBS (GIBCO Laboratories); DMEM, Trypsin, PBS (HyClone); rabbit anti-p65 (Caymen Chemicals), mouse anti-GAPDH (Santa Cruz Biotechnology); mouse anti-cytochrome C, rabbit anti-Bcl-2, rabbit anti-Bax and anti-histone H3 (Cell Signaling Technology).

Characterization of LP-CLA by GC-MS analysis

In order to characterize the conjugated linoleic acid produced from Lactobacillus plantarum, the lipids recovered from cell-free extract were dried with nitrogen gas at 70ºC and this dried sample was re-dissolved in 500μl of n-hexane. The lipids were derivatized to methyl esters by treating with 1ml of 5% methanolic HCL and the reaction mixture was vortexed for 20min and subjected to incubation for 60min at 100ºC. The methylated fatty acid esters were analyzed in GC-MS DSQ11 (Thermo Scientific). The methyl esters (1μl) of the L. plantarum sample was injected into the column with a flow rate of 1ml/min. Hydrogen gas was used as a carrier gas through zebran zb5 capillary column (30mx 250μm). The column temperature was set initially at 40ºC with a hold time of 2min and then increased to 300ºC with an increment of 10ºC/min and maintained for 37min. The *Lactobacillus plantarum* CLA (LP-CLA) isomer was characterized based on the retention time after co-chromatography with authenticated standards.

Docking of CLA isomers into active site of NF-κB

The three-dimensional X-ray diffraction structure of Nuclear factor kappa B (NF-κB, PDB_ID:1LE9 with X-Ray diffraction resolution of 3.0 Å) bound to Ig/HIV-κB site was retrieved from PDB . It consists of two p65 (A and E) subunits with 274 amino acid length and two p50 (B and F) subunits with 313 amino acid residues. The p50- p65 subunits (A and B) were considered for docking after removing the Ig/HIV-κB from the binding site. The binding pockets/sites were identified as mentioned above and the CLA isomers (cis-9,trans-11 and trans-10, cis-12) were docked into the active site using CDOCKER of Accelrys discovery studio.

Measurement of GSH and GSSG

The activity of GSH and GSSG was measured by enzymatic recycling assay (Anderson, 1985). MDA-MB-231 cells were treated with different concentrations of LP-CLA (20, 40 and 60μg/ml) for 24hr. The cells were washed with PBS and lysed with triton X-100. About 50μl of 10% sulfosalicylic acid was added to each aliquot of cell lysate (100μl), the samples were centrifuged (10,000 xg) for 5min at 4ºC. To assay total glutathione, an aliquot of supernatant (50μl) was added to a mixture of 0.7 ml of 0.3mM NADPH, 100μl of 6mM DTNB, and 175μl of H2O. The rate of change in optical density at 415ηm was assessed after addition of 10μl of glutathione reductase (50 units/ml). In order to assay GSSG, the GSH present in the sample was derivatized by adding 2μl of 2-vinylpiridine and 6μl of triethanolamine to 100μl of the supernatant. After 60min of incubation at 25ºC, the GSSG was measured in the same manner as GSH as described by Griffith (Griffith OW 1980). Glutathione levels were normalized to the protein content by BCA method.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-dipehnlytetrazolium bromide) assay

The LP-CLA induced cytotoxicity was measured by the MTT assay (Vijayarathna S, Sasidharan S 2012). Briefly, the MDA-MB-231 ($1x10^5$cells/well) cells were seeded and incubated for 24hr at 37ºC in 5% CO_2 atmosphere for the adherence of the cells to the flat bottom of the 96 well plates. The cells were treated with different concentrations of LP-CLA (20, 40, 60, 80 and 100μg/ml) and incubated for 24 and 48hr. At the end of the incubation, the media was removed and washed with 0.01M PBS (pH 7.4) and 25μl of MTT solution (5mg/ml in PBS was added to each well. After 3h of incubation at 37ºC, the purple-blue MTT formazan crystals were extracted using 0.1ml of extraction buffer (20% SDS in 50% dimethylformamide) and incubated for 30 min. The activity of the mitochondria, reflecting cellular growth and viability, was evaluated by measuring the optical density at 540ηm using microplate reader (Biotek-255907). Each sample with specific concentration was tested in three independent experiments run in triplicates. The means and the standard deviations were calculated and reported as the percentage of cell proliferation vs. control.

Percent of cell survival (%) = Mean OD value of test / Mean OD value of control × 100.

Detection of Apoptosis by Flow cytometry

The apoptotic nature of LP-CLA against MDA-MB-231 cells was detected by using Annexin V-FITC / Propidium iodide double staining by flow cytometry (Oosterhuis GJ et al., 2000). MDA-MB-231 cells (1x106 cells) were seeded into 60mm dishes containing DMEM media and cells were treated with LP-CLA at a concentration of 60μg and 1% ethanol (control) for 48h at 37ºC. After incubation, the treated cells were washed with PBS and harvested by trypsinization with trypsin-EDTA followed by centrifugation at 3000 rpm at 4ºC for 5min and the supernatant was decanted. These cells were resuspended in 1ml of 1X Annexin V binding buffer and

centrifuged at 3000 rpm for 5min. The cell pellet was resuspended with 100μl of Annexin V binding buffer and 5μl of Annexin V-FITC was added and incubated in dark for 15min at room temperature. The cells were washed with Annexin V binding buffer twice and the cells were fixed with ice-cold 70% ethanol (2ml) at 4ºC for 30min. The cell pellet was suspended in 200μl of Annexin V binding buffer, and the appropriate volume of stock RNase was added (final concentration of RNase being 10μg/ml) followed by incubation at RT for 40min. The cells were then stained with PI solution (50μg/ml) and incubated at room temperature for 30min in the absence of light and analyzed at 488ηm using flow cytometer at the rate of 1000-1,200 cells.

Analysis of DNA fragmentation

The MDA-MB-231 cells (5x10^{6}cells/well) were treated with different concentrations (20, 40, 60, 80 and 100 μg/ml) of LP-CLA and 1% ethanol (control) for 48hr. After treatment, the cells were scraped and pelleted by centrifugation for genomic DNA isolation. The DNA was electrophoretically resolved on 1.8% agarose gel in 1X TAE buffer and visualized by ethidium bromide (0.5μg/ml) staining under UV trans-illuminator (Navya et al., 2014; Sambrook J, F.E.F., Maniatis, 1989).

Detection of cytochrome-C release

The release of cytochrome C from mitochondria to the cytosol was measured by western blot analysis [Fujimura M et al., 1999]. Briefly, first the cells were treated with different concentrations of LP-CLA and after 24h, the cells were washed with ice cold PBS and gently lysed for 30s in 100μl of M-PER (Thermo-scientific). The Lysates were centrifuged at 12000xg for 15min at 4ºC to obtain the cytoplasmic extracts which were free from mitochondria. The supernatant was electrophoresed on 10% SDS-PAGE and then analyzed by western blot technique using anti-cytochrome C antibody.

Effect of LP-CLA on the expression of Bcl-2 and Bax

The impact of LP-CLA on the expression of Bcl-2 and Bax was analyzed by western blot analysis. The cells were treated with different concentrations of LP-CLA (20, 40, 60 and 80 μg/ml) for 24h. Upon treatment, the cells were washed twice with PBS and harvested with cold sterile PBS. The cell pellet was re-suspended in 200μl mammalian protein extraction reagent (M-PER, Thermo-scientific) with 2μl protease inhibitor cocktail containing 104mM, Aprotinin at 80µM, Bestatin at 4mM, E-64 at 1.4mM, Leupeptin at 2mM and Pepstatin A at 1.5mM for 30min on ice with intermittent vortexing for every 15s for the rupture of the plasma membrane. These contents were centrifuged at 10,000g for 15min at 4oC and the protein was estimated by BCA method. The cytoplasmic extract was analyzed for the expression of Bcl-2 and Bax proteins by western blot analysis as described above using Bcl-2 and Bax antibodies (Zhang et al., 2014). GAPDH was used as loading control.

Immunoprecipitation of p65

MDA-MB-231 cells were pre-incubated with specific concentrations of LP-CLA (20-80μg/ml) for 1h. Later the cells were stimulated with 1μg/ml of LPS for 12h. Upon treatment, the cells were washed twice with PBS and lysed in Nuclear protein extraction reagent (N-PER, Thermo-scientific) at 4°C for 20 min. Lysates were centrifuged at 16,000 ×g for 10 min and the protein concentrations in the supernatants were determined by BCA protein assay kit [24]. Nuclear proteins (50µg) were immunoprecipitated overnight at 4°C with 0.5µg of anti-p65 polyclonal agarose beads (Santa Cruz Biotechnology). The agarose beads were extensively washed with PBS (4 x 400µl) and subjected to SDS/PAGE (10% polyacrylamide) followed by Western blot analysis as described above. Histone was used as loading control.

Statistical analysis

All the experiments were performed in triplicates unless otherwise noted, and the resultant values represent the mean ± SEM. Statistical significance is denoted by an asterisk (*) when p values are *p<0.05, ** p<0.001 and ***p<0.0001, as calculated by Dunnet's test using Graph pad prism software version 5.0.

Results

Characterization of LP-CLA isomers produced by Lactobacillus plantarum (ATCC 8014)

The fatty acids extracted from the cell-free supernatant of *Lactobacillus plantarum* culture were analyzed by GC-MS after optimization of the cultural parameters. In the present experiment, the derived fatty acids were subjected to GC-MS after derivatization along with commercial standard linoleic acid (Sigma-Aldrich, 98% purity) and cis-9, trans-11 CLA isomer (Cayman 98% purity). GC-chromatogram of standard LA and cis-9, trans-11 CLA

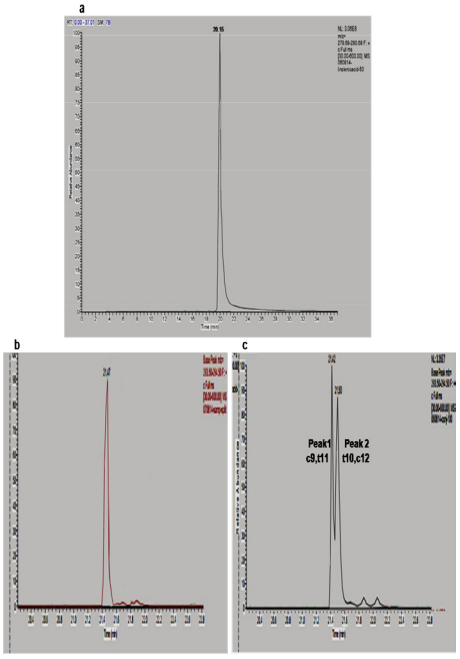

Figure 1. The GC- chromatograms of linoleic acid and conjugated linoleic acid isomers. a) Standard linoleic acid b) Standard cis-9,trans-11 CLA. c) Partial GC chromatogram of LP-CLA isomers extracted from Lactobacillus plantarum

Table 1. CDOCKER Energies and the Number of Hydrogen Bonds between the ligands and NF-κB

Receptor	Ligand	No. of Hydrogen Bonds	Amino acids involved in Hydrogen bonding	Bond distance (Å)	CDOCKER interaction energy (kcal/mol)
	cis-9, trans-11 CLA	2	LYS241	1.83	-51.75
			LYS272	1.94	
NF-κB	trans-10, cis-12 CLA	1	LYS218	1.85	-54.85

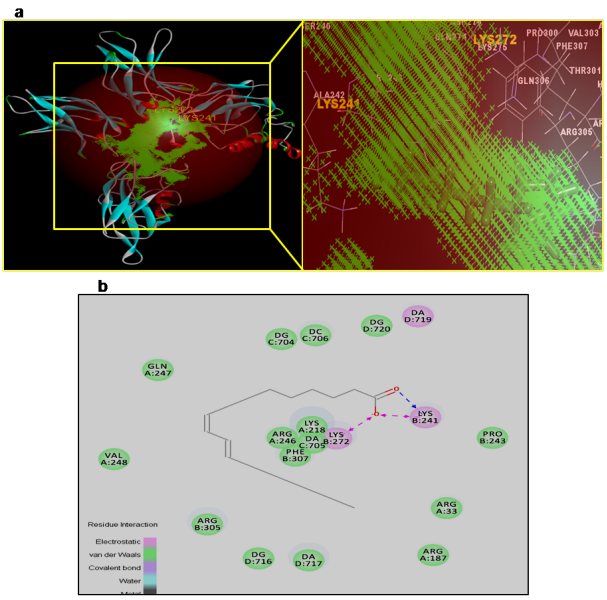

Figure 2. Binding Interaction of CLA Isomer cis-9, trans-11 with p50-p65 Heterodimer. a) The Ribbon structure of p50-p65, NF-κB with cis-9, trans-11 (green) CLA isomer. b) The hydrogen interaction of cis-9, trans-11 isomer with p50-p65, NF-κB

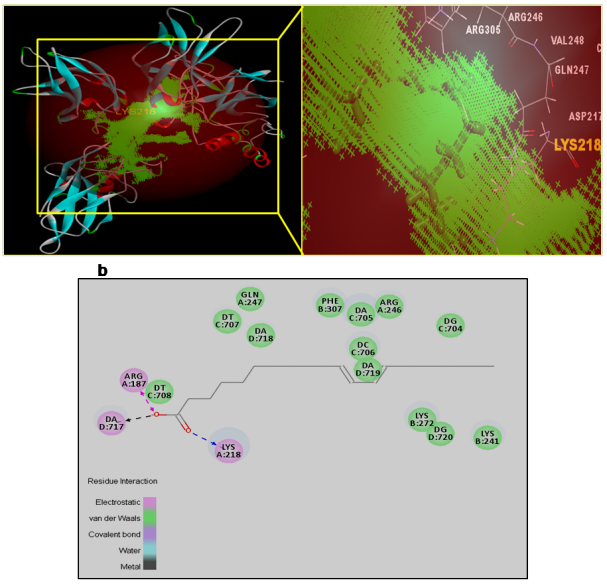

Figure 3. Binding confirmation of CLA isomer trans-10, cis-12 with p50-p65 heterodimer. a) The ribbon structure of p50-p65, NF-κB with trans-10, cis-12 (green) CLA isome. b) The 2D hydrogen interaction of trans-10, cis-12 isomer with p50-p65, NF-κB

isomer showed peaks at 20.15 and 21.45 respectively (Figure 1a and 1b). Fatty acids extracted from the L. plantarum showed two major peaks, with retention time of 21.42 and 21.50 (Figure 1c) and these fatty acids were

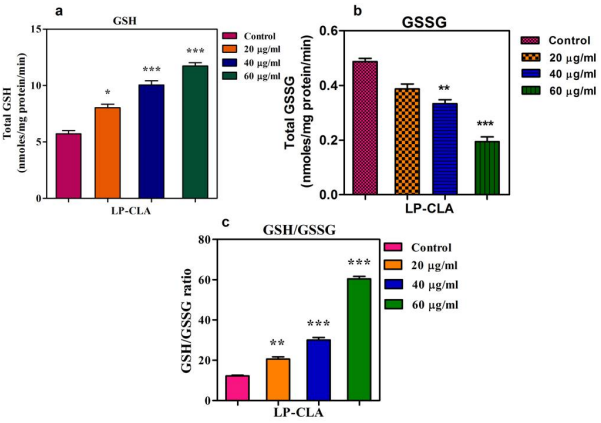

Figure 4. Effect of LP-CLA on GSH and GSSG in MDA-MB-231 Treated Cells. Cells were treated with LP-CLA (20, 40 and 60μg/ml) for 24 hr and measured for GSH and GSSG levels. a GSH content b GSSG levels. c GSH/GSSG ratio. Data are shown as mean ± SEM of three independent experiments. Statistical significance *p<0.05, **p<0.001 and ***p<0.0001 when compared with the control

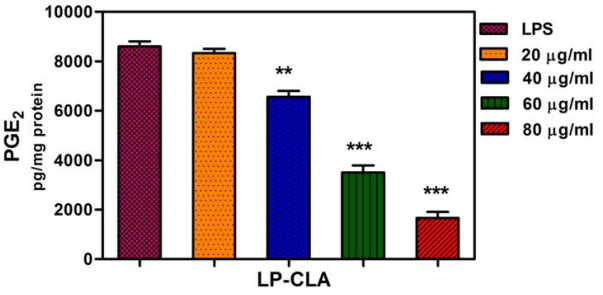

Figure 5. Photomicrograph of Optical Inverse Microscopy Showing the Effect of LP-CLA on MDA-MB-231 Cells Proliferation by MTT assay. From 'a' to 'g' are control (untreated cells), 1% ethanol treated control cells to 20, 40, 60, 80 and 100μg of LP-CLA treated cells after 48hr

confirmed as cis-9, trans-11 CLA and trans-11,cis-12 CLA respectively based on the comparison with standard CLA and verification with NIST database. From the results, it can be inferred that L. plantarum (ATCC:8014) is potential to convert linoleic acid into both cis-9, trans-11 CLA and trans-11,cis-12 isomers and the relative abundance of these two isomers was found to be in the ratio of 60:40.

Docking of cis-9, trans-11 and trans-10, cis-12 CLA isomers into the active sites of NF-κB

NF-κB is a central transcriptional factor responsible for regulating the transcription of inflammatory genes. Higher NF-κB activation fairly leads to rapid changes in the gene expression and results in chronic inflammation, cancer

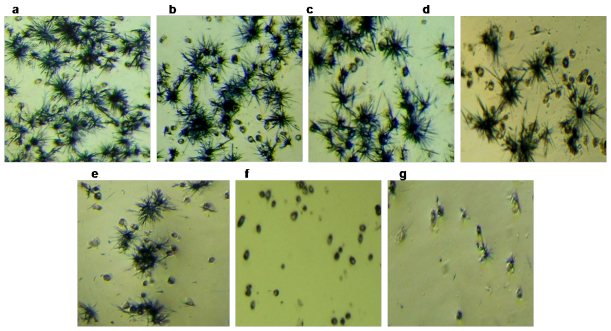

Figure 6. Antiproliferative Potential of LP-CLA. MDA-MB-231 cells were treated with 20, 40, 60, 80, 100μg/ml of LP-CLA and the cell survival was determined after 24hr and 48hr by MTT uptake. The percent of viable cells were calculated with comparison to the untreated cells. The number of cells in control was taken as 100%. Each data point represents the mean ± SEM of triplicate determinants and there is a significant difference with comparison with the control ($p<0.01$)

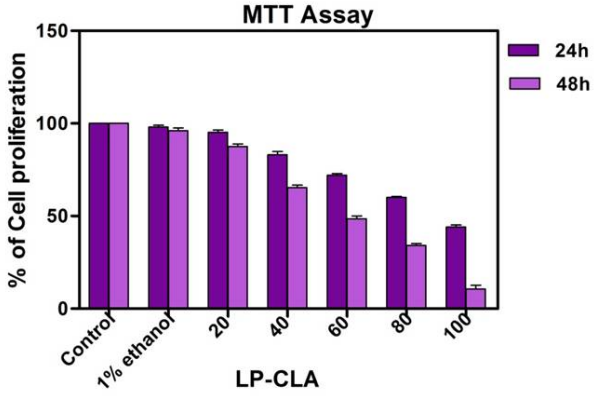

Figure 7. DNA Fragmentation Analysis of LP-CLA treated MDA-MB-231 Cells. After 48hr treatment with and without LP-CLA, DNA was isolated and analyzed on 1.8% agarose gel. Lane M: Φ DNA marker, Lane C: untreated (control) cells, Lane '1' 1% ethanol treated, Lane '2' to '6' are 20, 40, 60, 80 and 100μg/ml LP-CLA treated cells

development, and its progression. A deep understanding of the interaction of LP-CLA with this protein perhaps may lead to modulate the pro-inflammatory genes COX-2 and iNOS. The three-dimensional structure of NF-κB (PDB_ID: 1LE9) (RelA, p50-p65) with a resolution, of 3.0 [Å] of Mus musculus was retrieved from PDB. Three active site pockets were identified; the site with SER276 was selected for docking with cis-9, trans-11, and trans-10, cis-12 CLA isomers. The docking of CLA isomer cis-9, trans-11 resulted in the formation of 2 hydrogen bonds with LYS241 and LYS 272 of the p50 subunit with interaction energy of -51.75, whereas docking with trans-10, cis-12 CLA isomer resulted in a single hydrogen bond with LYS218 of the p65 subunit with -54.75 CDOCKER energy (Table 1). The docking of ligands at active site and the hydrogen bond interaction are shown in Figure 2 and Figure 3.

Glutathione redox status in LP-CLA treated cells:

The glutathione plays an important role in maintaining the redox status of the cell. The cells were treated with different concentrations of LP-CLA and the cellular extract was collected to determine the levels of GSH and

Figure 8. LP-CLA induced Apoptosis of MDA-MB-231 Cells. Annexin V-FITC staining is represented on the horizontal axis and PI on the vertical axis. a Control and b LP-CLA (60μg/ml induced early apoptosis after 48hr (LL1: Live cells, LR1: Early apoptotic cells, UR1; Late apoptotic or necrotic cells and UL1: debris)

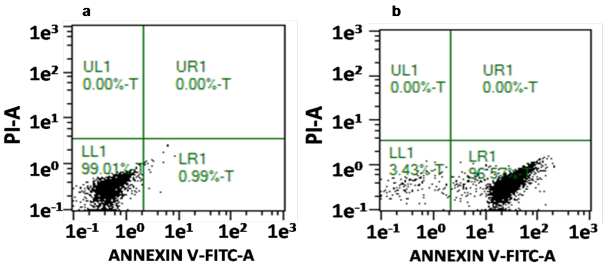

Figure 9. Expression Pattern of Cytochrome-C Release in LP-CLA Treated MDA-MB-231 Cells. a MDA-MB-231 cells were treated with LP-CLA (20-80μg/ml) and incubated for 24hrs at 37ºC. An equal amount of protein (50μg) from LP-CLA treated MDA-MB-231cells were analyzed by 10% SDS-PAGE. The proteins were transferred to nitrocellulose membrane and probed with anti-cytochrome C antibody. b The densitometric data of cytochrome-C release

GSSG. LP-CLA treatment led to the increase of GSH levels in the dose dependent manner and the GSH level was found to be 2 folds higher than the control cells at 60μg (Figure 4a), whereas the GSSG levels decreased in all treatments (Figure 4b). The GSH/GSSG ratio increased in correlation with the concentration of LP-CLA (Figure 4c). These results demonstrate the significance of LP-CLA in maintaining the redox status of the cells.

Antiproliferative potential of LP-CLA

The cell viability was determined through mitochondrial dehydrogenase activity by MTT assay. It is observed

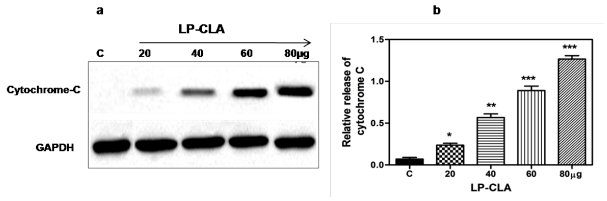

Figure 10. Effect of LP-CLA on the Expression of Bcl-2 and Bax. a MDA-MB-231 cells were treated with 20, 40, 60, 80 and 100μg/ml of LP-CLA and incubated for 24hrs at 37ºC in 5% CO2 atmosphere. An equal amount of protein (50μg) from LP-CLA treated MDA-MB-231 cells were analyzed by 10% SDS-PAGE and after electrophoresis. The proteins were transferred from the gel to the nitrocellulose membrane and probed with specific primary and secondary antibody b The densitometric data of Bax protein expression representing the relative density of the western blot bands normalized with GAPDH

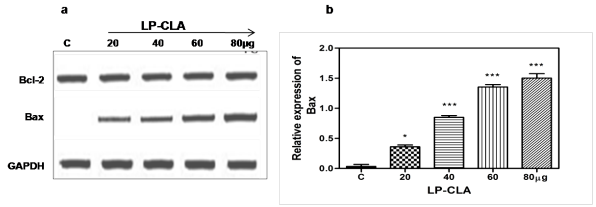

Figure 11. Effect of LP-CLA on Phosphorylation of IκBα. MDA-MB-231 cells, pre-incubated with different concentrations of LP-CLA for 1hr, were stimulated with 1 μg/ml of LPS and incubated for 12hrs at 37ºC. The cytoplasmic extract proteins were extracted and analyzed for phosphorylated IκBα using anti-IκBα by western blot analysis. The protein levels were normalized by GAPDH. Data shown are representative expression patterns from duplicate independents

that LP-CLA treated (20-100μg/ml for 24h and 48h) MDA-MB-231 cells showed inhibition of cell growth in concentration dependent manner. Time course analysis showed the drastic difference in cell viability between 24hr and 48hr, indicating that breast cancer cells respond to LP-CLA treatment after 24hr (Figure 5). The IC50 value was noticed as 60μg/ml and 80μg/ml at 48h and 24h respectively (Figure 6). Thus, LP-CLA displayed a strong cytotoxic activity on MDA-MB-231 breast cancer cell lines.

DNA fragmentation induced by LP-CLA in MDA-MB-231 breast cancer cells

In order to delineate the mechanism of cell death mediated by LP-CLA, DNA fragmentation analysis, which is an apparent marker for apoptosis, was conducted. MDA-MB-231 cells treated with different concentrations (20, 40, 60 80 and 100µg/ml) of LP-CLA for 48hr indicated the generation of oligo nucleosomal fragments of DNA on 1.8% agarose gel. The degree of nuclear DNA fragmentation was correlated to increased concentrations of LP-CLA (Figure 7).

LP-CLA induces apoptosis of MDA-MB-231 cells

The induced apoptosis in MDA-MB-231 cells treated with LP-CLA was further confirmed by flow cytometry analysis. The Annexin V-FITC/PI double staining assay

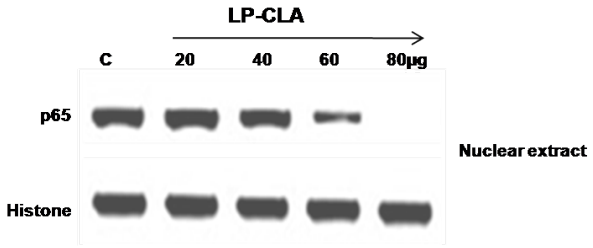

Figure 12. LP-CLA Inhibits the Nuclear Translocation of p65. MDA-MB-231cells were treated with the indicated concentrations of LP-CLA for 1hr followed by LPS stimulation for 12hrs. The nuclear extracts were immunoprecipitated and probed with anti-p65 by western blotting. Equal loading was confirmed by stripping the blot and reprobing it for Histone

was used for the identification of cellular apoptosis. The cells were stained with PI to distinguish early apoptotic cells from necrotic cells. The red-fluorescent PI dye is impermeable to live cells and apoptotic cells, but stains dead cells with red fluorescence by binding tightly to the nucleic acids in the cell. As shown in Figure 8a, the lower left quadrant demonstrats the presence of viable cells in the untreated control cells. The increase in the proportion of cells undergoing early apoptosis was significantly higher following LP-CLA (60μg/ml) treatment in MDA-MB-231 cells as represented in the lower right quadrant (Figure 8b).

LP-CLA activates the release of cytochrome-C

The release of cytochrome-C from the mitochondria in to the cytoplasm is the hallmark for the cells undergoing apoptosis [Luhrmann A, Roy CR (2007)]. To specify the molecular basis of apoptosis and to detect the release of cytochrome-C into the cytosol, western blot analysis with cytochrome-C antibody was performed in MDA-MB-231 cells treated with LP-CLA. The release of cytochrome-C into the cytoplasm was not detected in the untreated cells (control). In contrary, LP-CLA stimulated the release of cytochrome-C into the cytoplasm in dose-dependent manner indicating the LP-CLA induced cell death (Figure 9).

LP- CLA induces the expression of Bax:

The different proteins of the BCL-2 family have been implicated in triggering or preventing apoptosis. Bcl-2 and Bax are the proteins associated with the mitochondrial membrane and their ratio is crucial for cell survival. Bcl-2 is a mitochondrial membrane associated protein and plays an important role in the proliferation of cells. The changes in the expression levels of Bcl-2 and Bax upon treatment with LP-CLA were observed by western blot analysis. As shown in the Figure 10, Bcl-2 expression was not altered both in control and treated cells in response to LP-CLA (20-80μg). However, the level of pro-apoptotic protein Bax increased significantly in concentration-dependent manner in comparison with untreated cells.

LP-CLA inhibits the phosphorylation of IκBα and p65 nuclear translocation

Transcription factor NF-κB (p50/p65) is generally localized in the cytoplasm by its inhibitor IKB. One of the important events in the activation of NF-κB is

the dissociation and degradation of IκBα from NF-κB heterodimer and the process is mediated through the phosphorylation of IκBα. The cytoplasmic phosphorylated IκBα levels were determined by western blot analysis. Application of LPS stimulated the phosphorylation of IκBα in the control cells and the level of phosphorylated IκBα was suppressed significantly by LP-CLA pre-treatment in a dose-dependent manner (Figure 11). Finally, the level of p65, the functionally active subunit of NF-κB, was investigated in nuclear extracts by immunoprecipitation. As represented in the Figure 12, the control showed increased levels of p65, which was blocked by LP-CLA in the dose-dependent manner. These results suggest that LP-CLA inhibits the LPS-induced translocation of p65 to the nucleus by blocking the phosphorylation of IκBα. The cytotoxic effect of CLA in MCF-7 cells is through suppression of NF- ϰ B and production of ROS (Rakib et al., 2013).

Discussion

In the present study, the focus of the study is on unraveling the mechanism of probiotic action through the production of CLA. It is demonstrated that probiotic bacteria, *Lactobacillus plantarum* have the capacity to convert linoleic acid into conjugated linoleic acid isomers and the CLA derived from L. plantarum was able to promote the apoptosis process through the downregulation of NF-κB pathway. Quantitative analysis by GC-MS showed the presence of both cis-9, trans-11 and trans-10, cis-12 isomers in 60:40 ratio. *Lactobacillus plantarum* produced mixed isomers of two bioactive CLA isomers including cis-9, trans-11 (0.38 mg/ml)) and trans-10, cis-12 (0.42 mg/ml) from 8 mg/ml sunflower oil by bacterial lipase conversion to linoleic acid [Hosseini ES et al., 2015] L. plantarum NCUL0050 (Zeng Z, Lin J, Gong D 2009; Van Nieuwenhove CP et al., 2007), L. plantarum APIA 50 (Liu P et al., 2011) exhibited the presence of both cis-9, trans11 and trans-10, cis-12-CLA.

Reduced glutathione is considered to be one of the most important scavengers of ROS and its ratio with oxidized glutathione (GSSG) can be taken as a marker of oxidative stress. As glutathione plays an important role in maintaining the redox status of the cell, the level of glutathione in LP-CLA treated MDA-MB-231 cells was measured. LP-CLA treatment led to increase in the GSH levels in a concentration gradient manner and two folds increase in GSH level was seen at 60μg/ml. The over expression of GSH content in the cell after exposure to LP-CLA indicates that LP-CLA interferes with the cell proliferation. Since, the ratio of GSH/GSSG determines the redox status of a cell, the level of GSSG in cells treated with CLA was also measured. MDA-MB-231 cells treated with LP-CLA showed higher GSH and lower GSSG levels when compared to untreated cells .These results suggest, LP-CLA as a potent antioxidant. The earlier findings showed that t10,c12 CLA exerts its anti-proliferative activity mainly through ROS generation in colon cancer cells(Pierre et al., 2013).

Nuclear factor kappa B (NF-κB), a redox transcription factor, is inhibited in the cytoplasm by association with IκBα. Stimulation with bacterial products, particularly LPS, triggers a signal that leads to the ubiquitination of the IκBα followed by translocation of NF-κB to the nucleus where it induces the transcription of pro-inflammatory and cell proliferating genes. The NF-κB complex is constitutively expressed in all tumor cell and there are ample evidences of its oncogenic role especially p65 and p50 components and are considered to be important targets for inhibition. Amino acid residues 33,35, 36, 38, 39, 41-44, 122-124, 187, 218, 220, 246, and 247 are resided in DNA binding region of NF-κB p65 and residues 56, 58, 59, 61, 62, 65-68, 143, 146, 243, 274, 307 and 308 are located in the p50 subunit (Chen et al., 1998). Biochemical assays and molecular docking analysis showed that inhibiting serine 276 phosphorylation of p65 subunit of NF-κB can prevent angiogenesis and metastasis (Law et al., 2010). Docking of with anolide into the p50 subunit revealed the possibility of inhibiting the DNA binding site targeted by NF-κB (Nithya et al., 2009). In this study, the binding of CLA isomers with NF-κB revealed that the carboxylate group of cis-9, trans-11 made an hydrogen bond with protonated NH3 group of LYS241 and another bond with LYS272. From the reports, it is evident that LYS241 is the key residue involved in the binding of NF-κB with DNA at the DNA binding confirmation of the protein. From these results, it can be deduced that cis-9, trans-11 CLA interferes with the DNA binding confirmation of p50 component of NF-κB. Therefore, NF-κB is indeed an interesting target macromolecule in the design of anti-inflammatory and anti-cancer drugs.

Apoptosis/programmed cell death is recognized by a characteristic pattern of morphological, biochemical and molecular changes. CLA isomers cis-9, trans-11 CLA, trans-10, cis-12 CLA and mixed isomers of CLA tested against HepG2 cells were able to induce characteristic apoptotic changes and higher proportion of cells in G0/1 and lower proportion in G2/M phases of the cell cycle suggested the effect of CLA isomers in the reduced viability and proliferation of HepG2 cancer cells in relation with cell cycle arrest and induction of apoptosis (Achenef et al., 2012). El Roz et al (2013) reported the apoptotic potential of t9,t11-CLA against MCF-7 breast cancer cells.

To gain insight into the effect of LP-CLA on proliferation of MDA-MB-231 cell lines and to understand the mechanism underlying apoptosis, the effect of LP-CLA on the proliferation and apoptosis of MDA-MB-231 cells was studied by MTT assay at two-time points. A dose-dependent decrease in cell proliferation was observed until 48hr of LP-CLA treatment with a maximum decrease in cell proliferation at 100μg/ml with an IC_{50} at 60μg/ml

In order to confirm the induction of apoptosis by LP-CLA treatment in MDA-MB-231 cell lines, DNA fragmentation analysis and Flow cytometric analysis were performed. The data showed nuclear fragmentation of MDA-MB-231 cells in a dose-dependent manner after 48h of incubation. Based on ANNEXIN V/PI flow cytometric analysis, this study found an increase in early apoptotic cell population from the control cells to LP-CLA treated cells. Apoptosis is associated with the antiproliferative effect of LP-CLA as the inhibition of cell proliferation and

the induction of apoptotic proportion was increased with increased concentrations of LP-CLA in MDA-MB-231 cell lines. These research results reveal that the anti-proliferative effect of LP-CLA against MDA-MB-231 cells was primarily through the induction of apoptosis.

The release of cytochrome-C into the cytoplasm in response to LP-CLA treatment was measured in MDA-MB-231 cells by western blot analysis. The cytochrome-C release into the cytoplasm was increased in tune with increased concentration of LP-CLA and our results correlate with earlier findings of disruption of normal mitochondrial membrane potential in HT-29 colon cancer cells treated with CLA of derived milk fat (Cho et al., 2003).

The cytoplasmic extract of LP-CLA treated sample was analyzed for the expression of Bcl-2 and Bax. The results showed no apparent changes in the expression levels of Bcl-2, when compared with untreated cells. However, the levels of Bax increased significantly with an increase in concentrations of LP-CLA. It suggests that even though the Bcl-2 protein level did not alter, it is the relative ratio of Bcl-2 to the Bax that determines apoptosis. The unaffected expression of Bcl-2 in LP-CLA treated MDA-MB-231 cells resulted in a higher ratio of Bax to Bcl-2 suggests that LP-CLA induces Bax mediated apoptosis in MDA-MB-231 cells. Kim KJ et al (2015) demonstrated that t10, c12-CLA induces apoptosis in human colorectal cancer cells through ATF3-mediated pathway.

The present study clearly demonstrated that LP-CLA downregulated the inhibition of NF-κB in LPS stimulated MDA-MB-231 cells. Analysis of nuclear NF-κB p65 and IκBα suggested that the inhibition of NF-κB p65 translocation into the nucleus was associated with inhibition of IκBα degradation by blocking the phosphorylation of IκBα.

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