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Cytotoxicity and Apoptosis Induction of *Lactobacillus acidophilus* Cell-free Supernatant against Human Colon Carcinoma Cell Line

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ABSTRACT

Background: *Lactobacillus acidophilus* is one of the promising probiotics that attracts the attention of the scientific community to research its therapeutic characteristics that can provide an option to fight colon cancer.

Methods: The cytotoxic effect of cell-free supernatant of *Lactobacillus acidophilus* (LACFS) was evaluated by using the MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) test to measure cell *via*bility and the high content screening assay (HCS) to detect apoptotic cell death.

Result: LACFS was found to possess the highest anticancer activity on Caco-2 at 50 µg/ml. NCM425 cells were more tolerant toward LACFS than the Caco-2 when the Half-maximal inhibitory concentration (IC_{50}) was 161.8 µg/ml on NCM425 compared to 30.63 µg/ml on Caco-2. When 6.25,12.5, 25 and 50 µg/ml concentrations of LACFS were applied, a substantial drop in mitochondrial membrane potential occurred. A significant rise in cytochrome C release and total nuclear intensity was recorded at 25 and 50 µg/ml concentrations and the viable cell count was decreased at 12.5, 25 and 50 µg /ml, while membrane permeability was dropped at 50 µg/ml. The expression of caspase 3 was observed at 50 µg/ml only while caspase 9 expression was seen at 25 and 50 µg/ml which further indicates that the harmful effect on the Caco2 cells causes them to die through apoptosis.

Key words: Apoptosis, Colon cancer, Cytotoxicity, *Lactobacillus acidophilus*, Probiotics.

INTRODUCTION

The digestive system of humans is a receptacle for a diverse and dynamic microbial population that mostly contains bacteria that greatly impact the body in homeostasis and illness (Liśkiewicz *et al*., 2018). The intestinal flora grows and flourishes within the first three years of human childhood (Gonzalez *et al*., 2011). Probiotic bacteria include live microorganisms' dietary supplements that are classified as healthy ingredients. They improve a host animal's health by preserving gut microbial flora (Er *et al*., 2015). Probiotics could help stop colorectal cancer through several strategies such as modifications in the microbiota of the intestine, inactivation of cancer-causing chemicals, improving the host's immunological response and anti-proliferative activities through apoptotic and cellular differentiation control, combat against harmful and putrefactive bacteria and undigested food fermentation (Uccello *et al*., 2012).

The link between the Lactobacillus-enriched diet and the lower risk of colon cancer has been demonstrated (Goldin *et al*.,1980). Among the most prevalent probiotics are the Lactobacillus species which possess health-improving characteristics that are firmly related to the reduction of allergy reactions. in addition to anti-tumor and anti-inflammatory properties (Ding *et al*., 2018; Kahouli *et al*., 2017; El-Deeb *et al*., 2018). They induce programmed cell death and prevent colonic tumorigenesis (*Escamilla et al*., 2012). *Lactobacillus acidophilus* inhibited the growth of both colonic and gastric cancer cells (Lee *et al*., 2004).

Cancer incidence is expected to increase from 11.5 billion in 2007 to 16.0 billion by 2030, driven by an ageing and rising global population (Hasan *et al*., 2024). As ranking third, colorectal cancer (CRC) is considered to be among

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the most frequent malignancies affecting both the colon and the rectum (Siegel *et al*., 2019). Colorectal cancer is often managed using surgery, radiation, chemotherapy, or immunotherapy (Sadreddini *et al*., 2019). In a study designed to examine how modified circulatory cells can be used as targeted carriers for anticancer drug delivery, traditional therapies might well be associated with unfavorable adverse effects, including chemoresistance, toxicities and cancer recurrence (Singh *et al*., 2020).

MATERIALS AND METHODS

Lactobacillus acidophilus cell-free supernatant (LACFS)

A *Lactobacillus acidophilus* isolate was obtained from the Department of Biology/College of Science/ Baghdad University. It was activated twice by incubating its stock solution anaerobically for 24 and 48 hours at 37°C while subculturing it in MRS broth (Oxoid). One hundred and twenty ml of MRS broth containing 1×10^8 CFU/ml of a 24-hour-grown *L. acidophilus* culture was incubated anaerobically for 24 hours at 37°C. The broth was then centrifuged at 6000 rpm for 15 m and filtered through 0.22 µm pore-size filter papers (Microlab) before being lyophilized and stored at -20°C until it was needed (El-Mokhtar *et al*., 2020).

MTT assay

Five concentrations (50, 25, 12, 5, 6.25 and 3.1 µg/ml) of the LACFS of L. acidophilus were prepared using an MTT kit (Intron Ltd) after 0.5 ml of distilled water was added to the lyophilised product. These concentrations were then tested on human colon adenocarcinoma Caco-2 (RRID: CVCL_0025) and NCM425 (RRID: CVCL_D876) cell cultures (Sigma-Aldrich). Cells were cultivated on RPMI1640 for each concentration at 37°C for 24 hours. They were then extracted using an EDTA/trypsin solution and resuspended in a medium that included 10-20% bovine serum albumin before being plated on a 96-well microtiter plate (All steps were done in triplicate). The MTT assay was used to examine the anticancer potential activity and the results were read at 517 nm after 24 hours (Vinken *et al*., 2015). The cell viability was measured by the formula below:

Percentage of cell viability =

$$\frac{\text{OD of sample - OD of blank}}{\text{OD of control - OD of blank}} \times 100\%$$

Cell death parameters

The high content screening (thermo fisher) approach was used to measure nuclear intensity, *via*ble cell count, membrane permeability, cytochrome C release and mitochondrial membrane potential on Caco-2 in order to estimate cell death parameters at doses of 6.25, 12.5, 25 and 50 µg/ml. This method determines morphological traits and molecular reactivity after chemical treatment using fluorescent markers (Hoechst 33342, SYTOX Green/Red, mito tracker red CMXRos and Alexa Fluor 488/594-conjugated antibodies) (O'Brien *et al*., 2017).

Caspase assay

The Caspase-Glo® 3/7 and Caspase-Glo® 9 Assay Kits (Promega) were used to measure Caspase-3 and Caspase-9 activities as stated by the manufacturer's instructions. Dimethyl Sulfoxide (DMSO) (Thermo Fisher, USA) and Tamoxifen (Dabur Pharma) were used as positive and negative controls, respectively (Hadid *et al*., 2022).

Statistical analysis

ANOVA and Dunnett's multiple comparisons tests were used to analyze the data. Statistical significance was established at a p-value of less than 0.05. Each and every statistical analysis was conducted using GraphPad Prism version 8.4.3.

RESULTS AND DISCUSSION

Cytotoxicity of Lactobacillus acidophilus cell-free supernatant

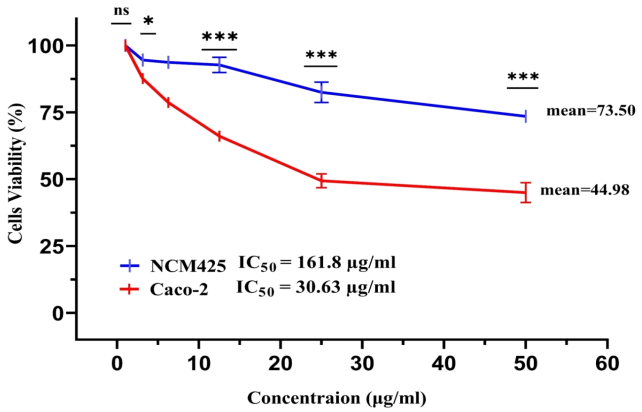
The cytotoxic effect of *L.* acidophilus was evaluated on the colorectal cancer cell line (Caco-2) by using an MTT assay. This experiment aimed to investigate the *via*bility of cells with various dosages of *L. acidophilus* LACFS on Caco-2 and it was shown in form of IC_{50}, which is the concentration required to reduce 50% of cell *via*bility. A lesser IC_{50} value indicates that the *L. acidophilus* supernatant is more effective in inhibiting cancer cell growth and vise-versa. Results indicated that *L. acidophilus* cell-free supernatant (LACFS) possessed cytotoxicity activity on colonic cancer with IC_{50}= 30.63 µg/ml (Fig 1). Earlier studies had demonstrated that L. *acidophilus* has cytotoxic activity against Caco-2 (Hasan *et al*., 2021 ; Shokryazdan *et al*., 2017). When tested at different incubation times, cytotoxicity had been shown on HepG2, Chang and MDA-MB-231 cell cultures (20). On the other hand, cytotoxicity on NCM425 cells was drastically low compared to those of cancer cell line with IC_{50} higher than on cancer cells by nearly 5 times; and it was insignificant with untreated (control) group when tested at all concentrations (Fig 1) (except at 25 and 50 µg/ml). This result agrees with earlier research (Nami *et al*., 2014). which described that *L. acidophilus* CFS has a negligible effect on normal cell lines when tested for cytotoxic activity.

The MTT assay results showed that cytotoxicity of LACFS as expected, was dose-dependent, with a maximum cytotoxic impact reported at a concentration of 50 µg/ml on both Caco-2 and NCM425. As shown in (Fig 1), the cytotoxicity of LACFS on Caco-2 was significantly higher than on NCM425 at all tested concentrations as well as in untreated (control) cell lines. These results were close to those found by (Dehghani *et al*., 2021; Alsadee *et al*., 2024). which revealed that *L. rhamnosus* supernatant suppressed the development of HT-29 cancer cells in a dose- and time-dependent manner. These outcomes indicate a remarkable finding that *L. acidophilus* CFS has safer activity on normal colonic cells than on cancer cells when cell viability recorded 73.5% and 26.5% at 50 µg/ml respectively. Similar results were reported by (21) when *L. acidophilus* CFS appeared to be safer on the normal cell line.

Cell death caused by *Lactobacillus acidophilus* cell-free supernatant (LACFS)

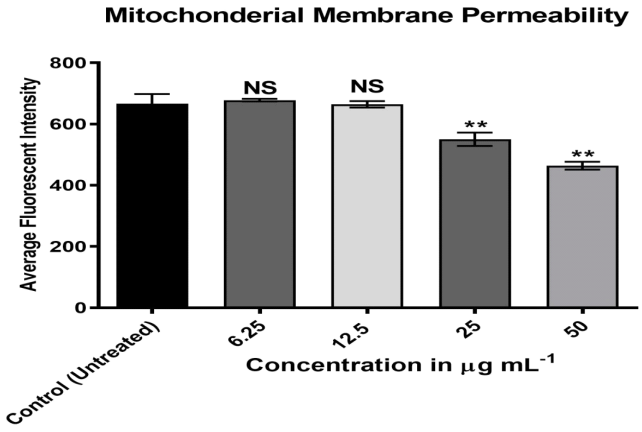
The high content screening (HCS) assay was performed on the colonic cell line to further understand the cell-health indicators. HCS gives several criteria for determining the cytotoxicity of a substance at the single-cell level (Abraham *et al*., 2008).The tested indicators were mitochondrial membrane potential (MMP) changes, cytochrome C discharge *via* mitochondria, the permeability of the cell membrane, *via*ble cell count and morphology of nucleus intensity.

The MMP reduction causes cytochrome C to leak from mitochondria to cytosol so MMP along with cytochrome C were tested by HCS assay and the results showed that there was an effect of LACFS on mitochondrial membrane potential (MMP) at concentrations of 25 and 50 µg/ml which had significant differences from that of untreated (control) group (Fig 2). At 25 and 50 µg/ml, cytochrome C levels rose considerably (Fig 3). In a study, by Madempudi *et al*. (2017); Al-Mashhadani *et al*. (2024). MMP decreased significantly evidenced with rising in cytochrome C levels on colonic cancer cells after treatment with supernatant of *Bacillus coagulans*. The reduction of mitochondrial membrane potential (MMP) and the induction of apoptosis may be ascribed to the downregulation of the anti-apoptotic gene Bcl-2 and an elevation in Bax and caspase-9 which are



Legend 1: Normal cell line (NCM425) of colon (in blue) was more tolerant to cell-free supernatant of *L. acidophilus* than Caco-2 (in red) which was more sensitive by approximately 5 folds measured by IC_{50} values. Results are presented as mean ± standard deviations and all treatments were done in triplicate.

Fig 1: Cytotoxic effect of *Lactobacillus acidophilus* cell-free supernatant (LACFS) on Caco-2 and NCM425 cell lines.



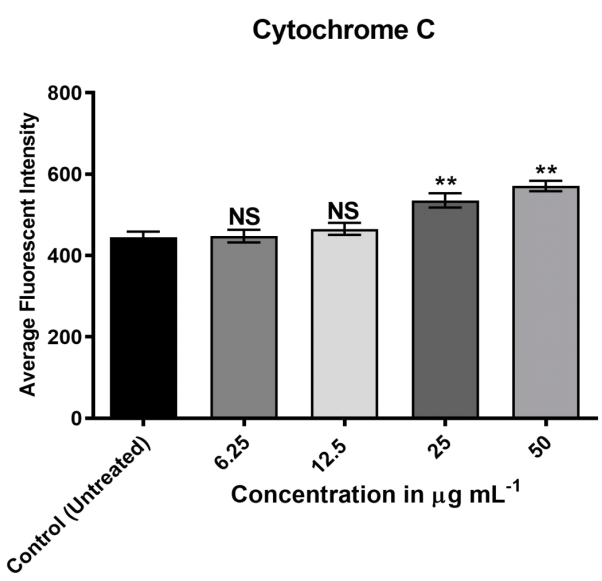
Legend 2: No impact of cell-free supernatant of *L. acidophilus* observed on mitochondrial membrane potential up to 25 µg/ml as evaluated by ANOVA test followed by Dunnett's multiple comparisons test; results are presented as mean±standard deviations and all treatments were done in triplicate.

Fig 2: Activity of *Lactobacillus acidophilus* cell-free supernatant (LACFS) on mitochondrial membrane permeability of Caco-2 cell line.

regarded as apoptotic signals that activate the endogenous route of apoptosis (Al-Saily *et al*., 2019). Such processes induce alterations in the membrane permeability as a result of the expansion of the transition pore for mitochondrial permeability, which allows minute molecules and ions such as Ca^{2+} ions to pass through leading the respiratory chain to decouple and release cytochrome C into the cytoplasm. Eventually, cytochrome C release triggers caspase-9, which leads to the creation of caspase-3 and the initiation of cysteine proteases, which are primarily responsible for the disintegration and digesting of the cell from within (Cha *et al*., 2010; Tafani *et al*., 2002). On cell membrane permeability (Fig 4) findings revealed that LACFS has activity only at high concentration (50 µg/ml) which was significant (P-value≤0.05) in comparison to the untreated (control) group. A previous study by reported a comparable impact of *L. acidophilus* on the permeability of the cells when experienced against the Caco-2 cell line. Modifications in cellular membrane permeability have been linked to apoptotic or toxic responses, in addition, the collapse of cell membrane integrity seems to be a typical morphological hallmark of severe cytotoxic effects (Hassan *et al*., 2018).

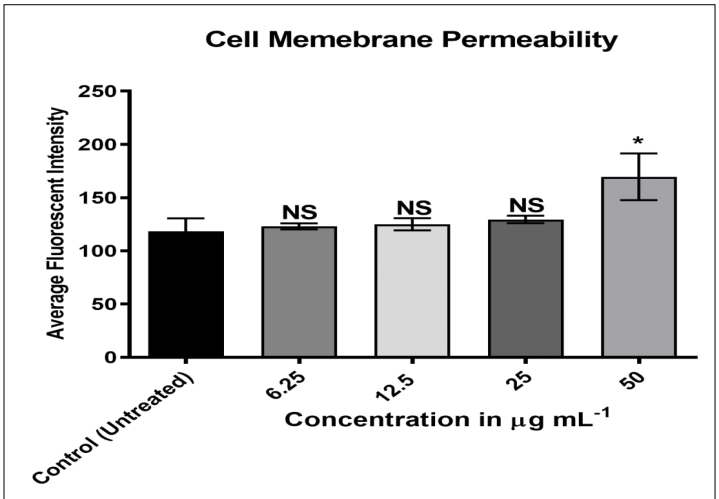
Results (Fig 5) showed that *L. acidophilus* cell-free supernatant (LACFS) has a highly significant effect on the viable count of Caco-2 at concentrations of 25 and 50 µg/ml, compared to the untreated (control) group. The distinction in such survivability findings as contrasted to MTT experiments would be that in HSC the findings convey the impact of previous exposure to the drug for the assessment of morphology and cellular defects in cultures after only a few hours of exposure. However, in MTT, the tested cells were exposed to the agent for a prolonged period . Apoptosis is marked by chromatin condensation and DNA fragmentation, followed by a decrease in nuclear intensity as fragmentation advances (Khan *et al*., 2010).

In this study breakdown of DNA was also seen by a substantial increase in overall nuclear intensity at 25 and 50 µg/ml as shown in (Fig 6), while no effect was noticed at other treatments in comparison to the control (untreated)



Legend 3: No impact of cell-free supernatant of *L. acidophilus* observed on cytochrome c release up to 25 µg/ml as evaluated by ANOVA test followed by Dunnett's multiple comparisons test; results are presented as mean±standard deviations and all treatments were done in triplicate.

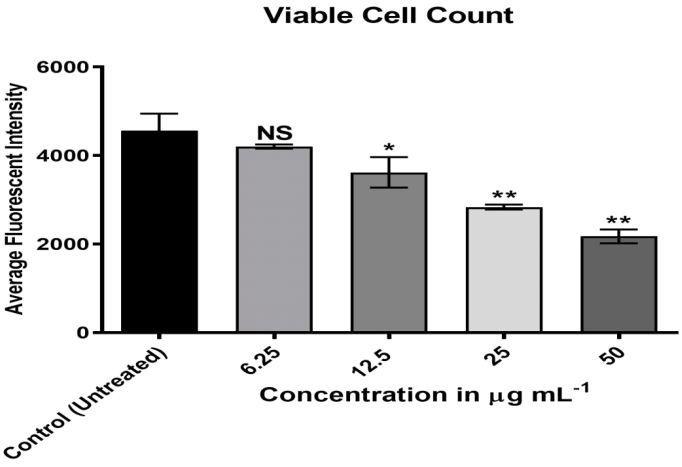
Fig 3: Activity of *Lactobacillus acidophilus* cell-free supernatant (LACFS) on cytochrome c releasing of Caco-2 Cell Line.



Legend 4: Effect of cell-free supernatant of *L. acidophilus* observed on cell membrane permeability only at 50 µg/ml as evaluated by ANOVA test followed by Dunnett's multiple comparisons test; results are presented as mean ± standard deviations and all treatments were done in triplicate.

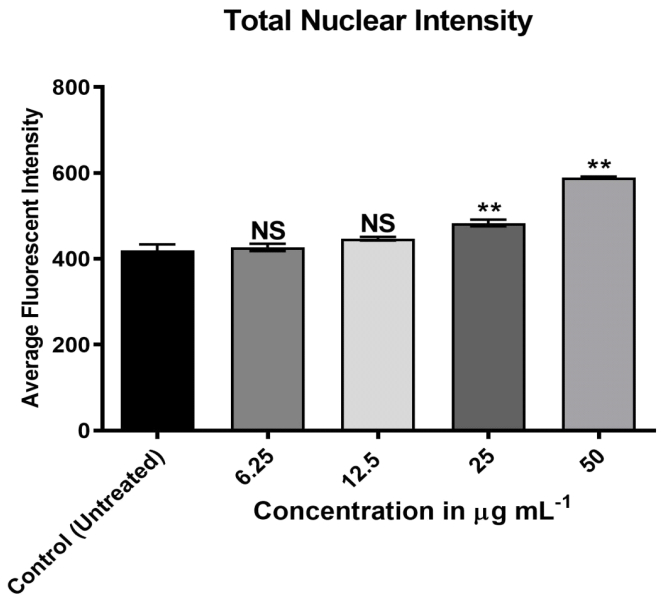
Fig 4: Activity of *Lactobacillus acidophilus* cell-free supernatant (LACFS) on cell membrane permeability of Caco-2 Cell Line.

group. The nuclear intensity outcomes of Kamil *et al*. (2021). also showed an increase when dextran extracted from probiotics Leuconostoc was applied to the MCF7 cell line. As shown in (Fig 7), the impact of LACFS on caspase 3 was only significant at 50 µg/ml concentration when it was extremely significant at 25 and 50 µg/ml concentrations on caspase 9 compared to DMSO (negative control) (Fig 8). It is worth noting that prior probiotic anti-cancer investigations have yielded similar outcomes. High doses of probiotic supernatant, cell walls and pellets might have a potent



Legend 5: Viable cell count of Caco-2 was affected by cell-free supernatant of *L. acidophilus* in all tested concentrations (except at 6.25 µg/ml) as evaluated by ANOVA test followed by Dunnett's multiple comparisons test; results are presented as mean±standard deviations and all treatments were done in triplicate.

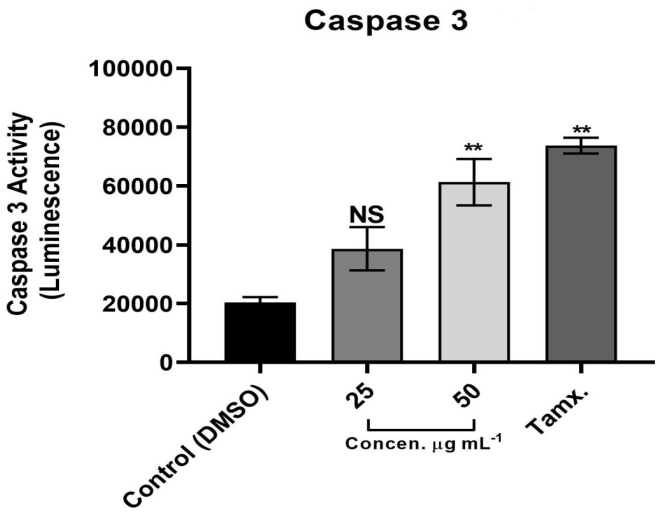
Fig 5: Activity of *Lactobacillus acidophilus* cell-free supernatant (LACFS) on Caco-2 viable count.



Legend 6: Total nuclear intensity of Caco-2 after treatment with cell-free supernatant of *L. acidophilus* no impact was recorded up to 25 µg/ml as evaluated by ANOVA test followed by Dunnett's multiple comparisons test; results are presented as mean± standard deviations and all treatments were done in triplicate.

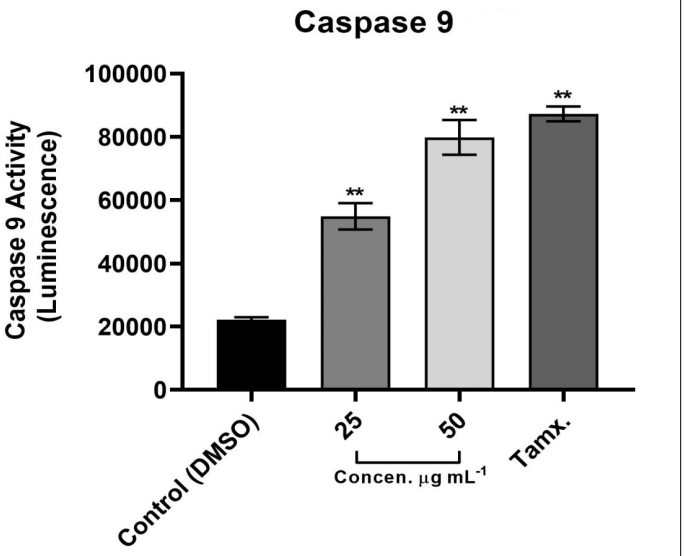
Fig 6: Activity of *Lactobacillus acidophilus* cell-free supernatant (LACFS) on the total nuclear intensity of Caco-2 Cell Line.

anti-tumor impact (Desrouillères *et al*., 2016 ; Rasheed *et al*., 2025). Probiotic *L. delbrueckii,* for instance, suppresses the development of colorectal carcinoma cells SW620 and triggers death *via* a caspase 3-dependent internal mechanism (Wan *et al*., 2014). When *L. acidophilus* CICC 6074 concentrations were raised in comparison to the untreated control group, the quantity of caspase-3 and caspase-9 rose dramatically (Guo *et al*., 2020; Ghiath *et al*., 2025).



Legend 7: Activity of caspase 3 was significant at 50 µg/ml compared to the negative control (DMSO: Dimethyl Sulfoxide) after treatment with cell-free supernatant of *L. acidophilus* as evaluated by ANOVA test followed by Dunnett's multiple comparisons test; results are presented as mean ± standard deviations, and all treatments were done in triplicate. Tamoxifen was used as a positive control.

Fig 7: Effect of *Lactobacillus acidophilus* cell-free supernatant (LACFS) on caspase-3 expression of Caco-2 Cell Line.



Legend 8: Activity of caspase 9 was significant at both tested concentrations (25 & 50 µg/ml) compared to the negative control (DMSO: Dimethyl Sulfoxide) after treatment with cell-free supernatant of *L. acidophilus* as evaluated by ANOVA test followed by Dunnett's multiple comparisons test; results are presented as mean ± standard deviations and all treatments were done in triplicate. Tamoxifen was used as a positive control.

Fig 8: Effect of *Lactobacillus acidophilus* cell-free supernatant (LACFS) on caspase-9 expression of Caco-2 Cell Line.

CONCLUSION

It is noteworthy that *Lactobacillus acidophilus* cell-free supernatant (LACFS) was cytotoxic on human colon cancer cells with lesser cytotoxicity on normal cells which may propose an alternative promising anticancer therapy. Also, LACFS promotes apoptosis in colonic cells as an antitumor combating mechanism.

Conflict of interest

All authors declare that they have no conflict of interest.

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