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ORIGINAL ARTICLE

The secondary metabolites produced by *Lactobacillus plantarum* downregulate BCL-2 and BUFFY genes on breast cancer cell line and model organism *Drosophila melanogaster*: molecular docking approach

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Abstract

Purpose The current study was designed to evaluate the toxicity of the secondary metabolites of *Lactobacillus plantarum* against the human breast cancer cell line (MCF-7) and the *Drosophila melanogaster.*

Methods In this study, toxicity analyses of secondary metabolites of *Lactobacillus plantarum* were analyzed on breast cancer cells, and the *Drosophila melanogaster.* After application, in the MCF-7 cell line, expression levels of RRAS-2, TP53, BCL-2, APAF-1, CASP-3, FADD, CASP-7, BOK genes; in *D. melanogaster*; expression levels of RAS64B P53, BUFFY, DARK, DECAY, FADD, DRICE, and DEBCL genes were determined by RT-PCR. In addition, analysis of *L. plantarum* secondary metabolite was performed by GC–MS method and molecular binding poses of secondary metabolites and human enzymes were investigated in silico.

Results *Drosophila melanogaster* being used as a model organism where some of the human genes were preserved. The IC50 value of the secondary metabolite in the MCF-7 cell line was determined to be 0.0011 mg/ml. Lethal concentration 50 (LC_{50}) and 99 (LC_{99}) values of secondary metabolites against fruit fly adults were 0.24 mg/ml and 0.54 mg/ml, respectively. The expression levels of BCL-2 and BUFFY genes which are anti-apoptotic in human and fruit flies have been reduced, and at the same time, increased expression of DECAY, FADD, RAS64B apoptotic genes in *D. melanogaster*.

Conclusion The substance detected in the secondary metabolite content and encoded as L13 (3-phenyl-1, 2, 4-benzotriazine) has been observed to have high binding affinity in the studied genes.

Keywords *Lactobacillus plantarum* · *Drosophila melanogaster* · MCF-7 · Molecular docking · Apoptosis · Gene expression

Introduction

Today, studies on the effects of probiotics on human health are increasing rapidly. Probiotics are defined as useful, live microbial food contents of lactobacilli, bifidobacteria, enterococci, and streptococci that regulate intestinal microbial homeostasis [1]. *Lactobacillus plantarum* is known as a rod-shaped, Gram-positive lactic acid bacterium. It is usually found in human and other mammalian GI pathways, saliva, and various food products. It is a bacterium that can survive at temperatures between 15 and 45 °C and at temperatures as low as 3.2 pH [2].

Cancer is considered to be a general term used for diseases that affect all or part of the body. The characteristic feature of cancers is that the cells grow beyond the standard growth limits, invade the surrounding cells, and spread to the organs. The most common types of cancer in men are lung, prostate, colorectal, stomach, and liver cancer, while breast, colorectal, lung, cervical, and gastric cancer in women [3]. Breast cancer is the most common type

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of cancer among women. The method determined in the treatment of cancer usually depends on the stage of the tumor. The main treatment modalities are surgical treatment, chemotherapy, radiotherapy, hormonal treatment, and immunotherapy. Each treatment method has different applications, risks, and side effects [4]. These risks, side effects, and multidrug resistance have revealed the necessity to develop alternative methods. Thus, the disadvantages of the standard methods used in the treatment of cancer disease have increased the interest in alternative methods such as the use of probiotics as a supportive method.

The cross-resistance of several tumor cells against a variety of drugs, which are resistant to a particular agent, is described as multiple drug resistance [5]. Various mechanisms have been elucidated in relation to multiple drug resistance in cancer cells, such as irregularities in apoptotic mechanisms, changes in cell cycle control points, repair of damaged cellular targets, and reduction of intracellular drug deposition.

Numerous cellular mechanisms have been elucidated describing drug resistance. These mechanisms include increased excretion of drugs by human multidrug resistance-related protein (MRP), changes in drug targets such as DNA topoisomerase II, and increased detoxification of compounds [5]. In another mechanism, the substrate binds directly to the drug-binding region of the protein and is pumped into the extracellular medium by the energy derived from the hydrolysis of ATP [6].

Apoptosis is a genetically based programmed cell death that allows the destruction of cells that have lost their function, are overpopulated, irregularly developed, or genetically damaged. Apoptosis plays an important role in the development of normal breast cells. Apoptosis rates are related to tumor grade and more aggressive tumors have higher rates of apoptosis and proliferation. Disruption of the apoptotic mechanism overrides most of the control point pathways and leads to the expansion of neoplastic cells. The genes and proteins that control apoptosis can become manipulation targets to increase the death of cancer cells [7].

The investigation of the genetic basis of various diseases, especially cancer, is very important and it is known that *Drosophila* is a very good model organism for such studies [8]. In the present study, *Drosophila* was used as the model organism and the changes in the expressions of some genes on apoptosis were studied by determining the effect of secondary metabolites on breast cancer cell line and *Drosophila*. Besides, molecular docking was studied. The docking process involves the binding of the target ligand structure to the active site of the predicted protein and the prediction of interactions during binding. In our study, we also demonstrated suitable confirmations of the L13 compound in secondary metabolite as a ligand within human apoptotic and anti-apoptotic proteins binding sites in silico using Autodock vina114 [9].

Materials and methods

Lactobacillus plantarum

In this study, the bacterium of *L. plantarum,* isolated from animal sources, was obtained from the Molecular Biology and Genetics Laboratory of Kırşehir Ahi Evran University MRS (De Man Rogosa Sharpe, Merck) medium was used in the activation and development of *L. plantarum* bacteria used in the study. The bacterium is incubated in MRS solid media in an incubated anaerobic jar at 37 °C for 48 h. In the preparation of secondary metabolites, the bacteria in the stationary phase (after the 18 h incubation at 37 °C) were centrifuged at 5000 × g for 15 min. Bacteria supernatants were separated from the pellet and adjusted to pH 7.2 and sterilized by 0.22 µm (Millipore, USA) filters. The lyophilization of the sterilized supernatants was carried out in a lyophilization apparatus at − 86 °C. The GC–MS analysis of the secondary metabolite was performed at the Scientific and Technological Research and Application Center of Aksaray University.

Drosophila melanogaster

Drosophila melanogaster fly used in this study was obtained from Erzurum Technical University Basic Genetic Laboratory. The culture medium for fruit flies was prepared [10] and four different doses of 0.07 mg/ml 0.14 mg/ml 0.27 mg/ml 0.54 mg/ml secondary metabolite applications were performed. After 24 h, mortality rates were recorded and the LC_{50} and LC_{99} values were determined by Probit analysis.

Breast cancer cell line

The MCF-10A and MCF-7 breast cancer cell line used in this study were obtained from the Cancer and Stem Cell Laboratory of the Molecular Biology and Genetics Department of Kırşehir Ahi Evran University.

The stock-extracted MCF-7 cells were cultured in a 37 °C incubator containing 5% CO_2 in 75 cm^2 flasks in RPMI-1640 medium-containing 10% fetal bovine serum and 1% penicillin–streptomycin. The stock-extracted MCF-10A cells, a non-tumorigenic epithelial cell line, were cultured in a 37 °C incubator containing 5% CO_2 in 75 cm^2 flasks in Dulbecco's modified Eagle medium F-12 (DMEM/F-12) supplemented with 5% horse serum, 20 ng/ml epidermal growth factor, 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 µg/ml insulin, and 1% penicillin/streptomycin. Cytotoxicity analysis was performed to determine the cytotoxic effects of the

secondary metabolite on the MCF-10A and MCF-7 cells. In this study, XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium) method was used for cytotoxicity analyses. In this method, cells were cultured in 96 well plates with 5000 cells per well. After 72 h incubation of the metabolite with cells, XTT solution was added and after standing for 2–3 h, optical densities were determined using ELISA (enzyme-linked immunosorbent assay), a reader. The IC_{50} value was calculated as a result of these densities.

FTIR spectroscopy of secondary metabolites in cancer cells

Fourier transform infrared spectroscopy (FTIR) is an analytical method used to determine the chemical bonds and the biochemical compounds at the molecular level. In this study, cells were treated with different concentrations of seconder metabolites of *L. plantarum* for 24 h and 48 h. Afterward, the culture medium was collected and centrifuged, and the cells were trypsinized for 1 min at 37 °C. Cells (5×10^6) were collected by centrifugation (1000*g*, 2 min) and washed three times with sterile PBS solution to ensure complete removal of the growing medium. FTIR spectroscopic analyses of secondary metabolites were performed at Kırsehir Ahi Evran University.

GC–MS analysis of secondary metabolites

GC–MS is a device used in the analysis and structure analysis in which GC (gas chromatography) and MS (mass spectrometry) units work together. Gas chromatography performs the separation of the components in the mixture, while the mass spectrometer performs the structural analysis of each separated component. The GC–MS analysis of the secondary metabolite was performed at Aksaray University Scientific and Technological Application and Research Center.

Total RNA isolation and cDNA synthesis

RNA isolation was performed from the cells after metabolite application to determine the expression levels of the genes studied after the IC_{50} value applied to the MCF-7 cell line. Similarly, RNA was isolated from dead adults of *Drosophila* after LC_{50} and LC_{99} concentration applications.

For *Drosophila,* Zymo QuickRNA kit was used, while the GeneAll (Cat. No. 605-005) HyperScript™ kit was used for MCF-7 cells. Applications were made according to the manufacturer's protocols. cDNA synthesis was performed using the GeneAll (Cat. No. 605-005) HyperScript™ kit according to the manufacturer's protocol. Real-time PCR reactions of cDNAs synthesized to determine the expression changes of genes were performed.

Apoptotic genes

Breast cancer-related genes have been identified from the Qiagen commercial firm's data bank (https://www.qiagen.com) from the list of apoptosis genes. The orthologs of the human genes found in *Drosophila* are available from Flybase.org (Table 1).

Molecular docking studies

Ligand and protein preparation

The three-dimensional structures of the 18 active substances identified as a result of GC–MS analysis of the *Lactobacillus plantarum* secondary metabolite were obtained from the PubChem (https://pubchem.ncbi.nlm.nih.gov) data bank. The gene access is provided from RCSB PDB protein database [RRAS-2 (2ery), TP53 (1tup), BCL-2 (1g5m-1gjh), APAF-1 (1cy5), CASP-3 (3edq), FADD (3ezq), and CASP-7 (4fdl)] (https://www.rcsb.org/).

Molecular docking calculations were obtained from two different programs; Autodock Vina, VMD, and Free Academic Maestro (Schrödinger) [11, 12]. Water molecules and cofactors were removed from the proteins to provide the interaction between only ligand and receptor. The Lamarckian genetic algorithm was used as a score function to guess the best interaction between ligand and anti-apoptotic proteins. The highest binding score refers to the most stringent binding between protein and ligand.

Results

The present study demonstrates that exposure of MCF-7 cells to secondary metabolites of *L. plantarum* for 24 h and 48 h induces overall biochemical changes. Figures 1, 2, 3 shown that the cells are disintegrated and nucleic acids are spread to the environment with the application time (200–1200 cm^{-1}). These results support the cytotoxic effect of the metabolite to the cell line.

Table 1 Studied genes in *Drosophila melanogaster* and *Homo sapiens*

Drosophila melanogaster	*Homo sapiens*
RAS64B	RRAS-2
P53	TP53
BUFFY	BCL-2
DARK	APAF-1
DECAY	CASP-3
FADD	FADD
DRICE	CASP-7
DEBCL	BOK

Fig. 1 FTIR spectrum of non-treated MCF-7 cells (control)

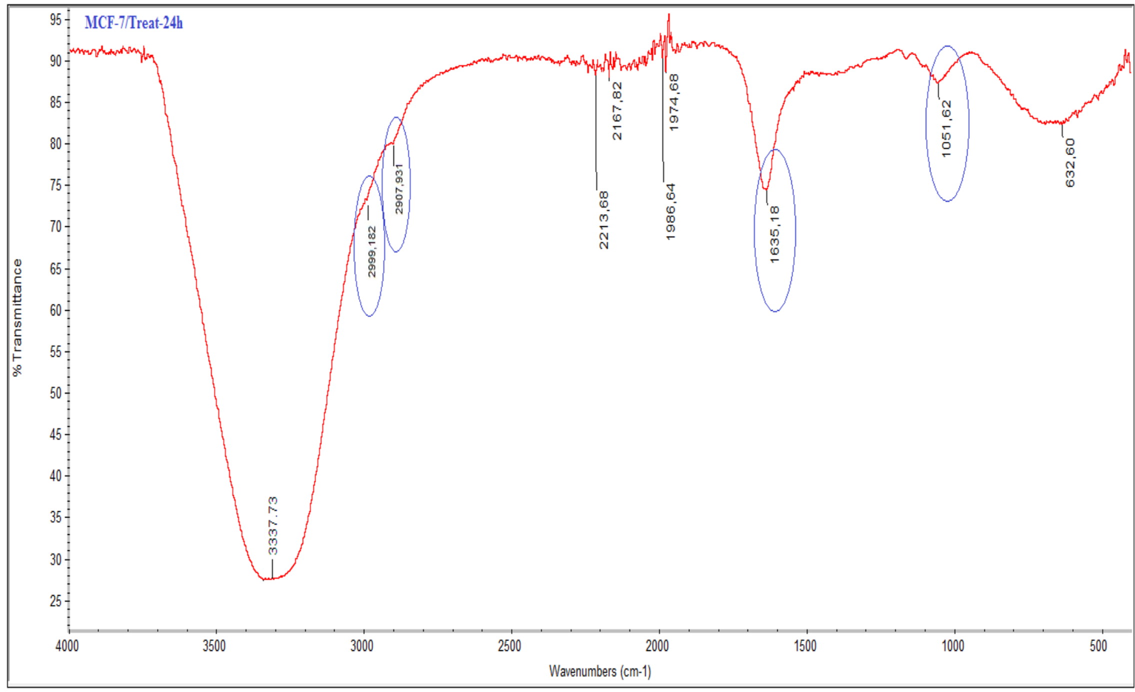


Fig. 2 FTIR spectrum of metabolite treated MCF-7 cell (24 h)

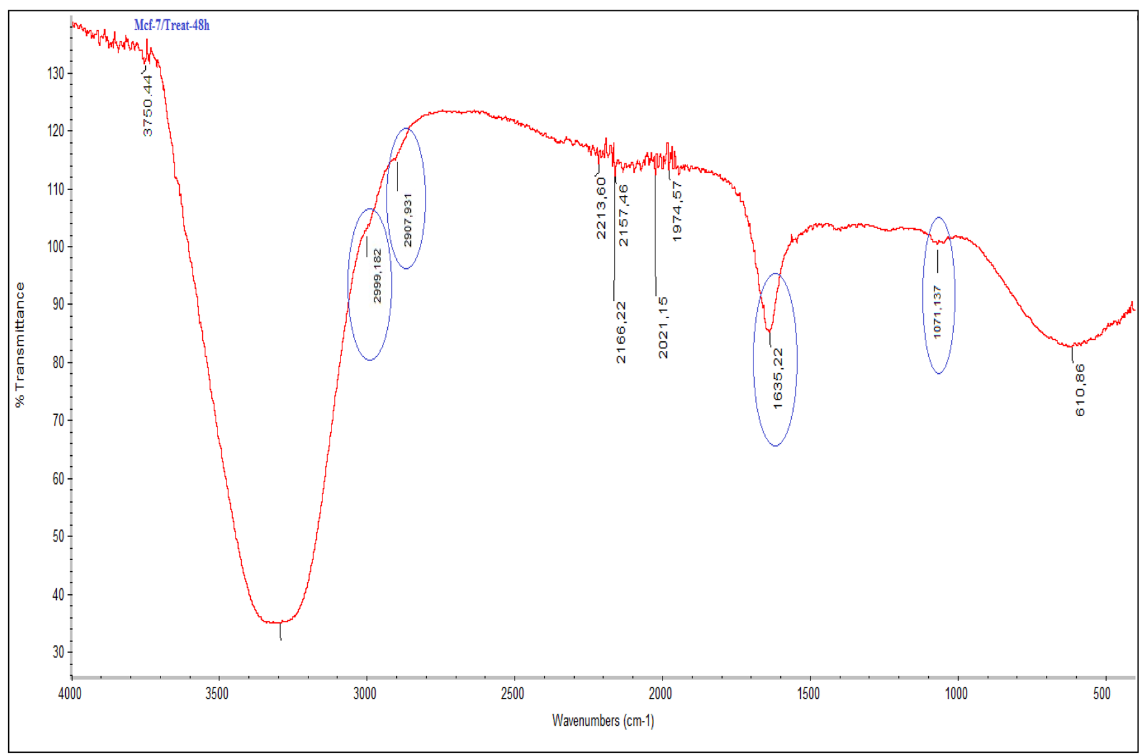


Fig. 3 FTIR spectrum of metabolite treated MCF-7 cell (48 h)

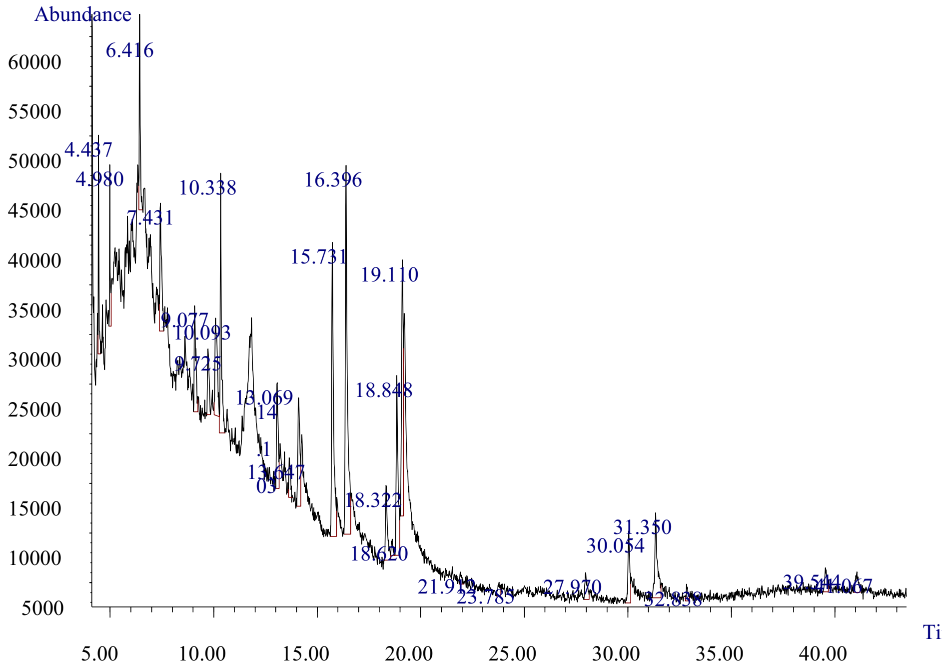


Fig. 4 GC–MS analysis spectrum of secondary metabolites of *L. plantarum*

In Fig. 4, the results pertaining to GC–MS analysis of *L. plantarum* lead to the identification of a number of compounds. These compounds were identified through mass spectrometry attached with GC. As a result of the GC–MS analysis, many substances from the PubChem databanks (https://pubchem.ncbi.nlm.nih.gov/) were scanned, and peer and similar substances were found (Table 2).

The anti-cancer mechanisms associated with secondary metabolite may explain the cytotoxic potency of these compounds. Anti-cancer agent selectivity is significant,

Table 2 The substances determined by GC–MS analysis

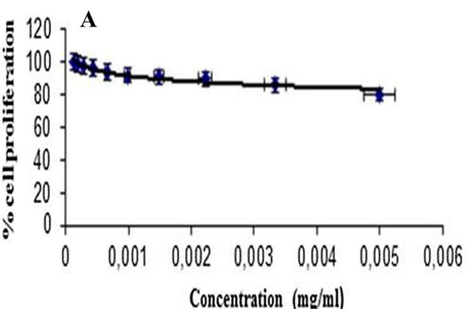
Code	Chemical name	Pubchem Number	Chemical structure
L 1	2-Ethyl-3,6-dimethylpyrazine	25916	
L2	2-Butyl-3,5-dimethylpyrazine	528127	
L3	P-Tolyl methyl sulfone	18521	
L4	2-Amino-4-methylpyridin-3-ol	580054	
L5	5-Methyl-1,3-diazaadamantan-6-one	580197	

Table 2 (continued)

L6	1-Phenylpyrrole	12480	
L7	3-Methylisoquinoline	14306	
L8	2,6-Dimethylcyclohexanone	17780	
L9	1-Amino-2-methylnaphthalene	16733	
L10	7-Ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]oct-3-ene	536095	

Table 2 (continued)

L11	L,L-Cyclo(leucylprolyl)	102892	
L12	2,3,5,6-Tetrafluorophenyl isothiocyanate	140779	
L13	3-Phenyl-1,2,4-benzotriazine	239695	
L14	Phenylalanyl-prolyl diketopiperazine	99895	
L15	2-(5-Nitrothiophen-3-yl)pyrimidine	901433	
L16	2-(2-Nitrothiophen-3-yl)pyrimidine	12399594	



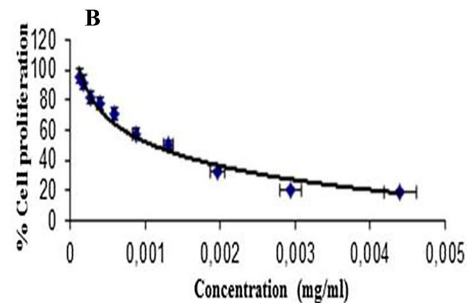


Fig. 5 Graphical representation of the antiproliferative effect of *Lactobacillus plantarum* secondary metabolite products on **a** MCF-10A and **b** MCF-7 cells using XTT cell proliferation kit

Table 3 LC_{50} and LC_{99} doses of secondary metabolite against to *Drosophila* adults

Duration (24 h)	*N*	LC_{50}	LC_{99}	DF	C2
	10	0.24 mg/ml	0.56 mg/ml	2	0.694

Since the goodness-of-fit Chi-square was significant ($P < 0.05$), a heterogeneity factor was used in the calculation of confidence limits

N number of the tested stages

because most anti-cancer drugs currently in use induce serious adverse effects. In this study, secondary metabolites of *L. plantarum* were determined to investigate whether the cytotoxic activity was specific to cancer cells (Fig. 5).

Drosophila adults were exposed to a mixture of secondary metabolites at doses determined for 24 h. Mortality rates were determined in the applications and probit analysis was performed to determine LC_{50} and LC_{99} doses [13]. The indicated doses are shown in Table 3.

The substance with L13 code was found to be docked all tested proteins with good confirming. The results indicated that this molecule may be successful in inhibiting these proteins. In this case, this molecule showed a great affinity against tested proteins and may play an important role as an anti-cancer agent. In the study, we showed the docking results of L13 with proteins (Table 4).

FADD and L13 interactions can be observed in Fig. 6. In the FADD and L13 compound, non-covalent molecular interaction occurred only with amino acid residue C:300 LYS.

In BCL2 and L13 docking result, non-covalent interactions were observed with PHE 109, MET 112, PHE 150, ALA 146, PHE 101, and LEU 134 amino acid residues (Fig. 7).

In our gene expression results; a significant decrease was observed in the expression of BUFFY, an anti-apoptotic gene, according to the gene expression results obtained by LC_{50} application in *Drosophila*. A decrease in the expression level of the anti-apoptotic gene, BUFFY, can be interpreted as contributing to the progression of the cell to apoptosis. Significant decrease of expression in apoptotic genes, DARK and DEPCL, was also seen in *Drosophila*. The increase of expression in DECAY and FADD is an important result.

Decreased gene expression in BCL-2, an anti-apoptotic gene, as a result of IC_{50} dose application in MCF-7 cell line. Decrease in the expression level of BCL-2 can be interpreted as the contribution of the applied substance to the cell's apoptosis. It is noteworthy that there is decreased expression of apoptotic genes, APAF-1 and TP53. These results suggest that the apoptotic process can be run through different receptors and through different gene families. In our study, the effect of the related substance on the expression of these genes has been studied for the first time.

Discussion

Lactobacillus plantarum is an important species of lactic acid bacteria (LAB). There is limited information about the anti-cancer properties of LAB on cytotoxic and antiproliferative activity of metabolites that produced this important probiotic bacterium. However, the benefits of LAB against many diseases have been extensively studied. These include inflammatory bowel diseases, immunomodulating functions, control of serum cholesterol, and various types of cancer [14]. In our study, L13 coded ligand was found as the most effective substance of the secondary metabolite produced by *L. plantarum* as a result of molecular docking. We found the chemical name of this substance as 3-phenyl-1,2,4-benzotriazine from the PubChem (ID: 239695). Same substance has been studied by Kotovskaya et al. to determine its antiviral and cytotoxic activity on Vero cell cultures [15]. Kotovskaya et al. concluded that this substance is active against serious diseases caused by smallpox and some other pathogenic viruses [15].

In the past, cancer research has been conducted in mammalian-based systems, from tissue culture to animal studies. However, in recent years, flies have been used as a model organism. One of the greatest contributions of flies to the study of cancer biology was the elucidation of the Ras signal transduction cascade defined more than 20 years ago [16–18]. It was found that this pathway was preserved in mammals.

Table 4 Docking-binding energy results of ligands and proteins

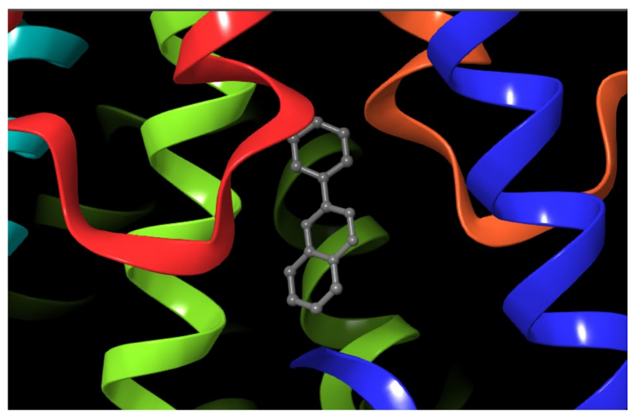
Protein	RRAS-2		
Number of ligand	Binding energy (kkal/mol)	Number of ligand	Binding energy (kkal/mol)
L01	−5.4	L10	−5.2
L02	−5.4	L11	−5.8
L03	−6.2	L12	−5.5
L04	−5.3	L13	−6.0
L05	−5.3	L14	−6.1
L06	−5.1	L15	−5.4
L07	−5.9	L16	−5.1
L08	−5.0		
L09	−6.1		
Protein	TP53		
Number of ligand	Binding energy (kkal/mol)	Number of ligand	Binding energy (kkal/mol)
L01	−4.8	L10	−5.2
L02	−5.0	L11	−5.8
L03	−5.5	L12	−5.5
L04	−5.4	L13	−7.1
L05	−5.3	L14	−6.8
L06	−5.3	L15	−5.5
L07	−5.8	L16	−5.3
L08	−5.0		
L09	−7.0		
Protein	BCL-2 (1g5m)		
Number of ligand	Binding energy (kkal/mol)	Number of ligand	Binding energy (kkal/mol)
L01	−4.7	L10	−4.8
L02	−5.4	L11	−6.2
L03	−5.5	L12	−5.4
L04	−4.5	L13	−6.9
L05	−5.2	L14	−6.8
L06	−5.0	L15	−6.1
L07	−5.5	L16	−5.8
L08	−4.8		
L09	−5.6		
Protein	BCL-2 (1gjh)		
Number of ligand	Binding energy (kkal/mol)	Number of ligand	Binding energy (kkal/mol)
L01	−4.7	L10	−5.1
L02	−5.3	L11	−5.4
L03	−5.1	L12	−5.2
L04	−4.5	L13	−6.5
L05	−5.0	L14	−6.4
L06	−5.1	L15	−5.8

Table 4 (continued)

Protein	BCL-2 (1gjh)		
Number of ligand	Binding energy (kkal/mol)	Number of ligand	Binding energy (kkal/mol)
L07	−5.5	L16	−4.8
L08	−4.9		
L09	−6.1		
Protein	APAF-1		
Number of ligand	Binding energy (kkal/mol)	Number of ligand	Binding energy (kkal/mol)
L01	−3.7	L10	−3.8
L02	−3.9	L11	−4.4
L03	−5.1	L12	−4.1
L04	−4.4	L13	−5.6
L05	−3.9	L14	−5.5
L06	−4.0	L15	−4.9
L07	−4.4	L16	−4.2
L08	−4.0		
L09	−4.7		
Protein	CASP-3		
Number of ligand	Binding energy (kkal/mol)	Number of ligand	Binding energy (kkal/mol)
L01	−4.8	L10	−5.3
L02	−5.4	L11	−5.8
L03	−5.7	L12	−5.4
L04	−4.5	L13	−7.7
L05	−4.8	L14	−6.7
L06	−5.3	L15	−5.9
L07	−5.8	L16	−5.3
L08	−4.9		
L09	−6.1		
Protein	FADD		
Number of ligand	Binding energy (kkal/mol)	Number of ligand	Binding energy (kkal/mol)
L01	−4.7	L10	−5.1
L02	−6.1	L11	−6.1
L03	−6.5	L12	−5.1
L04	−6.3	L13	−8.6
L05	−6.1	L14	−6.9
L06	−7.1	L15	–
L07	−5.8	L16	–
L08	−6.0		
L09	−6.0		

Table 4 (continued)

Protein	CASP-7		
Number of ligand	Binding energy (kkal/mol)	Number of ligand	Binding energy (kkal/mol)
L01	−5.1	L10	−5.2
L02	−5.3	L11	−5.9
L03	−5.6	L12	−5.2
L04	−4.8	L13	−7.9
L05	−5.0	L14	−6.9
L06	−5.5	L15	−5.8
L07	−6.1	L16	−5.7
L08	−5.0		
L09	−6.5		



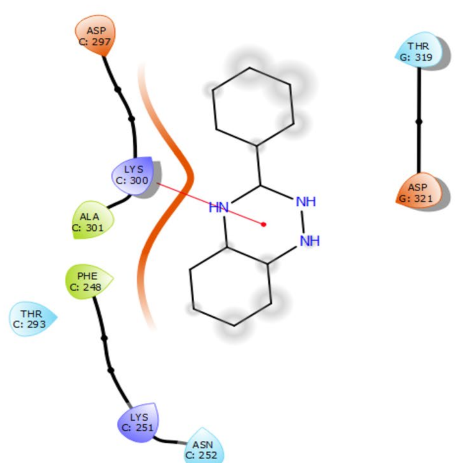
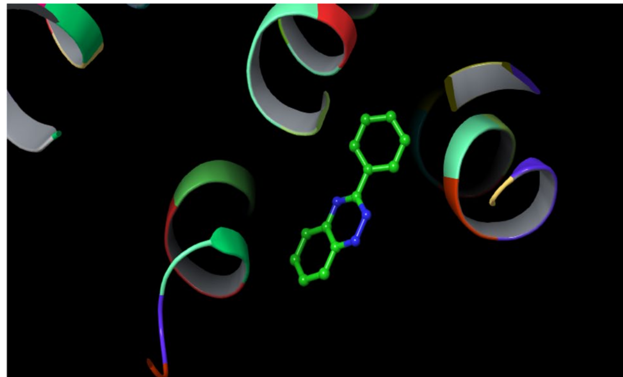


Fig. 6 Superimposition of L13 molecule at the active site of FADD protein in molecular docking



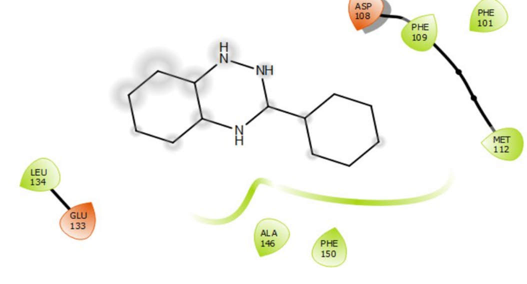


Fig. 7 Superimposition of L13 molecule at the active site of BCL2 protein in molecular docking

Caspases play a key role in the apoptosis mechanism. It is known that similar pathways are preserved in mammals and *Drosophila*. In *Drosophila*, DCP-1, DCP-2, DRICE, and DRONC caspases, as well as DECAY caspase have been identified. Dorstyn et al. pre-characterized the DECAY caspase in *D. melanogaster*. They reported that the caspase species described in this study was very similar to caspase-3-like effector caspases in mammals and shared a similar substrate specificity [19]. Studies show that 75% of human disease genes are protected in *Drosophila* [20].

Failure rates, high cost, and time-consuming experiments in the development of cancer drugs have created the need for alternative methods. The preservation of the signaling pathways, the ease of working in time, and the low cost of cultivation have made *D. melanogaster* a powerful tool for high-throughput screening of cancer drugs. The fact that *D. melanogaster* contains the homolog of genes linked to various human cancers has provided an advantage for the use of the organism in cancer drug discoveries. Thus, fruit fly, *D. melanogaster*, has been used as a vehicle for testing and developing cancer therapeutics in vivo [21]. For example, the HIPPO signaling pathway, known as cell proliferation and regulation of apoptosis, was first described in *D. melanogaster* [22]. Similarly, the role of the JAK-STAT signaling pathway in human leukemia has been shown to cause an overgrowth of hemocytes in *D. melanogaster* before the discovery of its role in human leukemia [23].

In our study, a decrease in anti-apoptotic genes and an increase in apoptotic genes both in *Drosophila* and cancer cell line indicate the correlation of *Drosophila* as a model organism with the cell line. Molecular docking study was performed in each of the 16 materials determined by the analysis of the secondary metabolite and the active substance in the secondary metabolite was obtained from the binding values of L13-coded ligand structure. In the light of the results obtained from our study, it is thought that secondary metabolites obtained from this probiotic bacterium can be used as an anti-cancer agent. The results from *Drosophila* as an in vivo model organism also support this assessment. The most effective L13 will be purified and the anti-cancer effect will be tested by further studies.

Compliance with ethical standards

Conflict of interest All the authors declare no conflict of interest.

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