Iranian Journal of Pharmaceutical Sciences
2022: 18 (1): 47-54
www.ijps.ir

Original Article

Effect of Solvent Polarity on the Extraction Yield of Antioxidants from *Lactobacillus* Supernatants

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Abstract

There is increasing evidence to suggest that the *Lactobacilli* species possess antioxidant activities, however, there are a few reports of optimization of solvent systems for the separation of their antioxidant compounds by thin-layer chromatography. In the current study, we explore the efficiency of four organic solvents (aqueous, methanol, ethyl acetate, and n-hexane) for the extraction of antioxidant materials from *Lactobacillus* supernatants. According to the results, methanol extraction significantly increased the antioxidant properties of *Lactobacillus* supernatants. In addition, the methanol extract of *Lactobacillus* supernatants was fractionated using thin-layer chromatography (TLC). A solvent system consisting of methanol/chloroform is a promising approach for TLC analysis of *Lactobacillus* supernatant. The partial purification of the antioxidant components using thin-layer chromatography demonstrated a drastic rise in the antibacterial and antioxidant properties in comparison to the crude methanol extract of the same sample. In this study, the TLC fractionation of *Lactobacillus* extracellular materials was described for the first time. Further isolation and purification are essential to identify these bioactive compounds.

*Keywords***:** Antibacterial, Antioxidant, Extraction, *Lactobacillus*, Supernatant, Thin-Layer Chromatography.

1. Introduction

In the last two decades, lactobacilli have been used in medicines, and pharma foods to prevent infection by pathogenic bacteria [1], as well as disease prevention and treatment such as; cancer, urogenital tract infections, allergy, inflammatory diseases, bowel syndromes, and diarrhea [2].

It was shown that *Lactobacillus* has various beneficial effects on the host's health including digestion, and bone health, vitamin, and mineral production, elimination of carcinogens, lowering cholesterol, immuno-stimulation, allergy, and lactose intolerance [3]. In addition, the antioxidant properties of *Lactobacillus*

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Cite this article as: Pourramezan Z., Oloomi M., Kasra Kermanshahi R., Effect of Solvent Polarity on the Extraction Yield of Antioxidants from *Lactobacillus* Supernatants, 2022, 18 (1): 47-54.

species could decrease the risk of accumulation of ROS during ingestion of food [4], so can prevent various degenerative diseases that are the consequence of cellular injury caused by free radicals [5].

Some literature illustrated the antioxidant activities of probiotics [6, 7]; however, optimization of solvent systems for the separation of antioxidant compounds by thin-layer chromatography has not been described yet [8].

In our previous study, we reported that most *Lactobacillus* species isolated from kefir and camel dough have antioxidant and antibacterial traits [9]. The objective of the present study was to compare the different extraction solvents by using water, methanol, ethyl acetate, and n-hexane for the isolation of antioxidant components. To achieve this goal, different solvent systems were evaluated for the ability to separate antioxidant compounds by thin-layer chromatography. Finally, the best solvent system for semi-purification of *antioxidant fractions of each lactobacillus strain* by TLC was suggested.

2. Materials and Methods

2.1. Bacterial Strains and Culture Media

Samples collection, cultivation, bacterial identification, and screening of antioxidant isolates were described previously [9]. The *Lactobacillus* strains were grown anaerobically in 100 mL of MRS (de Man, Rogosa and Sharpe) or PMM5 (Plantarum Minimal Medium) broths for 48 h at 37 °C [10].

2.2. Cell-Free Supernatant Preparation

The supernatant of fermented broth was acquired by centrifuging the broth at 4,000 ×g for 15 min. Centrifuged supernatants were passed through sterile 0.22 μm pore-size filters (Sartorius, Germany), and freeze-dried (Pishtaz Engineering, Iran) [11]. The crude extracts were kept in a sealed container at -20 °C [9].

2.3. Preparation of Aqueous, Methanol, Ethyl Acetate, and N-hexane Extracts from Lactobacillus Supernatants

For extraction, the freeze-dried sample was dissolved in the selected solvent (water, methanol, ethyl acetate, and n-hexane) and the solute was transferred to a glass vial. The solvent was evaporated using a rotary device at 40 °C, leaving only the active ingredient in the vials. Then, the different concentrations (10, 20, 40, 80, and 120 μg / ml) of the crude extract were prepared from the stock sample containing 1000 μg / ml of the selected solvent.

2.4.Determination of Antioxidant and Antibacterial Activities

For the determination of antioxidant and antibacterial activities, the DPPH and MIC methods were used as described previously [8].

2.5. Thin-Layer Chromatography and Bio-autography Analyses

Bioautographic analysis was conducted to check the antioxidant activity of separated compounds on the TLC plate [11]. A fixed concentration of each extract (10 μL of 10 mg/mL) was applied 2 cm from the base of a pre-coated silica gel F254 TLC plates (E. Merck, Darmstadt, Germany) with capillary pipettes. The antioxidant compounds were separated by ascending development for 17 cm in the selected solvent system (Table 1, and Table 2).

Table 1. Solvent systems for TLC separation of antioxidant in methanolic extract of PMM5 broth fermented by different *Lactobacillus* strains.

Methanol: dichloromethan: ethyl acetate	Dichloro methan: ethyl acetate	Methanol : ethyl acetate	Chlorof orm: ethyl acetate	Methanol: chloroform:	Methanol : Dichloromet han: chloroform	Methanol : Dichlorom ethan	Methanol : chlorofor m	Solvent systems
3:2:1	2:1	2:1, 3:1	1:3	7:1:2, 3:2:2	3:1:1	3:1	3:4, 3:3, 3:2, 3:1, 9:1*	*Lactobacillus* strain AG2
1:3:1	3:1	1:1, 1:2	1:2	1:1:3	1:3:1*	1:2, 1:3	1:1, 2:1, 3:7	*Lactobacillus* strain AG12a
3:1:1	1:1	3:1	1:1	3:1:7	3:1:1*	3:1, 3:2, 2:1	1:1, 1:2, 5:1, 7:3	*Lactobacillus* strain AG20
3:2:1	1:3	3:0.5	2:3	3:2:1	3:3:1	3:2	3:2*	*Lactobacillus* strain AG32a
3:2:1	2:3	4:1	3:1	3:1:0.5	3:1:1, 3:1:0.5*	3:2	3:2	*Lactobacillus* strain AG32b
3:1:1	4:5	4:1	3:4	3:1:1	3:1:1	3:2, 4:1	7:4, 5:4*	*Lactobacillus* strain AG12b1
3:1:1	2:3	3:2	3:1	3:1:1	3:1:1	3:2	3:2*	*Lactobacillus* strain YA2
4:1:1	1:2	4:1*	1:4	3:1:1	3:1:1	3:2	3:2	*Lactobacillus* strain AG12b2
2:1:1	1:3	2:1	1:3	3:3:1, 3:2:1, 3:4:1	3:1:1	3:1, 3:2	3:1, 3:2*	*Lactobacillus* strain K1C

Table 2. Rf values of antioxidant fractions isolated from extracellular materials of *Lactobacillus* strains cultured in PMM5 medium at 37˚C for 48 h using optimized solvent systems by *TLC-DPPH* method.

L. kimchii strain YA2	*L. durianis* strain AG32b	*L. suebicus* strain AG20	Lactobacillus casei strain K1C		*Lactobacillus hilgardi* strain AG12a										Strain
Methanol: chloroform (3:2)	Methanol: Dichloro-methane: Chloroform (3:1:1.5)	Methanol: chloroform: Ethyl acetate (7:1:2)	Methanol: chloroform (3:2)		Methanol:Chloroform:Dichloromethane(3:1:1)										Solvent system used
F1	F2	F1	F1	F3	F2	F1	F9	F8	F7	F6	F5	F4	F3	F2	F1	TLC fraction number
0.63	0.66	0.54	0.72	0.34	0.20	0.03	0.65	0.64	0.53	0.51	0.46	0.41	0.35	0.3	0.11	R_f Value of Antioxidant active TLC spots
-	-	-	-	-	-	+	+	-	-	+	-	+	-	-	-	Antibacterial activity

† *w: Comparatively weak in activity; +: comparatively strong in activity; - : no activity*

Spots and bands were visualized by visible and UV irradiation (240 and 300 nm). In order to test the repeatability of the method, TLC assays were performed in triplicate and Rf values were measured in each experiment. In all the TLC analyses, the antioxidant extracellular materials produced by the *Lactobacillus* cultures represented the same Rf value that demonstrated the repeatability of the test (Table 2).

Since different supernatant extracts showed different TLC band patterns using a common mobile phase (Figure 1), it was implied that the samples have different antioxidant ingredients. Therefore, to optimize the solvent systems in TLC, different mobile phases were used (Table 1), and then the exact location of the antioxidant on TLC paper was determined using the DPPH reagent. For the screening of the antioxidant band, the developed air-dried plate was sprayed with a methanol solution of 0.1 % DPPH antioxidant reagent, and the active antioxidant constituents were detected as yellowish-white spots on the purple background (Figure 1). All detected active antioxidant constituents were noted according to their R_f values [12].

For solvent systems' optimization, the ratio of solvents was changed in a way that the antioxidant spot achieves the best separation and resolution from the other spots. To achieve this goal, various mobile phases with different ratios were tested (Table 1). There may be several antioxidants in the bacterial culture itself. Therefore, the extract of the uncultured broth was used as a control in each experiment to exclude the false results.

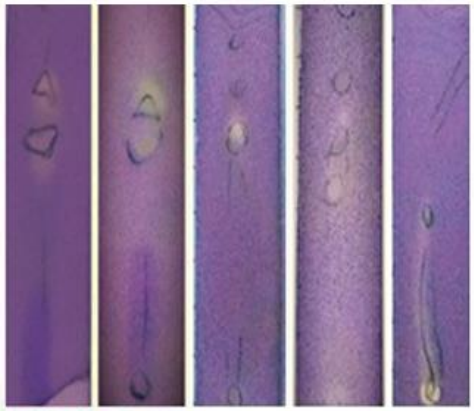

Figure 1. Optimization of the solvent system (mobile phase) for isolation of antioxidants from methanolic extract of the of *Lactobacillus casei* strain K1C supernatant. From right to left: Methanol: chloroform: ethyl acetate (3:4:1), methanol: chloroform: ethyl acetate (3:3:1), methanol: chloroform: ethyl acetate (3:2:1), Methanol: chloroform (3:2), methanol: chloroform (3:1).

2.6. Statistical Analysis

Results are presented as mean ± standard error of the mean. The data were analyzed by one-way ANOVA. A *p-value* less than 0.05 was considered significant.

3. Results and Discussion

Extraction temperature is an important factor that can affect the antioxidant capacity of an extract. The increasing temperature during extraction raises the IC_{50} level significantly [13]. In addition, it was suggested that lyophilization before extraction could be considered an innovation in the use of probiotic products [11]. Therefore, we used lyophilization and 40 °C in the extraction procedure.

The antioxidant activity of two *Lactobacillus (L. hilgardii* strain AG12a and *L. casei strain* K1C) supernatants extracted by different solvents were plotted in Figures 2 and 3.

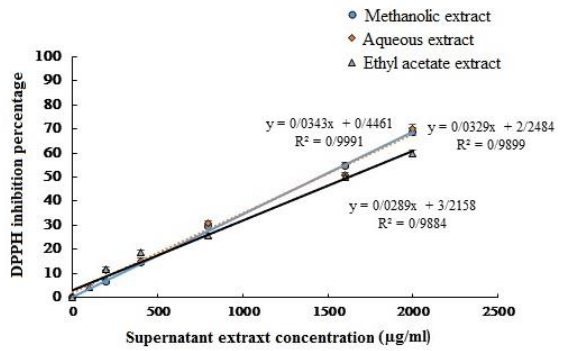

Figure 2. DPPH assay results for aqueous, methanolic and ethyl acetate extracts of the MRS supernatants fermented anaerobically by *L. hilgardi* strain AG12a at 37 ° C for 48 hours. IC_{50} of the samples based on the linear equation of methanolic (y = 0.0343x + 0.4461), aqueous (y = 0.0329x + 2.2484) and ethyl acetate (y = 0.0289x + 3.2158) extracts that are equal to 1444.72, 1451.42, and 1618.83 µg/ml, respectively.

As illustrated in Figures 2 and 3, the antioxidant activity of aqueous and methanol extracts was very similar, but methanol extracts on average possessed more antioxidant properties. On the other hand, due to its reasonable price, good solubility for most compounds, and ease of evaporation at low temperatures (boiling point 65 ° C), methanol is the most efficient solvent in antioxidant extraction. A comparison of antioxidant properties in the methanol extract of MRS broth fermented by *Lactobacillus* strains was shown as IC_{50} values in Figure 4.

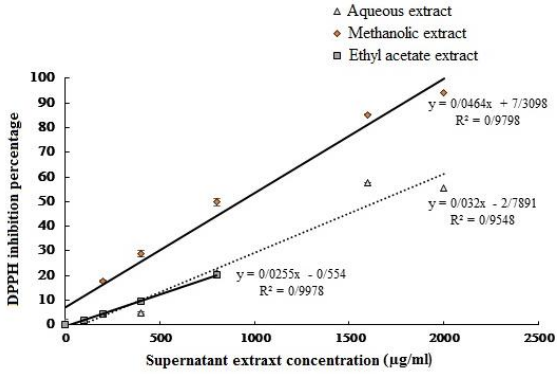

Figure 3. DPPH assay results for aqueous, methanolic and ethyl acetate extracts of the MRS supernatants fermented anaerobically by *L. casei strain* K1C at 37 ° C for 48 hours. IC_{50} of this sample is based on the linear equation of methanolic (y = 0.0464x + 7.3098), aqueous (y = 0.032x – 2.7891) and ethyl acetate (y = 0.0255x – 0.554) extracts that are equal to 920.047, 1649.66, and 1982.51 µg/ml, respectively.

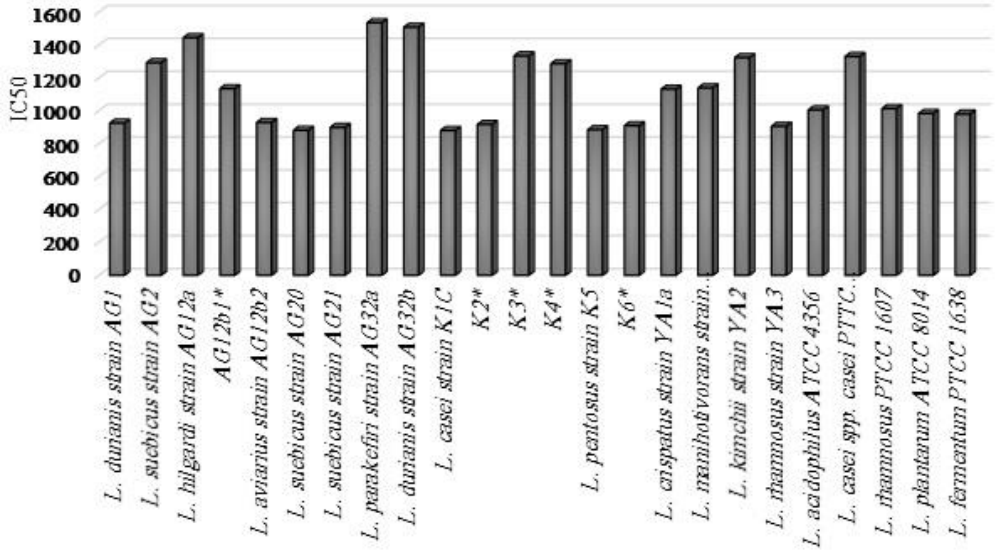

Figure 4. DPPH scavenging activity (IC_{50} µg/mL) of methanol extract of Lactobacillus supernatants.

There is no report of antioxidant fractionation of *Lactobacillus* by TLC, so there is a need for mobile phase optimization. Since methanol extracts of different samples using a constant mobile phase showed different patterns in TLC (Figure 1, 5), different mobile phases were used for the fractionation of various samples (Table 1). We could not fractionize other methanol extracts, either because of the poor separation and resolution or because of an overlapping antioxidant spot with the control. The suitable solvent systems in which the antioxidant spot showed the best separation and resolution were highlighted by an asterisk (*) in Table 1.

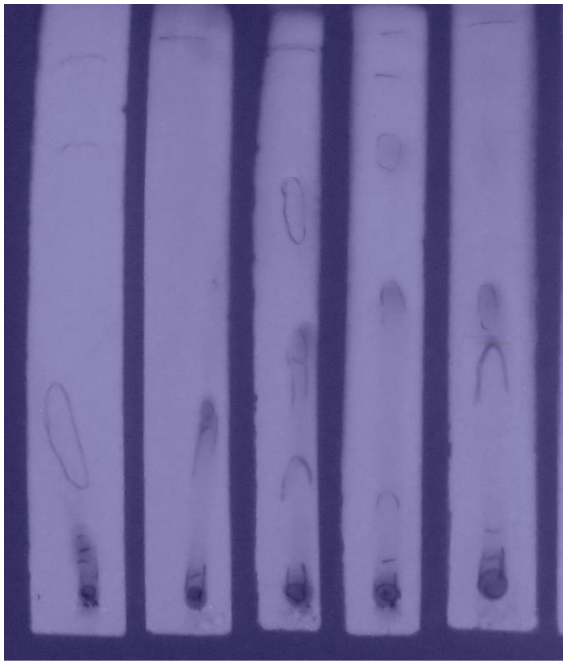

Figure 5. Thin-layer chromatography of methanolic extract of the supernatant of different Lactobacillus isolates grown in MRS at 37 ° C for 48 hours. All samples were subjected to 5: 1 methanol chloroform solvent system by TLC chromatography, which showed different stains in different samples. From right to left correspond to isolates AG12a, AG20, AG12br, AG2 and AG32a.

In this work, among the compounds isolated on the TLC plate, only 16 bands showed antioxidant properties and were considered the most active compounds in *Lactobacillus* strains extracellular extract (Table 2).

To determine the antibacterial activity of the probiotics supernatants, most of the researchers used disk diffusion or spot-on-lawn methods [14]. The most important problem of these two methods is that their results are not comparable in different laboratories. In addition, the disk diffusion method underestimates the antimicrobial activity, because some compounds do not diffuse into the agar pores [15]. In the current research, the minimum inhibitory concentration levels of bioactive components were determined that may assess the potential use of these compounds *in vivo*.

The antibacterial activity of antioxidant fractions against both gram-positive and gram-negative bacteria is presented in Table 3. The MICs of TLC fractions extracted from *Lactobacillus casei* strain K1C supernatant against *E. coli* ATCC 11303 and *Staphylococcus aureus* ATCC 6538, were 500 and 1000 µg/ml, respectively. We previously reported the MICs of *Lactobacillus casei* strain K1C supernatant extract against *E. coli* ATCC 11303 and *Staphylococcus aureus* ATCC 6538, were 25 and 25 mg/ml, respectively [9]. As expected, MICs were reduced after the extraction of antioxidant fractions.

The secondary metabolites provide survival functions for the microorganisms and these compounds are used as competitive weapons against other bacteria and fungi [16].

Table 3. MICs of fractions isolated from extracellular materials of *Lactobacillus* strains cultured in PMM5 medium at 37˚C for 48 h, against both gram-positive and gram-negative bacteria.

Lactobacillus strains	TLC fraction number	MIC (µg/mL)							
		S. aureus	*E. coli*	*S. typhi*	*S. pyogenes*	*Y. entrolitica*	*S. epidermidis*	*B. licheniformis*	*K. pneumonia*
L. hilgardi strain AG12a	F4	-	-	250	250	250	1000	>1000	>1000
	F6	-	-	1000	>1000	>1000	>1000	>1000	>1000
	F9	-	-	250	1000	>1000	1000	500	>1000
L. casei strain K1C	F1	1000	500	31.25	250	1000	500	250	250

In the current study, the partial purification of the antioxidant components using thin-layer chromatography raised the antibacterial and antioxidant properties of the fraction in comparison to the crude methanol extract of the same sample, which indicates the presence of respective secondary metabolites in the isolated fraction that may play a role in the observed biological activity.

Nyanzi et al. (2015) determined the minimum inhibitory concentration of probiotic methanol extracts against some pathogenic bacteria. According to their results, the MICs of lyophilized probiotic extract for *E. coli* and *S. aureus* were 0.062 mg/mL and 0.25 mg/mL, respectively. According to our previous results, the MIC of *Lactobacillus hilgardii* strain AG12a against *E. coli* ATCC 11303 and *Staphylococcus aureus* ATCC 6538, were 8 and 17 mg/ml, respectively [9]. The difference between the results of the present study and the results of Nyanzi et al. (2015) is quite expected, because they used freeze-dried methanol of whole-cell extract, while we used freeze-dried supernatant methanol extract. As expected, there are more antioxidants in the whole cell of *Lactobacillus* than in the fermented supernatant alone.

4. Conclusion

According to the results, n-hexane extract of *Lactobacillus* supernatants did not show any antioxidant properties (Figure 1S), so it can be concluded that the extracellular antioxidant materials are not among non-polar compounds. Moreover, ethyl acetate extracts showed the lowest levels of antioxidant properties. The bioactive compounds secreted from *Lactobacillus* species in PMM5 medium after 48 h fermentation are mainly polar and can be extracted by solvents such as methanol and chloroform.

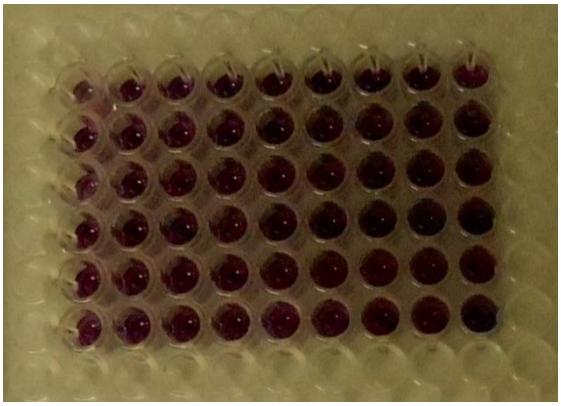

Figure 1S- DPPH assay for n-hexan extraction did not show any antioxidant activities in different *Lactobacillus* strains.

This is the first report on the TLC solvent systems' optimization for the isolation of antioxidants from the methanolic extract of *Lactobacillus* supernatants. A solvent system consisting of methanol/chloroform can be used for TLC analysis of antioxidants from methanolic extract of each *Lactobacillus* supernatant. Considering that the samples used in antioxidant/antibacterial tests were semi-purified fractions, it is worth mentioning that the pure active compound(s) would possibly show stronger cytotoxic and antibacterial effects.

In conclusion, these findings prove the presence of biologically active compounds in *Lactobacillus* culture. These *Lactobacilli* strains and bioactive compounds could be used as starters and preservatives in the food industry and/or as drugs in the pharmaceutical industry. Further studies on the bioactivities of lactobacilli extracts are valuable to be done in the future.

Acknowledgments

The authors are thankful to Dr. Atousa Aliahmadi, Department of Biology, Medicinal Plants, and Drug Research Institute, Shahid Beheshti University, Tehran, Iran, for showing keen interest in this work and for support.

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