

Therapeutic effects of *Mycobacterium phlei* cell wall extracts

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Aim: To investigate the cytokine-stimulating activity of several *Mycobacterium phlei* cell wall extracts. The bacillus Calmette-Guérin application is the gold standard treatment for some types of superficial bladder carcinoma. However, its usage is limited due to its harmful side effects. Intensive research has been carried out to find less toxic and more potent therapeutic agents for treatment. Researchers focus on *M. phlei* for this purpose. The cell wall extract of *M. phlei* is sufficient for antitumoral activity.

Materials and methods: In this study, *M. phlei* cells were cultured and several different cell wall extracts were prepared by physicochemical procedures. Human monocytic cell lines were incubated with these extracts, and tumor necrosis factor- α and interleukin-12 were determined in the samples.

Results: Various cell extracts showed tumor necrosis factor- α and interleukin-12 stimulating activity. Matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) profiles of the samples were also obtained. The MALDI-MS spectra of the samples showed that peptides of 3808, 9207, 11,533, 12,460, and 21,587 Da may be involved in the immunostimulation activity.

Conclusion: This study showed that peptides obtained from mycobacteria cell wall extracts may be used to prepare new antitumoral chemicals for the treatment of superficial bladder carcinoma.

Key words: *Mycobacterium phlei*, cell wall, tumor necrosis factor- α , interleukin-12, MALDI-MS spectra

Mycobacterium phlei hücre duvarı ekstralarının terapötik etkileri

Amaç: Bacillus Calmette-Guérin uygulaması bazı yüzeysel mesane kanserlerinin tedavisinde altın standart yöntemdir. Ancak, zararlı yan etkileri nedeniyle uygulaması sınırlıdır. Daha az toksik ve daha güçlü etkiye sahip terapötik ajanların geliştirilmesi amacıyla yoğun çalışmalar yapılmaktadır. Araştırmacılar *Mycobacterium phlei*'ye odaklanmaktadır. *M. phlei*'nin hücre duvarı ekstresi antitumoral aktivite için yeterlidir. Bu çalışmanın amacı çeşitli *M. phlei* hücre duvarı ekstralarının sitokin uyarıcı etkilerini incelemektir.

Yöntem ve gereç: Bu çalışmada, *M. phlei* hücre kültürü yapılmış ve fizikokimyasal yöntemlerle çeşitli hücre duvarı ekstraları hazırlanmıştır. İnsan monositik hücre hatları bu ekstralarla inkübe edilmiş ve bu örneklerdeki tümör nekroz faktörü- α ve interlökin-12 ölçülmüştür.

Bulgular: Çeşitli ekstraların tümör nekroz faktörü- α ve interlökin-12 uyarma aktivitesi gösterilmiştir. Örneklerin MALDI-MS profilleri de çıkarılmıştır. Bu örneklerin MALDI-MS spektrumları 3808, 9207, 11.533, 12.460, and 21.587 Da molekül ağırlığındaki peptidlerin immünstimülasyon aktivitesinde rolü olabileceğini düşündürmüştür.

Sonuç: Bu başlangıç çalışması, mikobakteri hücre duvarından elde edilen peptidlerin yüzeysel mesane karsinoması tedavisi amaçlı yeni antitümöral bileşiklerin geliştirilmesinde kullanılabileceğini göstermektedir.

Anahtar sözcükler: *Mycobacterium phlei*, hücre duvarı, tümör nekroz faktörü- α , interlökin-12, MALDI-MS spektrumu

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Introduction

Bladder cancer is the second most common cancer type in men over 55 years of age in Turkey (1). Intravesical bacillus Calmette-Guérin (BCG) application is the gold standard treatment for some types of superficial bladder carcinoma (2). The pathogenicity of BCG limits its usage in human beings (3). Several researchers have looked for less toxic and more potent therapeutic agents. One of the promising alternatives is *Mycobacterium phlei*. The cell wall extract of *M. phlei* has immunostimulating and antitumoral activity (4-6). Researchers report that the immunostimulation activity of *M. phlei* is found in the DNA (7) or protein-rich cell wall extracts (8).

The aim of this study was to search for the components of *M. phlei* cells that have high immunostimulating and possible antitumoral activity. For immunostimulating activity, we measured the interleukin-12 (IL-12) and tumor necrosis factor- α (TNF- α) response of the subcellular components.

Materials and methods

Microorganisms

Mycobacterium phlei ATCC 11758 was obtained from Hacettepe University's Faculty of Medicine, Department of Medical Microbiology. The Middlebrook 7H9 broth was from Difco, USA; the ELISA plates and cell culture plates were from NUNC A/S, Denmark; and the human monocytic cell line U937, TNF- α , and IL-12 kits were from Matriks Biyoteknoloji, Turkey. All of the chemicals were of analytical grade.

Growth of mycobacteria

Mycobacterium phlei cells were grown in Middlebrook 7H9 liquid broth containing 0.2% glycerol for 28-40 days at 37 °C. At the end of the growth period, samples were taken for viability testing with acid-resistant bacilli (ARB) and Gram staining. Cells were harvested by filtration, washed twice with 50 mM potassium phosphate (pH 7.2), and stored at -20 °C until use (9,10).

Preparation of the cell extracts

Different cell extracts were prepared with a modification of the method of Morales and Chin (7,8,11).

In 30 mL of 50 mM potassium phosphate (pH 7.2), 10 g of cells were suspended, and the mixture was then sonicated with a thermo-controlled sonicator with a 1.9-cm probe at 55 W for 20 min (Sample 1). Sample 1 was centrifuged at 27,000 $\times g$ with a Sorvall Superspeed centrifuge. Sample 2 was the precipitate and Sample 3 was the supernatant of Sample 1. For the preparation of Sample 4, Sample 2 was mixed with an equal volume of 2% sodium dodecyl sulfate (SDS) and the mixture was kept at 95 °C for 1 h. Sample 4 was centrifuged at 35,000 $\times g$ for 1 h. The precipitate was washed twice with distilled water and centrifuged, and the supernatants were combined (Sample 6). The precipitate was dissolved in a minimal volume of 50 mM phosphate buffer, pH 7.0 (Sample 5). Sample 6 was acidified and mixed with an equal volume of acetone. The precipitate was removed by centrifugation at 8000 $\times g$ for 1 h. The supernatant was evaporated and dissolved in 50% ethanol (Sample 7). These procedures are summarized in the Table.

The sugar content of the samples was measured using the phenol sulfuric acid method (12). Glucose was used as the standard.

Monocyte cell culture and incubation with samples

Human monocytic cell line U937 was stimulated with phorbol myristate acetate (PMA) for 2 days in 24-well plates. After incubation, the medium was changed and 1 mL of the sample, containing 5 $\mu\text{g}/\text{mL}$ sugar, was added to each well. The wells were incubated at 37 °C for 48 h under 5% CO₂ and 100 μL of sample was taken from each well for cytokine measurement.

Measurement of TNF- α and IL-12

TNF- α and IL-12 responses of the macrophages were measured using ELISA at Ankara University's Biotechnology Laboratory. The human TNF- α and IL-12 measurement kit prospectus was followed (13). To each well of the 96-well ELISA plate, 100 μL of the sample was added; the plates were coated with TNF- α (or IL-12) monoclonal antibody and incubated at room temperature for 2 h. After incubation, the wells were washed, and biotin-labeled monoclonal

Table. Treatment, cytokine-producing activities, and major MALDI-MS peaks of each sample.

Sample	Treatment	Total TNF- α (pg)	Total IL-12 (pg)	Major peaks obtained with MALDI-MS (Da)
1	Cells were sonicated for 15 min at 100 W and centrifuged at 35,000 \times g for 1 h.	40,359	326	1183, 1328, 1491, 2091, 3808, 4180, 4243, 5419, 5691, 5753, 9202, 10,021, 10,712, 11,449, 11,525, 12,461, 14,706, 21,584
2	Precipitate of Sample 1.	43,945	0	1491, 1729, 2092, 3858, 4182, 5391, 7188, 9207, 10,269, 10,794, 11,454, 12,460, 14,346, 16,017, 21,587
3	Supernatant of Sample 1.	39,169	262	1155, 1284, 1324, 2841, 3804, 5686, 5749, 9202, 10,662, 10,716, 11,456, 11,533, 12,468, 21,589
4	Sample 2 was boiled with 2% SDS for 1 h.	225	270	551, 567, 628, 644, 762, 854, 1471, 2190, 6527, 7910, 10,135, 10,787, 10,816, 10,875, 11,089, 16,018, 21,596
5	Precipitate of Sample 4 (35,000 \times g for 1 h).	0	60	1162, 1200, 1471, 1487, 1724, 1767
6	Supernatant of Sample 4 (precipitate of 4 was washed twice with water and the washes were included in this sample).	15,484	588	551, 668, 684, 700, 762, 895, 2272, 2627, 2957, 3349, 4260, 6438, 7913
7	Sample 5 was treated with acetone and centrifuged at 8000 \times g for 1 h; supernatant was evaporated and precipitate was dissolved in water.	0	0	608, 649, 651, 653, 655, 876, 953, 956

antibody conjugate was added and incubated at room temperature for 1 h. The mixtures in the wells were then reacted with 3,3',5,5'-tetramethylbenzidine for 30 min and the reaction was stopped by the addition of 0.6 M H₂SO₄. Absorbances were recorded at 450 nm. The measurements were done in triplicate, and the results were calculated with regression analysis.

MALDI-MS analyses

For matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) analysis, all of the samples were analyzed using a Voyager-DE PRO mass spectrometer (Applied Biosystems). Samples were prepared by the dried droplet method. Each sample was resuspended in alpha-cyano-4-hydroxycinnamic acid and 0.1% trifluoroacetic acid in a 50:50 mixture of water and acetonitrile, and then spotted onto a MALDI plate (14).

Results

The Table shows the TNF- α and IL-12 stimulating activities of the samples, starting with 10 g of cells, and the main peaks obtained by MALDI-MS. The MALDI-MS spectra of the samples are shown in Figures 1-3. TNF- α stimulating activity was highest in the parent sample (Sample 1). It contained several MALDI-MS peaks.

When the MALDI-MS spectra were investigated, it was observed that peptides and proteins with molecular weights of 3808, 9207, 11,533, 12,460, and 21,587 Da were commonly found in the parent sample (Sample 1) and the precipitate (Sample 2) and supernatant (Sample 3) of this sample (Figure 1). These peptides and proteins might be involved in the stimulation of TNF- α secretion.

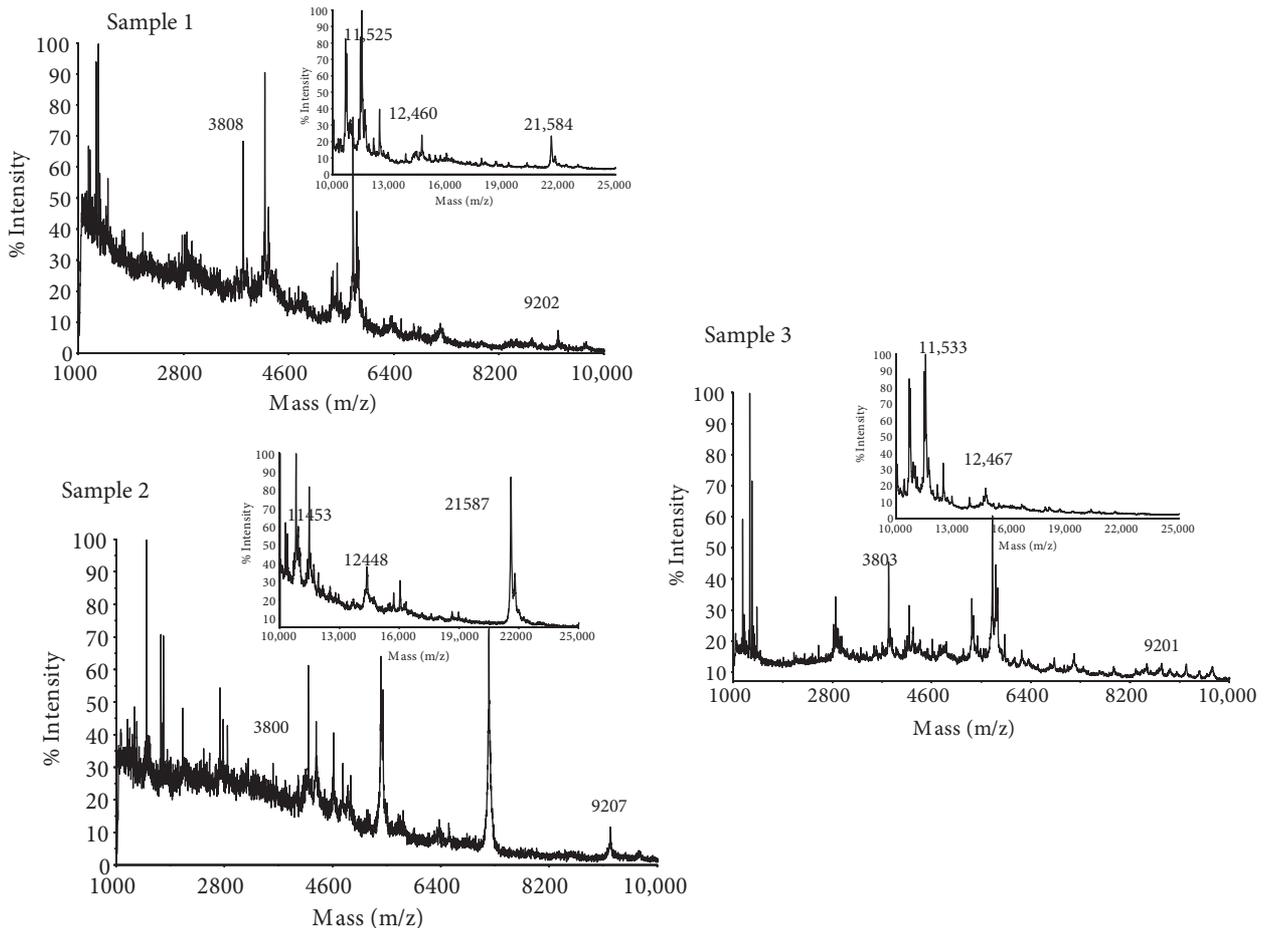


Figure 1. MALDI-MS spectra of Samples 1, 2, and 3. Insets show a region of high molecular weight (10,000-25,000 Da).

The TNF- α activity of Sample 4 was about 0.005% of that of the parent sample. The MALDI-MS spectrum of this sample had only a 21,584-Da peak with low intensity among the peaks common for Samples 1, 2, and 3 (Figure 2). The precipitate of Sample 4 (Sample 5) had only peaks below 2000 Da (Figure 2). The supernatant of Sample 4 is Sample 6. The precipitate of Sample 4 was washed with water and centrifuged twice, the supernatants were combined with Sample 6, and about 35% of the original activity was recovered. In the MALDI-MS spectrum of this sample, most of the peaks suspected for TNF- α production were present, although at low intensity (Figure 2).

Sample 7 is the acetone extract of Sample 6. This sample did not contain any TNF- α stimulating activity, nor did it contain any measurable quantities

of protein. The MALDI-MS spectrum showed peaks with molecular weights of less than 2000 Da (Figure 3).

Discussion

TNF- α activities were more widely distributed among the samples as compared to IL-12. When the parent sample was centrifuged, TNF- α stimulating activity was distributed almost equally between the precipitate (Sample 2) and the supernatant (Sample 3). The IL-12 value for the parent sample was much lower as compared to the TNF- α value. These findings are in accordance with previously published results (15).

Sample 1 had many MALDI-MS peaks, which made it difficult to say which peaks were responsible

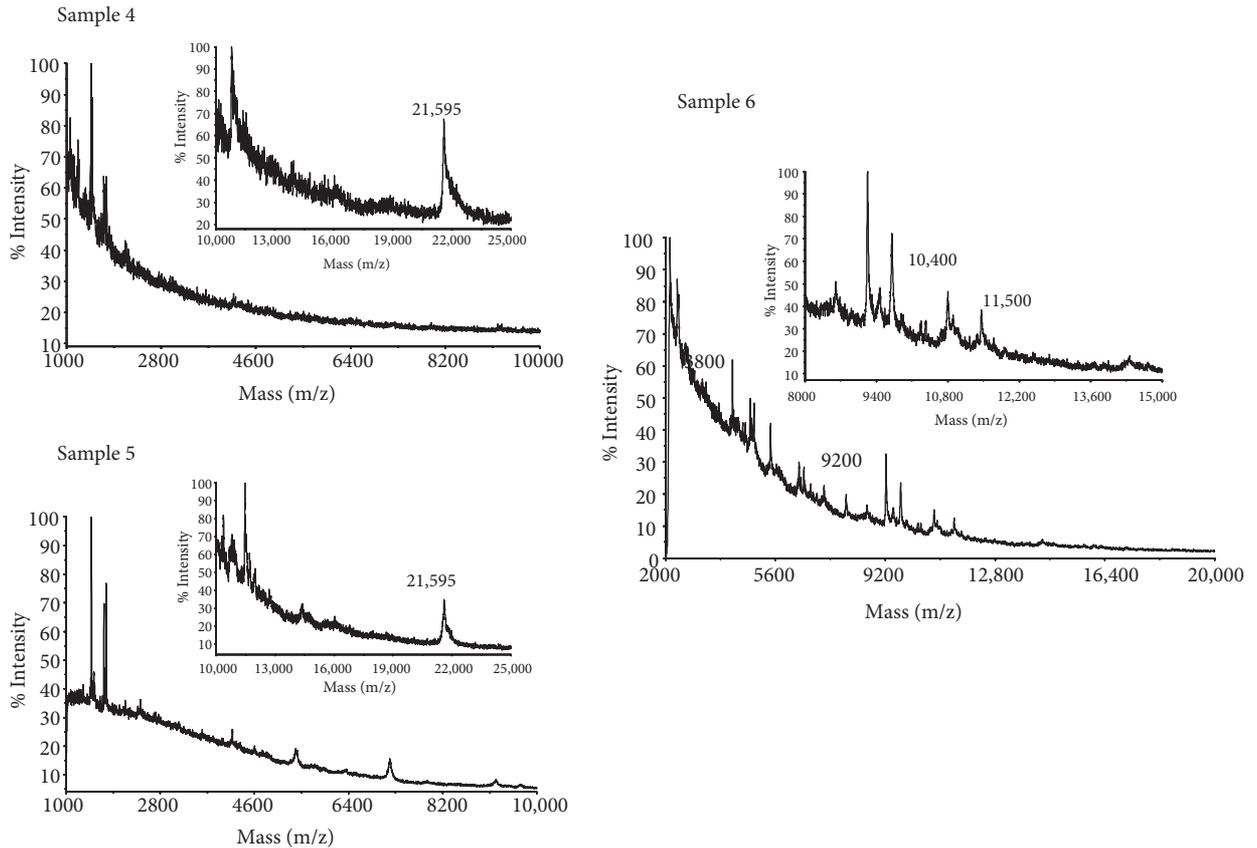


Figure 2. MALDI-MS spectra of Samples 4, 5, and 6. Insets of Samples 4 and 5 show a region of high molecular weight (10,000-25,000 Da). Inset for Sample 6 shows the region between 8000 and 16,000 Da.

for the TNF- α stimulating activity. Although the precipitate (Sample 2) was washed twice with distilled water and the supernatants were combined with Sample 3, it was not possible to remove all of the TNF- α stimulating activity from the precipitate. The peaks common to all 3 samples were suspected of having TNF- α stimulating activity. These peaks had molecular weights of 3808, 9207, 11533, 12,460, and 21,587 Da.

The purpose of the preparation of Sample 4 was to extract the proteins from the lipid environment of Sample 2 by boiling with 2% SDS solution. However, the denaturation of the proteins by SDS caused almost a total loss of TNF- α activity. This loss might be due to denaturation and/or subunit dissociation of the peptides and proteins. The MALDI-MS spectrum of this sample had only a 21,584 Da peak with low intensity among the peaks common for Samples 1,

2, and 3 (Figure 2). The loss of these peaks in the spectrum supports the possibility of the dissociation of the peptides into subunits. When a similar sample was boiled with distilled water, these peaks were not lost. Moreover, the MALDI-MS spectrum showed the presence of the peaks found in Samples 1, 2, and 3, and the TNF- α stimulating activity of this sample was about 83% of that of Sample 2 (11). These findings showed that the components responsible for TNF- α stimulating activity were resistant to high temperatures but sensitive to SDS treatment. On the contrary, the IL-12 stimulating activity of the sample was increased by SDS treatment, but this increase was thought to be caused by the SDS itself.

The absence of peaks above 2000 Da in Sample 5 might be due to experimental difficulties, since high SDS concentrations reduce the resolution of MALDI-MS spectra (16). It might be possible that

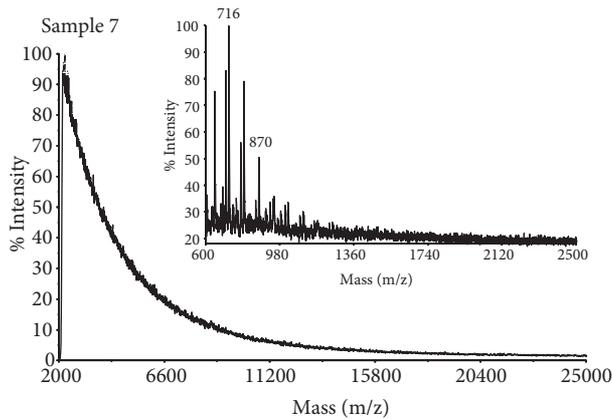


Figure 3. MALDI-MS spectrum of Sample 7. Inset shows the region between 600 and 2500 Da.

high-molecular-weight proteins and peptides could not be observed due to a high SDS concentration, and since they were denatured, they lost their activity, as well. Similarly, the gain in the protein peaks and the TNF- α stimulating activity in Sample 6 might be due to the dilution of SDS in the medium as a result of the washing process.

The mycobacterial cell wall is rich in lipid and mycolic acid content. Mycobacterial cell wall components, such as lipoproteins, may stimulate toll-like receptors of host cells to cause cytokine production (17). The purpose of the preparation of Sample 7 was to isolate the lipid- and lipoprotein-containing structures in Sample 6. The MALDI-MS spectrum of this sample showed low molecular-weight peaks, which were probably mycolic acids (Figure 3).

In summary, the factors that cause TNF- α secretion appear to be peptides or proteins found both in soluble and insoluble forms. This finding is in accordance with reports by Keskin (8) and contrary to the findings of Morales et al. (7), who

claimed that the immune-stimulating activity was due to an extract rich in DNA. Results showed that this immune-stimulating activity might be due to peptides with molecular weights of 3808, 9207, 11,533, 12,460, and 21,587 Da. The factors were resistant to boiling but sensitive to SDS. Our findings mirror those of Wang et al. (18), who reported on a hot-water extract from *Mycobacterium bovis*, or the BCG vaccine, that showed significant antitumor activity in vivo against a mouse bladder tumor model and a murine sarcoma. Their isolated material was a glycan with a molecular weight between 60 and 90 kDa, which had low solubility in acetone or ethanol and was heat-stable. The only difference with our results was that the molecular weights of the candidates that we obtained were much lower. This difference could be due to species differences, or due to differences in extract preparation or molecular-weight determination methods.

This was a beginning point for the development of new antitumoral chemicals for the treatment of superficial bladder carcinoma. A more detailed proteomics study that employs HPLC or 2D electrophoresis may isolate these active fragments of the cell wall completely. By determining the amino acid sequences of the isolates and by comparison of the fragments that show activity, better knowledge can be gained. A further study with animal models may also be necessary to show the in vivo effects of these active compounds.

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