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Antioxidant, Antimicrobial and Anticancer Activities of the Aspergillin PZ and Terphenyllin Secondary Metabolites: An *in vitro* Study

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Abstract

The importance of secondary metabolites obtained from fungi is increasing day by day in terms of human health. Many physiological and pharmacological effects, mainly antimicrobial, anticancer and antioxidant properties of these compounds have been reported. Aim of this study is to determine the anticancer, antioxidant and antimicrobial effects of aspergillin PZ and terphenyllin compounds, which are isolated from *Aspergillus* and also subjected to limited number of studies. The antimicrobial activity of aspergillin PZ and terphenyllin compounds was determined by using disc diffusion method using different bacteria. The antioxidant property of the compounds was determined by measuring the level of DPPH free radical scavenging. Cytotoxic activity was determined by experiments on human prostate cancer cell lines (PC3 and LNCaP) and over cancer cell line (A2780). Both compounds showed low antimicrobial activity on test bacteria (approximately 2-3 mm zone). High concentrations of applied compounds showed apparent DPPH free radical scavenging activity, while % scavenging activity was quite low at low concentrations. Both compounds showed significant anticancer activity on cancer cell lines ($p < 0.05$). Our results suggest that these two compounds have important biological properties due to their antioxidant and anticancer activities.

1. INTRODUCTION

Secondary metabolites are compounds that have a structurally low molecular weight and are highly diverse, not required for the growth and survival of the producer organism. Contrary to the fundamental role of primary metabolites, secondary metabolites serve to increase the suitability of the producer organism or to reduce the suitability of environmental organisms [1]. The fungi are regarded as therapeutic agents with antiviral, anticancer, antimicrobial and antioxidant capacity through their secondary metabolites as well as an important dietary supplement that have been grown increasingly in the world for 40 years [2]. Pharmaceutically important fungal secondary metabolites such as penicillin, cyclosporin, ergot alkaloids are quite significant for mammalian health [3].

Aspergillus fungi commonly found throughout the world; have pathological and therapeutic importance. Pathogen species may cause lung and cutaneous infections on the other hand as antibiotics and lovastatin producing therapeutic species are also available. According to 2017 *Aspergillus* Secondary Metabolites Database (A2MDB) data, 807 different secondary metabolites from 675 *Aspergillus* species were recorded [4]. The main groups of secondary metabolites in *Aspergillus* include polyketides, ribosomal and nonribosomal peptides and terpenoids [5]. Many metabolites synthesized by *Aspergillus* are very important for humans: The cholesterol-lowering drug lovastatin, the antibiotic penicillin and also the potent mycotoxins aflatoxin and gliotoxin are a small sample of the pharmacopenia encoded and produced by the *Aspergillus* genome [5, 6]. Aspergillin PZ is a secondary metabolite of isoindole-alkoid structure produced

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by fermentation of *Aspergillus awamori* (Nakazawa) [7]. Terphenyllin is a metabolite in p-terphenyl form produced by *Aspergillus candidus* [8]. Despite the availability of a number of studies on the characterization of aspergillin PZ and terphenyllin compounds [7, 8], the number of studies evaluating their biological activity is rather limited. In this study it is aimed to investigate cytotoxic activity of aspergillin PZ and terphenyllin substances on human prostate and over cancer cell lines (LNCaP, PC3 and A2780) and to evaluate antimicrobial activities on different bacteria (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae* and *Enterococcus faecalis*). Besides, it is aimed to measure the potential antioxidant properties of these substances by determining the free radical scavenging activity.

2. MATERIAL METHOD

2.1. Preparation of Test Compounds

The commercially available aspergillin PZ (Santa Cruz Biotech., USA) and terphenyllin (Santa Cruz Biotech., USA) compounds used in the study were purchased at 99% purity. Concentrations of compounds at 1, 5, 25, 50 and 100 µM were prepared in distilled water (for antimicrobial and antioxidant assays) or medium (for cell culture studies) and maintained at +4 ºC throughout the experiment.

2.2. Investigation of Biological Activity

2.2.1. Determination of antimicrobial activity

In this study, 3 gram negative (*Klebsiella pneumoniae* FMC 5, *Pseudomonas aeruginosa* DSM 50071, *Escherichia coli* ATCC 25922) and 2 gram positive (*Enterococcus faecalis* ATCC 700802 and *Staphylococcus aureus* Cowan I) bacterial strain obtained from Molecular Biology and Genetics Department of Bartin University were used. Bacteria were activated in Luria Bertani (LB) Broth (Merck, Germany) 24 h before the experiment. In order to measure the antimicrobial effect of aspergillin PZ and terphenyllin chemicals, bacteria were inoculated in petri dishes by pour plate method and antimicrobial properties of chemicals were investigated by disc diffusion method [9]. 15 µL of each activated microorganism suspension was equilibrated to 0.5 McFarland standard and poured into petri plates (9 cm diameter) aseptically. 20 ml sterile Mueller-Hinton Agar (Merck, Germany) medium was poured on the microorganisms and shaken gently for homogenous bacteria-agar mixture. 20 µL of prepared concentrations of aspergillin PZ and terphenyllin were soaked onto blank antimicrobial susceptibility test discs (Bioanalyse, Turkey) and all discs placed on the inoculated agar plates and incubated (37 ºC, 24 h). Once the incubation ended, diameters (mm) of inhibition zone around the discs were measured.

2.2.2. Antioxidant activity

Antioxidant activity of aspergillin PZ and terphenyllin compounds was determined according to Brand-Williams et al. [10]. 25 mg/mL DPHH as free radical was prepared in methanol. 100 µL of prepared concentrations of aspergillin PZ and terphenyllin was added to 3.9 mL of DPPH solution placed in test tubes. After incubating for 30 min, absorbances were measured (517 nm) against blank using spectrophotometer.

Decreased absorbance is determined as the remaining amount of DPPH. The results were calculated according to the following formula:

$$\% = [(\text{Control ABS} - \text{Sample ABS}) / \text{Control ABS})] \times 100.$$

2.2.3. Anticancer activity

Cell Culture

In our study, 3 different cancer cells were used including human ovarian cancer cell line (A2780) and human prostate cancer cell lines (LNCaP and PC3). All cells were grown in RPMI-1640 medium (prepared in 10% FBS, 1% penicillin and streptomycin solution) in 25 cm^2 cell culture flasks. Cells were maintained in CO_2 incubator (%5 CO_2, Panasonic, Japan) at 37 °C and media of the cells were changed twice a week. Once the cells reach 90% confluency, they were removed from the flasks using 0.25% trypsin-EDTA solution and added to 96-well plates.

Treatment with Aspergillin PZ and Terphenyllin

Test compounds were added to cell-grown wells and incubated (24 h in CO_2 incubator). After the incubation, the cytotoxic activities of both aspergillin PZ and terphenyllin were determined by using 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

MTT Assay

MTT solution (0.5 mg/mL) prepared in sterile PBS was added to 96-well plates. After 3 h incubation, the OD_{550} of the cells were measured by ELISA microplate reader (Synergy HT USA). The mean value of the OD_{550} of the control wells accepted as 100% viability. The percentage of cell viability was calculated as absorbance ratio of the experimental group to control group [11, 12].

2.3. Statistical Analysis

Measurements of antimicrobial zone were expressed as mean values. The free radical scavenging effects of compounds were calculated in percentage (%) and the mean ± standard deviation (SD) values of replicates were shown in the bar graph. For evaluation of cytotoxic analyses, the suitability of the groups to normal distribution was evaluated using Kolmogorov Smirnov test. One-way ANOVA was used to compare the groups. The homogeneity of the variances was analysed using the Levene's test. TAMHANE T2 test was performed for multiple comparisons and also when the variances are not homogeneous after one-way variance analysis. $p<0.05$ was considered statistically significant and the data were expressed as mean ± SD.

3. RESULTS

3.1. Antimicrobial Activity

The antimicrobial activities of aspergillin PZ and terphenyllin compounds are shown in Table 1 and Table 2, respectively. Accordingly, the concentrations of aspergillin PZ and terphenyllin were not effective on *E. coli*, *E. faecalis* and *S. aureus*, while they showed very weak antimicrobial effects on *K. pneumoniae* and *P. aeruginosa.* The lowest concentration (1 μM) of the compounds did not show any antimicrobial activity on microorganisms.

Table 1. *Antimicrobial activity of aspergillin PZ on test microorganisms (mm/zone)*

Test microorganisms	Aspergillin PZ				
	1 µM	5 µM	25 µM	50 µM	100 µM
Klebsiella pneumoniae	-	2	2	-	2
Pseudomonas aeruginosa	-	3	1.5	2.5	1.5
Escherichia coli	-	-	-	-	-
Enterococcus faecalis	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-

Table 2. *Antimicrobial activity of terphenyllin on test microorganisms (mm/zone)*

Test microorganisms	Terphenyllin				
	1 µM	5 µM	25 µM	50 µM	100 µM
Klebsiella pneumoniae	-	-	-	-	2
Pseudomonas aeruginosa	-	2	3	2.5	2
Escherichia coli	-	-	-	-	-
Enterococcus faecalis	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-

3.2. Antioxidant Activity

The antioxidant capacities of the compounds were investigated by measuring DPPH free radical scavenging effects. Increasing concentrations of aspergillin PZ and terphenyllin showed (%) increase in DPPH free radical scavenging effect (Figure 1A, B) In general, the free radical scavenging effect of the terphenyllin compound was slightly higher than that of aspergillin PZ.

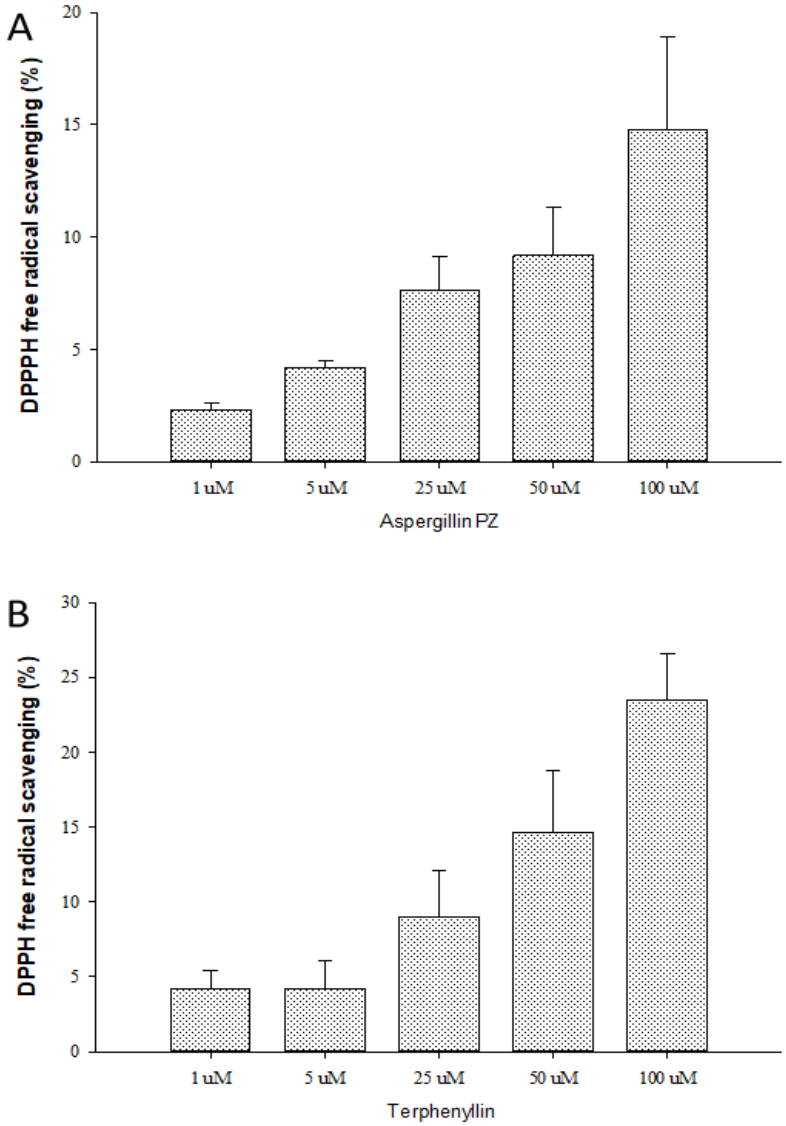

Figure 1. *Free radical scavenging effect (%) of aspergillin PZ (A) and terphenyllin (B) compounds*

3.3. Anticancer Activity

The cytotoxic effect of the compounds used in the study on human ovarian and prostate cancer cell lines is shown in Figure 2. aspergillin PZ at concentrations of 25, 50, and 100 µM decreased cell viability significantly in all cell lines when compared with solvent group ($p < 0.05$). As shown in Figure 2, 100 µM concentration of aspergillin PZ reduced the viability by 80% in the A2780, LNCaP and PC3 cell lines. 50 µM and 100 µM concentrations of terphenyllin resulted in significant reductions in cell viability in human ovarian and prostate cancer cell lines compared to the solvent ($p < 0.05$). The effect of this compound on prostate cancer cell lines was higher in the LNCaP cell line compared to the PC3 cell line.

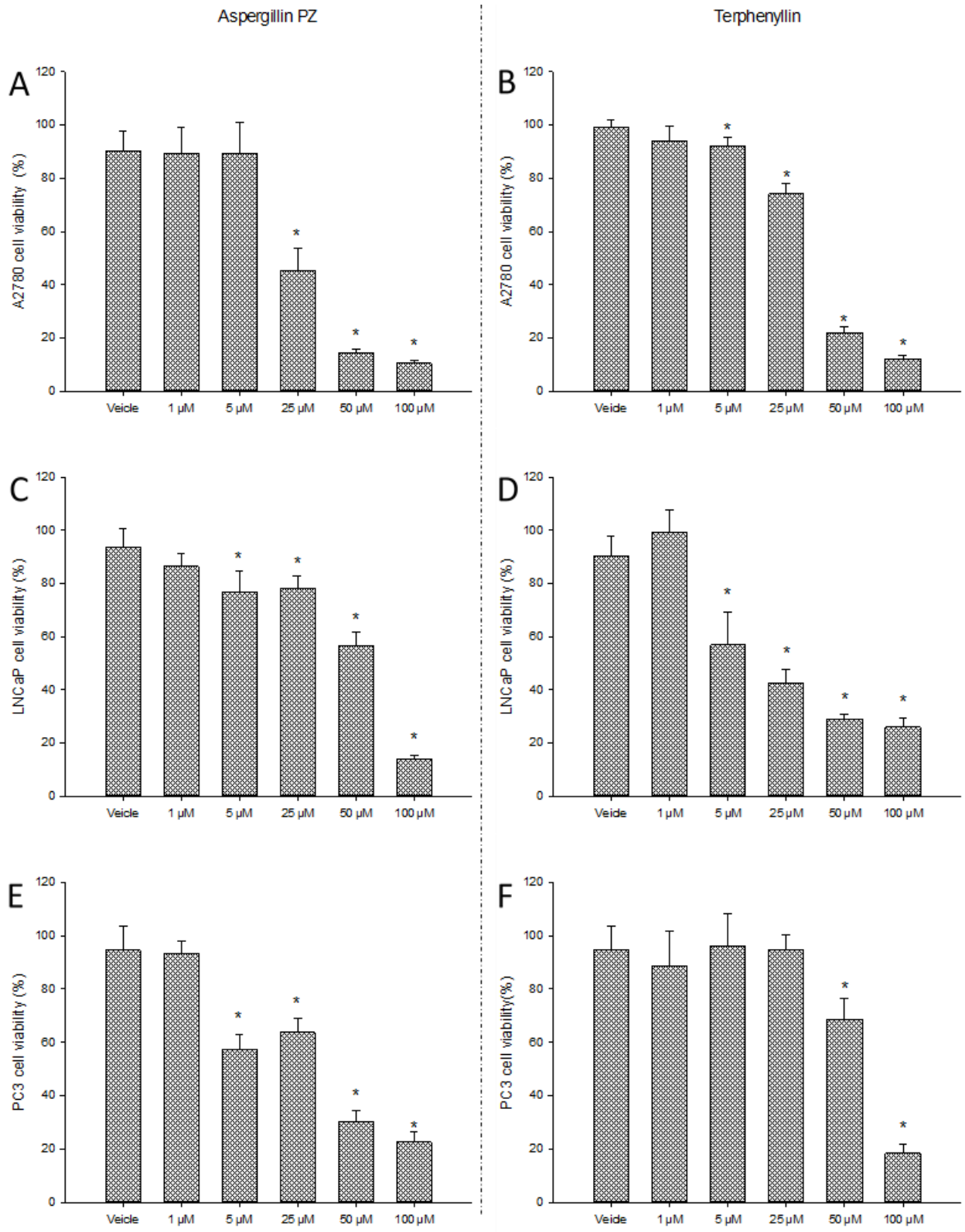

Figure 2. *Cytotoxic effect of aspergillin PZ (A, C, E) and terphenyllin (B, D, F) on human ovarian and prostate cancer cell lines (%). High concentrations of aspergillin PZ and terphenyllin compounds (50-100 µM) reduced viability in all three cell lines significantly. *p<0.05 vs other groups*

4. DISCUSSION

Secondary metabolites have an extremely broad biological activity. While some benefit the society, most of them cause problems that cannot be handled. This dichotomy is an indication of the diversity of natural products produced by fungi. The fungal metabolites, which have the greatest negative effect, including mammalian toxins commonly known as mycotoxins. However, many important drugs have been discovered with fungal chemical studies. Natural products are still very important therapeutic agents and used as precursors in drug development. Especially, they play very important roles in the development of effective therapies for cancer, bacterial and fungal infections, malaria, as well as neurological, cardiovascular, and autoimmune diseases [13]. Fungi are efficient bioactive secondary metabolite sources and contribute to exceptional ways to improve human and animal health [14]. Therefore, it is important to identify new secondary metabolites and to determine their biological activity.

Aspergillin PZ and terphenyllin used in the experiment have been subjected in quite limited studies so far and current studies emphasis on isolation and identification of these compounds [7, 15, 16]. A few studies provide information about the antimicrobial, antioxidant and cytotoxic activity of these compounds. For example, aspergillin PZ analogues isolated from *Aspergillus* have been reported to deform the *P. oryzae* cone moderately and show anti-tumor activity against MH-60 and HL-60 cancer cells [7]. Yen et al. in their study investigating the antioxidative activity of 3,3″-di-OH-terphenyllin and 3-OH-terphenyllin compounds on H_2O_2-induced cellular stress, reported that H_2O_2- increased DNA damage and lactate dehydrogenase leakage in Int 407 cells, while this increase was significantly inhibited in treatment groups [17]. In addition, it has been reported that intracellular reactive oxygen species in the cells treated with terphenyllin compounds before experimental stress reduces the formation by 30% and 35%, respectively, and increases the catalase and glutathione peroxidase activity by 33% and 25%, respectively, thus showing antioxidant activity [18]. In another study, it was shown that aspergillin PZ isolated from *Trichoderma gamsii* had cytotoxic activity on MDA-MB-231, A549, and PANC-1 cell lines [19]. Canham et al. reported that effect of synthetic sample of (+)-aspergillin PZ on two highly invasive tumor lines (DU145 prostate cancer and A2058 melanoma) was determined. The IC_{50} value of this compound on the cells was calculated to be >10 μM [20].

In the present study, we aimed to determine the biological activities of aspergillin PZ and terphenyllin compounds isolated from *Aspergillus* fungi. We have shown that both compounds generally have increased cytotoxic effects on cancer cells depending on the dose. In addition, the dose-dependent increased free radical scavenging effect of the test compounds suggests that they can be evaluated as potential antioxidants. However, the antimicrobial effect of these compounds was very poor. These anti-tumor and antioxidant effects, which are similar to the studies in the literature, increase the biological importance of both compounds. The determination of the effects of the compounds at the molecular level is important for explaining particularly the antitumor mechanism.

5. CONCLUSION

As a result, high concentrations of aspergillin PZ and terphenyllin compounds (50 and 100 μM) significantly reduced viability in human ovarian and prostate cancer cell lines. Both compounds showed free radical scavenging effect in parallel with increasing concentration. However, these compounds were not effective on microorganisms in which antimicrobial activity was tested. Current studies have been insufficient in number to determine the biological activity of aspergillin PZ and terphenyllin compounds. The present study aimed to determine the different biological activities of these compounds and to contribute to the literature. Our results suggest that these two compounds show strong cytotoxic properties. It will help further studies to demonstrate mechanism of cytotoxic effect and affected cellular processes (apoptosis, DNA damage, etc.).

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CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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