Cytotoxicity and DNA protective effects of the *Terfezia* and *Picoa* species from the eastern region of Turkey

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ABSTRACT

Background and Aims: This study aims to investigate the cytotoxicity and DNA protective effects of semi-arid, arid, or desert truffles such as the *Terfezia* and *Picoa* species.

Methods: The DNA protective effects of the *Terfezia* and *Picoa* species was evaluated using plasmid pBR322 DNA treated with UV and H₂O₂. The cytotoxic effects of water and methanol extracts of the *Terfezia* and *Picoa* species on H1299 and HUVEC cells were evaluated using the MTT assay.

Results: Higher concentrations of *T. olbiensis* and *P. lefebvrei* extracts have demonstrated a DNA protective effect. The water extract of *P. juniperi* only at a 40 mg/mL concentration demonstrated a DNA-protective effect, whereas *T. boudieri* extract did not show any DNA-protective effect at all concentrations tested. H1299 cells showed more sensitivity to the water extract of *T. olbiensis* (4.66%), *P. juniperi* (12.04%), *P. lefebvrei* (21.93%), and the methanol extract of *T. boudieri* (20.93%). In general, the water and methanol extracts of the *Terfezia* and *Picoa* species demonstrated the least cytotoxic effects on HUVEC cells, except for the water extract of *P. juniperi* (38.46%).

Conclusions: In conclusion, results obtained from this study show that the *Terfezia* and *Picoa* truffle species have potential DNA protective and cancer prevention properties.

Keywords: arid-semi arid truffle, DNA protective activity, cytotoxicity effect, mushroom, *Terfezia*, *Picoa*

INTRODUCTION

Several studies have reported that food components and a balanced diet play a key role in maintaining human health integrity; however, when imbalanced, it can cause many chronic diseases (Ames, 2010). Despite extensive advances in treatment development over the past decade, the global prevalence of various diseases, cancer incidence, and the cancer-related mortality rate remain high (Ferlay et al., 2012; Torre et al., 2015). Genetic predisposition, sedentary lifestyle, poor dietary habits, diabetes, alcohol consumption, smoking, and other various external factors significantly increase the risk of developing various cancer types such as tumors of the liver, pancreas, lung, cervical, prostate, colorectal, skin, and breast (DeSantis et al., 2014; Siegel, Miller, & Jemal, 2016; Sheikh, Sarker, Kamarudin, & Ismail, 2017).

Some mushrooms species are edible for humans and can be used as dietary supplements. Not only the quality of their taste but also their biological properties are of significant value. Truffles are rich in many nutritional compounds such as protein,

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unsaturated fatty acids, carbohydrates, mineral substances, vitamins, amino acids, acids, dietary fibers, volatile organic compounds (Corrêa, Brugnari, Bracht, Peralta, & Ferreira, 2016; Ma et al., 2018). Mushrooms are also known as a significant source of biologically active compounds such as glycoproteins, beta-glucans, lectins, peptides, polysaccharides, vitamins, phenolic and polyphenolics, terpenoids, alcohols, and ergosterols. In the past thirty years, isolation of these compounds from various macrofungi has been performed and investigated as therapeutic agents in several studies (Aprotosoae et al., 2017; Reis, Martins, Vasconcelos, Morales, & Ferreira, 2017). These biomolecules of epigeous mushrooms are thought to be responsible for the biological and medicinal activities such as antioxidant (Özyürek, Bener, Güçlü, & Apak, 2014; Souilem et al., 2017; Akyüz, Kireççi, Gökçe, Kirbağ, & Yilmaz, 2019), detoxification, antigenotoxic, anticancer, angiostatic, radical scavenging, DNA protective (Kim & Kim, 1999; Shameem, Kamili, Ahmad, Masoodi, & Parray, 2017), antiviral, antimicrobial, antiparasitic, antifungal, antiinflammatory, hypoglycemic, cardiovascular, hypocholesterolemic, antihypertensive, hepatoprotective and immunomodulatory, as well as cytotoxicity effects on cancer cells (Wasser & Weis 1999; Roupas, Keogh, Noakes, Margetts, & Taylor, 2012; Souilem et al., 2017; Kothari, Patel, & Kim, 2018). Furthermore, the most common uses of edible fungi are for the treatment of fatigue resistance, low immunity, microbial infection, heart disease, stroke, hypertension, diabetes, obesity, fatty liver, ageing, tumors, Parkinson and Alzheimer diseases (Valverde, Hernández-Pérez, & Paredes-López, 2015).

Among all edible mushrooms, truffles show some distinctive characteristics and recently received more attention than other epigeous mushrooms. Truffles are one of the oldest types of food and are consumed in large quantities because of their incredibly delicious taste and musky aroma (Wang & Marcone, 2011; Kagan-Zur, Roth Bejerano, Sitrit, & Morte, 2014; Dahham, Al-Rawi, Ibrahim, Majid, & Majid, 2018; Khalifa et al., 2019). The most commonly consumed truffles are the *Terfezia*, *Tuber*, and *Tirmania* species. They can be classified under two broad types: forest (*Tuber*) and arid, semi-arid, or desert truffles (*Terfezia*, *Tirmania*, and *Picoa*). Truffles are very rich in antioxidant compounds and can eliminate free radicals such as DPPH, ROS, and hydroxyl (OH) groups (Guo, Wei, Sun, Hou, & Fan, 2011; Beara et al., 2014; Ferlay et al., 2015; Patel, Rauf, Khan, Khalid, & Mubarak, 2017). Organisms have developed antioxidant defense and repair systems to protect their cellular environment from oxidative stress; however, these systems are insufficient to prevent damage completely (Wasser, 2011; Wasser, 2014; Sánchez, 2017). Therefore, antioxidant supplements or foods containing antioxidants can be used to help reduce the oxidative stress in the human body (Tadhani, Patel, & Subhash, 2007). Antioxidant compounds can eliminate free radicals. Various compounds such as vitamins, carotenoids, proteins, peptides, phenolic compounds, nucleotides, polysaccharides, alkaloids and organic acids have been reported to have antioxidant activities (Sarmadi & Ismail, 2010; Stajic, Vukojevic, Knezevic, Duletic Lau-sevic, & Milovanovic, 2013).

DNA protective properties and cytotoxicity effects of the Ascomycetes (*Terfezia* and *Picoa*) species grown in the eastern

region of Turkey are not well investigated because truffles have received less attention than epigeous fungi. Our aim in the present study is to investigate the cytotoxic effects and DNA protective properties of the *Terfezia* and *Picoa* species.

MATERIALS AND METHODS

Truffle sample preparation

The samples used in this study were obtained from previous work (Akyüz, Kirbağ, Bircan, & Gürhan, 2015). The *Terfezia* and *Picoa* species were collected from Malatya (Central, Kale, Battalgazi, Arguvan Districts and their vicinity) and Elazığ (Baskil district and its vicinity), Turkey (N 38° 19' - 43° 19' E 038° 19' - 51° with an altitude of 690-1375 m, the beginning of March to the end of May (rarely continue until mid-July). The samples were dried and sieved at room temperature, weighed (30 g each), and placed in the cartridges of the Soxhlet instrument (Gerhardt EV 14). Extraction was performed for 3 h in 150 mL methanol (Merck) and dH₂O separately in the Soxhlet device. Then, the solvents were removed by an evaporator device. The extracts obtained were stored at +4 °C. The day after extraction, studies were started with fresh extracts.

Evaluation of the DNA protective effect

The evaluation of the protective effect of the extracts on DNA damage was performed using the screening of plasmid DNA pBR322 (Vivantis) treated with UV and H₂O₂ in gel electrophoresis. Plasmid DNA's are widely used in DNA protective activity determination and give reliable results (Golla & Bhimathati, 2014; Verma, Shrivastava, & Kumar, 2015; Soumya et al., 2019). In the study, plasmid DNA samples were treated with the extracts, oxidized by H₂O₂ + UV treatment, and then, checked on 1.25% agarose gels, according to Russo et al. (2000). Each tube of the experimental samples contained 3 µL pBR322 plasmid DNA (172 ng/µL), 1 µL of 30% H₂O₂, and 5 µL of each extract at 40, 20, 10, and 5 mg/mL in a total volume of 10 µL. UV irradiation continued for five minutes on a UV transilluminator (DNR-IS) surface with an intensity of 8 mW/cm² at 302 nm at room temperature. After irradiation, 10 µL of the reaction mixture were mixed with loading dye (6X) and loaded on a 1.25% agarose gel for electrophoresis. Untreated pBR322 plasmid DNA and partially treated plasmids with only UV or only H₂O₂ were used as controls in each run of gel electrophoresis. Gels were stained with EtBr and photographed using the gel documentation system (DNR-IS, MiniBIS Pro) (Tepe, Degerli, Arslan, Malatyali, & Sarikurkcü, 2011).

Cell lines

H1299 (non-small cell lung cancer) and HUVEC (Human umbilical vein endothelial cell) cell lines were grown in the DMEM (Dulbecco's Modified Eagle Medium) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic solution (100 U/mL penicillin and 100 µg/mL streptomycin) and then incubated at 37°C in 5% CO₂ humidified incubator (Jin, Zhang, Kang, Wang, & Zhao, 2010). Cells were counted with a hemocytometer and diluted to the cell density required for the MTT assay.

The Cytotoxic activity assay

The cell viabilities of H1299 and HUVEC cell lines treated with various concentrations of extracts were evaluated using the

MTT assay. The MTT assay is a highly preferred method for determining cytotoxic activity. The MTT dye is a yellow colored compound which is reduced by mitochondrial dehydrogenase produced by the live cells to water insoluble blue colored formazan crystals. When formazan crystals dissolve, it gives absorbance at 570 nm in the spectrophotometer (Mosmann, 1983). Firstly, 5×10^3 cells/well were seeded in a 96 well-plate. After 24 hours, truffle extracts were added to cells at the concentrations of 6.25, 12.5, 25, 50, 100 $\mu\text{g/mL}$. Forty-eight hours later, the MTT solution was added and the cells were incubated at 37°C and 5% CO_2 for four hours. Then, DMSO was added to each well to dissolve the formed formazan crystals. The color intensity was measured using a spectrophotometer at a wavelength of 570 nm. Viability (%) of cells were calculated using absorbance readings (Berridge, Herst, & Tan, 2005). A statistical calculation was made with the Graphpad Prism program with nonlinear regression analysis and graphs were created. Also, the relative cell viability was calculated using the following formula: $\text{Viable cells (\%)} = (\text{As}/\text{Ac}) * 100$ (As: Absorbance of extraction treated sample cells, Ac: Absorbance of the control cell group).

RESULTS

The DNA protective effects of water extracts obtained from desert truffle on pBR322 plasmid DNA were evaluated. The DNA protective activity of these extracts as shown in Figure 1. The DNA protective effects of five different concentrations of water extracts obtained from the *Terfezia* and *Picoa* species were evaluated using the plasmid pBR322 DNA screening method in gel electrophoresis. The DNA derived from plasmid pBR322 showed three bands in agarose gel electrophoresis. The faster-moving band on the gel is scDNA, and the slower-moving band is ocDNA and linDNA, which is the result of the separation of the open-circular DNA (Figure 1, Lane 1-3). These bands were stable in the presence of UV and H_2O_2 at the four different concentrations of water extracts obtained from the *Terfezia* and *Picoa* species (Figure 1, Lane 4-19).

According to our results, the water extract of *T. boudieri* did not show any protection of plasmid pBR322 DNA from damage induced by UV radiation and H_2O_2 in all concentrations tested (DNA could not maintain its stable structure) (Figure 1, Lane 4-7). Water extracts of *T. olbiensis* (40, 20, 10 and 5 mg/mL) showed a protective effect to plasmid pBR322 DNA from the damage induced by UV radiation and H_2O_2 . Higher concentrations of this extract demonstrated remarkable protection effect on plasmid pBR322 DNA (Figure 1, Lane 8-11). Various concentrations of water extract obtained from *P. lefebvrei* have been found to have a DNA protective effect (Figure 1, Lane 12-15). The water extract of *P. juniperi* was found to prevent DNA damage induced by UV radiation and H_2O_2 at a concentration of 40 mg/mL (Figure 1, Lane 16), but the DNA could not maintain its stable structure at the concentrations of 20, 10, and 5 mg/mL (Figure 1, Lane 17-19).

Various concentrations of the methanol and water extracts of the *Terfezia* and *Picoa* species showed a cytotoxic effect on H1299 and HUVEC cell lines (Figure 2). The water and methanol extracts of *T. boudieri* had no effect on the viability of H1299 cells at concentrations of 6.25 and 12.5 $\mu\text{g/mL}$. However, the water extract showed cytotoxic effects of 92.84%, 63.32%, and 28.31% at 25, 50, and 100 $\mu\text{g/mL}$, respectively. The methanol extract of *T. boudieri* showed cytotoxic effects of 93.78%, 37.19%, and 20.93% at 25, 50 and 100 $\mu\text{g/mL}$, respectively on H1299 cell line. The water extract of *T. olbiensis* showed no cytotoxic effect on H1299 cell line at 6.25 and 12.5 $\mu\text{g/mL}$, whereas the cytotoxic effect on H1299 cell line at 25, 50, and 100 $\mu\text{g/mL}$ were found to be 93.71%, 79.28%, and 4.66%, respectively. The cytotoxic effects of the methanol extract of the same species on H1299 cell line were found to be 83.02%, 70.68% and 47.15% at 25, 50 and 100 $\mu\text{g/mL}$, respectively. The water extract of *P. juniperi* showed no cytotoxicity on H1299 cell line at the concentrations of 6.25, 12.5, and 25 $\mu\text{g/mL}$, whereas it was found to be 56.72% and 12.04% at 50 and 100 $\mu\text{g/mL}$, respectively. The methanol extract of *P. juniperi* did not show any effect on cell viability at the concentrations of 6.25, and 12.5 $\mu\text{g/mL}$ on the same cell line, however, it showed cytotoxic ef-

Figure 1. Electrophoretic pattern of pBR322 plasmid DNA after treatment with dH_2O , UV and H_2O_2 in the presence of water extracts. Lane 1: Plasmid DNA (3 μL) + dH_2O (6 μL), Lane 2: Plasmid DNA (3 μL) + dH_2O (6 μL) + UV, Lane 3: Plasmid DNA (3 μL) + dH_2O (6 μL) + H_2O_2 (1 μL) + UV, Lane 4-7: Plasmid DNA (3 μL) + 40, 20, 10 and 5 mg/mL of *T. boudieri* water extracts + UV + H_2O_2 (1 μL), Lane 8-11: Plasmid DNA (3 μL) + 40, 20, 10 and 5 mg/mL of *T. olbiensis* water extracts + UV + H_2O_2 (1 μL), Lane 12-15: Plasmid DNA (3 μL) + 40, 20, 10 and 5 mg/mL of *P. lefebvrei* water extracts + UV + H_2O_2 (1 μL), Lane 16-19: Plasmid DNA (3 μL) + 40, 20, 10 and 5 mg/mL of *P. juniperi* water extracts + UV + H_2O_2 (1 μL), respectively].

Figure 2. Normalized absorbances % of *Terfezia* and *Picoa* extracts on H1299 and HUVEC cell lines. (W: water, M: methanol, Pj: *P. juniperi*, Pl: *P. lefebvrei*, To: *T. olbiensis*, Tb: *T. boudieri*).

fects of 92.07%, 62.28%, and 36.09% at 25, 50, and 100 µg/mL, respectively. The water extract from *P. lefebvrei* did not show any cytotoxicity on H1299 cell line at a concentration of 6.25 µg/mL; however, it showed cytotoxic effects of 99.46% at 12.5 µg/mL, 64.32% at 25 and 50 µg/mL, and 21.93% at 100 µg/mL. The methanol extract of *P. lefebvrei* at a concentration of 6.25 µg/mL did not show any cytotoxic effect on the same cell line; however, it showed cytotoxic effects of 97.26%, 97.15%, 55.06% and 42.41% at 12.5, 25, 50, and 100 µg/mL, respectively.

The water extract of *T. boudieri* showed no cytotoxic effect on HUVEC cell line at the concentrations of 6.25, 12.5, 25 and 50 µg/mL; however, it showed a cytotoxic effect 96.02% at 100 µg/mL. The methanol extract did not show any effect on cell viability at the concentrations of 6.25, 12.5 and 25 µg/mL on the same cell line; however, it showed a cytotoxic effect of 98.9% and 69.77% at 50, and 100 µg/mL, respectively. The water extract of *T. olbiensis* had no cytotoxic effect on HUVEC cell line at the concentrations of 6.25, 12.5, 25, 50 and 100 µg/mL. The methanol extract of *T. olbiensis* had no cytotoxic effect on HUVEC cell line at the concentrations of 6.25, 12.5 and 25 µg/mL; however, it showed cytotoxic effects of 95.82% and 67.31% at 50, and 100 µg/mL, respectively. The water extract of *P. juniperi* on HUVEC cell line did not show any effect on cell viability at concentrations of 6.25 and 12.5 µg/mL, however it showed cytotoxic effects of 98.89%, 96.39% and 38.46% at 25, 50, and 100 µg/mL. The methanol extract showed no cytotoxicity on

the same cell line at 6.25, 12.5, and 25 µg/mL, while it showed cytotoxic effects of 83.02% and 62.28% at 50 and 100 µg/mL. There was no cytotoxic effect of water and methanol extracts obtained from *P. lefebvrei* on H1299 cell line at the concentrations of 6.25, 12.5, and 25 µg/mL. The water extract showed a cytotoxic effect of 87.22% and 64.32% at 50 and 100 µg/mL, respectively on the same cell line, while the methanol extract showed a cytotoxic effect of 85.17% and 55.14% at 50, and 100 µg/mL, respectively (Figure 2).

DISCUSSION

Several studies have demonstrated the antiangiogenic, anti-metastatic, chemopreventive, antitumor, antiproliferative, and neo-adjuvant properties of traditional medicinal plants in vitro, in vivo and in various clinical cancer models (Roupas et al., 2012; Siegel et al., 2016; Kothari et al., 2018; Khalifa et al., 2019). Many diseases, such as neurodegenerative diseases and cancer, arise as a result of the imbalance between oxidant and antioxidant defense mechanisms. Previous studies (Kim & Kim 1999; Guo et al., 2011; Beara et al., 2014; Xiao et al., 2015; Aprotosoaie et al., 2017; Shameem et al., 2017) have reported that these diseases are responsible for oxidative DNA damage caused by ROS, such as superoxide anion, hydroxyl radical and hydrogen peroxide. In the present study, results showed that all concentrations of water extracts obtained from *T. olbiensis* and *P. lefebvrei*, and the 40 mg/mL concentration of *P. juniperi* protected the ocDNA band

of pBR322 plasmid DNA. Some mushrooms can protect cellular DNA from oxidative damage. It has been suggested that antioxidant properties of fungi can be identified by various oxidation process stages and by different mechanisms as mentioned by previous studies (Tadhani et al., 2007; Sarmadi & Ismail, 2010; Wasser, 2011; Stajic et al., 2013; Wasser, 2014; Sánchez, 2017; Živković et al., 2019). Edible mushrooms can be proposed as a dietary supplement or traditional drug to confer prevention and/or treatment of conditions arising from oxidative damage. These edible fungi are also able to protect DNA from damage induced by hydroxyl, superoxide anion, and hydrogen peroxide radicals as reported by the previously mentioned studies. As a result, some edible mushrooms represent a valuable source of bio compounds with the potential to protect cellular DNA from oxidative damage in contrast to other mushroom species (Roupas et al., 2012). According to the data mentioned above, the DNA protective effect of both the *Terfezia* and *Picoa* species is strongly dependent on the truffle type and their origins. In this regard, our study results support the findings of the previously published studies, as mentioned above.

For evaluating the cytotoxic effect of the extracts, H1299 and HUVEC cell lines were used, which are the most relevant to perform cytotoxicity studies. The water extracts of *T. olbiensis*, *P. juniperi*, *P. lefebvrei*, and the methanol extract of *T. boudieri* has remarkably decreased the percentage of viability in H1299 cells. Intriguingly, the water extract of *T. olbiensis* at 100 µg/mL, in particular, showed a selective cytotoxic effect. The water and methanol extracts of the same species showed no strong cytotoxic effect on HUVEC cell line, however, the water extract of *P. juniperi* showed strong cytotoxic effect at 100 µg/mL. These results suggest that various species of *Terfezia* and *Picoa* have potent cytotoxicity effects on H1299 and HUVEC cell lines. Various solvent extracts such as aqueous, methanol, ethanol, hexane, ethyl acetate, and bioactive molecules such as polysaccharides, glucans, agaritine, cordycepin, ergosterol, triterpenes, polysaccharopeptides, lectin, lentinan, are extracted using multiple assays and techniques from the fruit bodies of various mushrooms such as *Agaricus* spp., *G. lucidum*, *L. edodes*, *G. frondosa*, *Pleurotus* spp., *H. erinaceus*, *C. militaris*, *Tuber magnatum*, *T. aestivum*, *Terfezia claveryi* and showed cytotoxic activities on MCF-7, SK-Hep1, A549, HepG2, L929, HeLa, HT-29, U-87 MG, PC3, T24 cells in a dose-dependent manner (Roupas et al., 2012; Beara et al., 2014; Souilem et al., 2017; Kothari et al., 2018; Dahham et al., 2018; Elsayed, Alsahli, El Enshasy, & Wadaan, 2019). Therefore, dietary supplementation of edible mushroom extracts may serve as a potential antioxidant or immunity-boosting agent that helps in cancer prevention. Some Ascomycetes and Basidiomycetes are clinically used for the prevention and treatment of several diseases in some countries. The activity of these mushrooms is generally related to the presence of biomolecules, which are considered as biological response modifiers, as reported by the previously published studies. Various assays and techniques have been used to investigate the efficacy of the biologically active compounds in vitro, in vivo and in clinical trials. The results of the studies performed on fungi species in various cancer cell lines are quite different from each other as stated in the aforementioned studies. We think that these different results may

depend on the species of fungi tested, sample preparation methods, the solvent used for extraction and the cancer cell line tested, various assays, techniques and analysis methods used, as shown in the above mentioned studies.

CONCLUSION

In conclusion, our findings suggest that the truffle (*Terfezia* and *Picoa*) species might contain some cytotoxic substances related to these metabolites. Given these results, the truffle (*Terfezia* and *Picoa*) species demonstrate potential DNA protective and cytotoxic activities, which may be responsible for their therapeutic effects. This study provides a useful basis for future studies on the DNA-protective and cancer-preventive properties of truffles.

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