

In vitro and in silico analyses of cytoprotective, antigenotoxic and antimutagenic potential of *Phlomis pungens* var. *hirta* extracts against oxidative damage

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Abstract

Cellular integrity depends mainly on the stability of DNA, which can be disrupted by genetic mutations and aberrations. Here, we show that the cell-protective activity and DNA damage prevention ability of *Phlomis pungens* var. *hirta* have been investigated. We found cell protective activity, healthy cell proliferation promoter activity and DNA damage preventing capacity in the controlled in vitro assays. Additionally, *P. pungens* was shown to possess the ability to suppress the genotoxicity of H_2O_2 as an antigenotoxic agent. Rutin hydrate and quercetin are the major flavonoids in *P. pungens*, which exhibit a wide range of biological activities, including antioxidant, neuro- and hepatoprotective and A β -oligomer reducing activities. *P. pungens* has shown impressive activity against Gram-positive and Gram-negative bacteria as well as strong scavenging activity against various radicals. Subsequently, *in silico* software tools PubChem, pkCSM and PASS Online were used for biological activity profiling and toxicity predictions of the major compounds in *P. pungens*. All evaluations of *P. pungens* could be suggested as a potential source for dietary supplements and therapeutic products.

1. INTRODUCTION

Cellular integrity mainly depends on DNA stability that can be disrupted by genetic mutations and aberrations. Reactive oxygen species (ROS) induced oxidative stress can threaten the DNA stability and this can lead to vigorous results on biological pathways governed by DNA (Dumont & Monari 2015). ROS (superoxide anion, hydroxyl radical, hydrogen peroxide) can induce both cytotoxic and mutagenic damages (Benhusein et al. 2010, Ray et al. 2012). Hydrogen peroxide (H_2O_2) is known to act as a spreader by transporting itself to regions where the extremely toxic $HO\cdot$ radical can form in the presence of free iron (Lenzen et al. 2022). Therefore, H_2O_2 and $HO\cdot$ are showing their toxic effects in concert and dependent on each other in oxidative stress (Lenzen et al. 2022). In fact, the main reason of the cell death is the iron-mediated oxidative DNA damage caused by the hydroxyl radical in eukaryotes/ prokaryotes (Perron et al. 2008). The reactive molecules can cause destruction of the bases and sugar-phosphate backbones of DNA, so base modifications (bulky like cyclo purine/etheno adducts and non-bulky like 8-oxoguanine/form amido pyrimidine), DNA crosslinks, a-basic sites, protein-DNA adducts and single-strand breaks can occur (Berquist & Wilson 2012).

The oxidative DNA damages should be repaired before the replication or transcription processes in order to prevent mutagenesis. Otherwise, several pathologies like carcinogenesis and neurodegeneration can occur. In order to protect the DNA damage, cells have important defense and repair strategies against ROS (Berquist & Wilson 2012). Homologous recombination, single strand annealing and non-homologous/microhomology mediated end joining pathways are the repair mechanisms for double strand DNA breaks while mismatch repair, base and nucleotide excision repairs (BER/NER) are the repair mechanisms for single strand DNA breaks (Kaur & Mojumdar 2021). NER and BER mechanisms are also responsible for the protection of DNA from the detrimental effects of base modifications like 8-oxo-7,8-dihydroguanine caused by oxidative stress such as ROS, ultraviolet light or genotoxic agents. 8-oxo-7,8-dihydroguanine is an oxidized form of guanine and it can cause transversion mutation such as Guanine-Thymine or Guanine-Adenine bonding (Ock et al. 2012). Therefore, the 8-hydroxydeoxyguanosine (8-OHdG), a nucleoside form of 8-oxo-7,8-dihydroguanine, is considered as a well-known biomarker of the oxidative damage (Emam et al. 2014). 8-OHdG accumulation was detected in various carcinoma cells and mutated genes (Kanazawa et al. 2006). Extensive DNA damages can also lead to the production of acentric chromatid/chromosome fragments which in turn cause tiny extra-nuclear bodies called

micronuclei (MN) and the identification of the MN is frequently used to determine genomic instability and genotoxic exposure levels in cells (Luzhna et al. 2013, Xu et al. 2014). Moreover, somatic mutation recombination test (SMART) is able to identify point mutations, deletions, rarely seen mitotic recombinations (a consequence of DNA damage repair process resulting in genome instabilities because of the heterozygosity loss), chromosomal aberrations and gene conversions (Lafave & Sekelsky 2009, Yilmaz Kardas et al. 2023). Thus, MN, 8-OHdG test and SMART are frequently used in literature for the detection of mutagens (Ceker et al. 2012a, Demir et al. 2013, Dirican et al. 2012, Haverić et al. 2018, Marković et al. 2020, Okada & Okada 2015, Patenkovic et al. 2009).

In addition to their usage for the nutrition, herbaceous plants have been consumed as medicine by people to take advantage of its useful features like protection against DNA damage and carcinogenesis (Lin et al. 2013). On the other hand, plants are known to have several phytochemicals or secondary metabolites identified as toxins which help plants to protect themselves against bacteria, fungi, insects or predators (Duan et al. 2019). Therefore, detailed toxicology analyzes should be done so that toxic constituents of the plant extracts are not ignored while investigating the beneficial effects. *Phlomis pungens* var. *hirta* (Lamiaceae) is a member of *Phlomis* L. genus whose members are known to have medicinal properties like ulcerogenic, anti-diabetic / microbial / inflammatory / nociceptive / mutagenic and immunosuppressive (Ulukanli & Akkaya 2011). Although *P. pungens* is known to be used by local people, there are limited number of studies in literature about it (Özcelik et al. 2010, Özkan et al. 2009, Ulukanli & Akkaya 2011). In one of those studies, researchers investigated the antibacterial activity of hexane, methanol and acetone extracts of *P. pungens* and found that hexane extract of the plant was antibacterial (Ulukanli & Akkaya 2011). In another studies, it was shown that petroleum ether and methanol extracts of *P. pungens* had antibacterial and antifungal effects (Özcelik et al. 2010, Özkan et al. 2009). Although it has a therapeutic importance, the cytotoxicities and antigenotoxic/mutagenic properties of this medicinal plant have never been tested. The cytotoxicity and genotoxicity of *P. pungens* were aimed to be evaluated in this study by MTS assay, micronucleus assay, 8-OHdG ELISA test and SMART. Other biochemical characterization techniques like liquid chromatography (LC), antibacterial and antioxidant activity tests of *P. pungens* extracts were also revealed here.

2. MATERIALS AND METHODS

2.1. Materials

Standards used for liquid chromatography and cell growth media were purchased from Merck-Sigma Aldrich. MTS assay reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium-inner-salt) was obtained from the Promega Company. Bacterial growth medium was obtained from Biomark. Bacteria (kwik-stik) were obtained from Microbiologics of France. *P. pungens* (the aerial parts) were collected when they are in flowering period between Lice and Kulp, Diyarbakir (Turkey) districts at 1100 m (altitude of ca.) in June 2020. In addition, a voucher specimen coded as MA 2019 is preserved in the Munzur University (Turkey).

2.2. Preparation of the plant extracts

Plant extraction was performed using standard methods found in literature using water, methanol and ethanol as the solvents (Dirican et al. 2012, Yilmaz Kardas et al. 2023).

2.3. Biochemical Characterization Techniques

2.3.1. LC analysis of the plant extracts

LC system (1260 Series, Agilent, USA) with UV-Visible detector was used for the analysis of phenolic compounds according to previous studies (De Villiers et al. 2004, Yilmaz Kardas et al. 2023).

2.3.2. Antioxidant activities of plant extracts

The antioxidant activity of the plant extracts were determined by three methods. Firstly, 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) test was performed according to the literature (Re et al. 1999, Yilmaz Kardas et al. 2023). The plant extract (0- 4000 mg/L) and ABTS⁺ solution were combined (1.66% v/v) and the IC₅₀ values were calculated according to the absorbances obtained at 734 nm. Secondly, 2,2'-diphenyl-1-picrylhydrazyl (DPPH) test was performed using standard methods (Allothman et al. 2009, Aminjafari et al. 2016, Yilmaz Kardas et al. 2023, Zhang et al. 2013). The plant extracts (0- 4000 µg mL⁻¹) and the control group were then mixed with 10% DPPH reagent (v/v). The mixture was kept in dark for 1 hour and the absorbances were measured at 517 nm. IC₅₀ values were calculated, accordingly. Thirdly, ferric reducing/antioxidant power (FRAP) assay was performed according to the literature (Benzie & Strain 1996). The absorbances were taken at 593 nm after the interaction of the plant extracts with the oxidant solution because it is known that the Fe(III)-2,4,6-tripyridyl s-triazine complex was reduced to the Fe(II) form at pH:3.6. With the help of the calibration curve obtained by Trolox (hydrophilic vitamin E analogue) the “µg g⁻¹ Trolox” values were calculated (Melilli et al. 2020).

2.3.3. Antibacterial activities

Standard disc diffusion method was used with *Escherichia coli* (ATCC-8739), *Aeromonas sp.* (ATCC-51107), *Pseudomonas aeruginosa* (ATCC-9027), *Enterobacter aerogenes* (ATCC-13048) and *Staphylococcus aureus* (ATCC-6538) (Yilmaz Kardas et al. 2023). 6 mm diameter discs were soaked in 4 mg mL⁻¹ of plant extracts or gentamicin and they were placed onto the agar (Muller Hinton) inoculated with the bacteria (10⁵ CFU mL⁻¹). After incubation at 37°C for 24h, area of the inhibitions were measured (Basile et al. 2006).

2.4. Cytotoxicity and Genotoxicity Tests

2.4.1. Lymphocyte isolation and cell culture conditions

The lymphocyte cells are important model systems for *in vitro* cytotoxicity and genotoxicity tests (Amani et al. 2019, Bailon-Moscoso et al. 2016). Therefore, the lymphocyte cells were used in this study and they were isolated from healthy human blood samples (obtained in heparinized tubes) by Ficoll-Paque technique (Jaatinen & Laine 2007). The cells were dissolved in a complete cell culture medium containing RPMI 1640 (500 mL), 2.5 mL of Penicillin-Streptomycin, fetal bovine serum (50 mL) and 1 g/L phytohemagglutinin. 37° C is used for the cell growth.

2.4.2. MTS assay

MTS test was performed to test cytotoxicity with healthy human lymphocytes. 24 h after the isolation step, the plant extracts prepared with cell culture media (0-100 mg/L final concentrations, no treatment for negative control) were added into the lymphocyte cells (2 × 10⁶ cells mL⁻¹). Then, the treated cells were kept in the

incubator further for 24 and 48 h. MTS was added into the cells (1:5) at the end of the periods. After 4 h, the absorbances were measured at 490 nm.

2.4.3. Micronucleus assay

For the genotoxicity analysis the micronucleus assay was used with healthy human lymphocyte cells (Ari et al. 2015, Celikler Kasimogullari et al. 2014, Yilmaz Kardas et al. 2023). The plant extracts were added into the lymphocyte cell cultures at the 24th hour of the culture (50 mg/L final concentrations, no treatment for negative control, $6.5 \mu\text{g mL}^{-1}$ H_2O_2 for positive control). The micronuclei were identified from the microscopic images (Fenech et al. 2003). The NDI (nuclear division index) was calculated using the Eq. (1) and micronuclei % observed among the two nuclei cells was calculated (Ari et al. 2015).

$$\text{NDI} = \frac{(\text{One nucleus cell} + (2 * \text{Two nuclei cell}) + (3 * \text{Three nuclei cell}) + (4 * \text{Four nuclei cell}))}{200} \text{ Eq. (1)}$$

2.4.4. 8-hydroxydeoxyguanosine assay (8-OHdG) Assay

The oxidative DNA damage levels in the lymphocyte cells treated with 50 mg/L plant extracts and $6.5 \mu\text{g/mL}$ H_2O_2 for 24 h was analyzed with an oxidative DNA damage ELISA kit (Oxiselect TM, Cell Biolabs STA-320, USA). No treatment was applied for negative control. Genomic DNA was isolated from the cells using the DNA extraction kit (Monarch Genomic DNA Purification Kit, New England Biolabs) and the concentration of nucleic acids was determined by measuring the absorbance at 260 nm against a blank. The 8-OHdG assay was performed according to the supplier's instructions by coating the plate with 8-OHdG conjugate, blocking with the assay diluent, adding DNA samples and treating them with primary/secondary antibodies. At the end the substrate was added (100 μL) and after 2 minutes of incubations the absorbance was measured at 450 nm.

2.4.5. The somatic mutation recombination test (SMART)

SMART was performed using the standard protocols with *flare-3*, *flr³/In (3LR) TM3, Bd^S* *D. melanogaster* strain and the multiple wing hairs strain with the genetic constitution *y; mwh j* (Graf et al. 1984, Patenkovic et al. 2009). All flies were fed with standard growth medium and kept at 22 °C (Chung et al. 2009, Yakovleva et al. 2016). *flr3* female flies (10 adults) were crossed with the *mwh* males (10 adults). Then, the trans heterozygous larvae (*mwh+/+flr3*) were collected after 72 ± 4 h of incubation and washed with distilled water. Plant extracts (50 mg/L), solvent controls (5%, v/v) and H_2O_2 (6.5 mg/L) were added into separate growth media with the larvae. The adult wings were examined under microscope (Sarıkaya et al. 2016). The wing spots were classified as small single spots (*flr3* or *mwh* phenotypes observed in ≤ 2 regions), large single spots (*flr3* or *mwh* phenotypes observed in > 2 regions) or twin spots (both of the phenotypes observed). The spot frequencies (*Fr.*) were determined as the spots (n) per number of wings (N) (Graf et al. 1984, Patenkovic et al. 2009, Sarıkaya et al. 2016). The inhibition % observed in the cotreatment groups were calculated by Eq. (2) (Abraham 1994, Mezzoug et al. 2006).

$$\text{Inhibition \%} = \frac{(\text{Fr. of } \text{H}_2\text{O}_2 \text{ alone} - \text{Fr. of Plant extract})}{\text{Fr. of } \text{H}_2\text{O}_2 \text{ alone}} \times 100 \text{ Eq. (2)}$$

2.5. Statistics

All of the experiments were performed in at least three biological repeats and standard errors (SE) were calculated according to them. Student's (paired) *t*-test was applied using Excel software and $p < 0.05$ was

determined as significant.

2.6. *In silico* analysis

Prediction of Activity Spectra for Substance (PASS) (<https://www.way2drug.com/passonline/predict.php>) is a publicly available internet-based software tool that predicts the biological activity profiles of drug-like organic compounds, such as organic or phytochemicals. The results of the software tool are given as "Pa; probably active" and "Pi; probably inactive". If the Pa value is greater than 0.7, the compound is predicted to have a high probability of experimental, biological, and pharmacological activity. Ames toxicity, skin sensitisation, hepatotoxicity, etc. the pkCSM (<https://biosig.lab.uq.edu.au/pkcsml/>) software tool was used to estimate toxicity-related values. The *in silico* analyses were done by adding SMILES formats from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) to these software tools.

3. RESULTS AND DISCUSSION

3.1 LC Results

Plant phenolics are the most abundant secondary metabolites in plants and have important roles in defense against pathogens, UV radiation, parasites and predators (Dai & Mumper 2010). It is also known that phenolic acids are the significant factors of odor and taste in plants and they are involved in the browning reactions, so it is important to identify those that occur in crops that have economic importance (Torres et al. 1987). The most significant property of the phenolic compounds is the protective role from the oxidative damage and related diseases like stroke, coronary heart disease and cancer. Thus, many analytical methods like LC–DAD, LC–MS and GC have been used for phenolic compound quantifications in plants (Matei et al. 2015). In this study, LC was performed to unveil the phenolic compounds of *P. pungens*. According to the findings (Table 1), rutin hydrate ($560.27 \pm 6.18 \mu\text{g/g}$) and quercetin ($110.22 \pm 63.14 \mu\text{g/g}$) were found in highest concentrations. Ferulic acid ($7.03 \pm 0.78 \mu\text{g/g}$), vanillic acid ($1.61 \pm 0.36 \mu\text{g/g}$) and *p*-coumaric acid ($0.45 \pm 0.05 \mu\text{g/g}$) were also found in the plant. Although their concentrations differ, it can be clearly concluded that *P. pungens* is very rich in phenolic compounds (Table 1). In many studies, these compounds were analyzed and their protective properties were revealed. For example, rutin hydrate was classified as an antioxidant flavonoid and its extraordinary ability to prevent neurotoxicity by inhibiting the endoplasmic reticulum stress was proven (Mostafa et al. 2019, Noon et al. 2020, Ojha et al. 2016, Sana et al. 2014). Rutin is a glycoside of quercetin. Because of its various protective biological properties, quercetin has been the focus of attention in many genotoxicity/carcinogenicity studies (Ozyurt et al. 2014). It was shown that quercetin has anti-inflammatory, antihypertensive, vasodilatory, antihypercholesterolemic, anti-atherosclerotic, antioxidant and anti-cancer properties (Lagunas-Rangel & Bermudez-Cruz 2020). Quercetin is known to protect DNA from oxidative damage and enhance DNA repair by modulating the DNA repair enzyme expressions (Min & Ebeler 2009). It is known that quercetin and rutin are able to interact with the hydrated electron at close to diffusion control rate as efficient scavengers (Cai et al. 1999, Zhao et al. 2003). Thus, quercetin and rutin can protect DNA from hydrated electron attack in addition to their capacity to repair DNA damage caused by radiation (Zhao et al. 2003). Moreover, ferulic acid is a well-known antioxidant molecule and it is known to suppress homologous recombination during DNA repair by inhibiting RAD51 production in breast cancer cells (Choi & Park 2015, Rahman et al. 2021). Oxidative damage protection capacities of vanillic acid and *p*-coumaric acid were also reported previously (Sevgi et al. 2015, Shen et al.

2019). To conclude, it was determined by the LC results that *P. pungens* has an excellent phenolic content and the protective effect of this plant on living systems may be due to this rich content.

Table 1
LC results of *Phlomis pungens*.

Compounds	Amounts ($\mu\text{g g}^{-1}$)
Caffeic acid	L.O.D.*
Gallic acid	L.O.D.*
Vanillic acid	1.61 ± 0.36
Rutin hydrate	560.27 ± 6.18
<i>p</i> -coumaric acid	0.45 ± 0.05
Ferulic acid	7.03 ± 0.78
Quercetin	110.22 ± 3.14

*L.O.D. indicates the values below the limits of detection.

3.2 *In silico* Results

Biological activity profiles and toxicity analysis of rutin hydrate and quercetin, which are the major phenolic ingredients detected in *P. pungens*, were determined *in silico* (Table 2). In particular, the results of the PASS software tool used to estimate the biological activity profile show that the two components have high effects in terms of antioxidant, free radical scavenger, lipid peroxidase inhibitor, hepatoprotectant, hemostatic, cardioprotectant, anticarcinogenic, apoptosis agonist, vasoprotector, antineoplastic, and chemopreventive (Filimonov et al. 2014, DE et al. 2015).

Table 2
Prediction of biological activity and toxicity based on major phenolic compounds in *P. pungens* via *in silico* analysis.

Activity	<i>In silico</i> analyses	Rutin hydrate	Quercetin
Prediction of biological activity	Antioxidant	Pa: 0,923 Pi: 0,003	Pa: 0,872 Pi: 0,003
	Free radical scavenger	Pa: 0,988 Pi: 0,001	Pa: 0,811 Pi: 0,003
	Lipid peroxidase inhibitor	Pa: 0,987 Pi: 0,001	Pa: 0,788 Pi: 0,004
	Hepatoprotectant	Pa: 0,986 Pi: 0,001	Pa: 0,706 Pi: 0,007
	Hemostatic	Pa: 0,993 Pi: 0,001	Pa: 0,771 Pi: 0,003
	Cardioprotectant	Pa: 0,988 Pi: 0,001	Pa: 0,833 Pi: 0,003
	Anticarcinogenic	Pa: 0,984 Pi: 0,001	Pa: 0,757 Pi: 0,007
	Apoptosis agonist	Pa: 0,747 Pi: 0,011	Pa: 0,887 Pi: 0,005
	Vasoprotector	Pa: 0,980 Pi: 0,001	Pa: 0,824 Pi: 0,004
	Antineoplastic	Pa: 0,849 Pi: 0,007	Pa: 0,797 Pi: 0,012
	Chemopreventive	Pa: 0,968 Pi: 0,001	Pa: 0,717 Pi: 0,006
Prediction of toxicity	Mutagenic (AMES toxicity)	No	No
	Max. tolerated dose (human)	0.451 (log mg/kg/day)	0.499 (log mg/kg/day)
	hERG I inhibitor	No	No
	hERG II inhibitor	Yes	No
	Oral Rat Acute Toxicity (LD50)	2.491 (mol/kg)	2.471 (mol/kg)
	Oral Rat Chronic Toxicity (LOAEL)	3.672 (log mg/kg_bw/day)	2.612 (log mg/kg_bw/day)
	Hepatotoxicity	No	No
	Skin Sensitisation	No	No
	<i>T. Pyriformis</i> toxicity	0.285 (log ug/L)	0.288 (log ug/L)
	Minnow toxicity	7.893 (log mM)	3.721 (log mM)
Pa = Probably active (> 0,70), Pi = Probably inactive, ERG: human Ether-a-go-go-Related Gene; LD50: lethal dose of 50%; LOAEL: lowest observed adverse effect level			

3.3 Antioxidant Activity Results

Antioxidants constitute the molecules that are able to inhibit or quench the free radical reactions which lead to delay or prevention of cell damages. Every species has its own antioxidant mechanism and plants are known to be extremely rich in compounds with antioxidative activities (Dumanovic et al. 2020). The antioxidant capacities of water, methanol and ethanol extracts of *P. pungens* were investigated in this study and the data obtained as a

result of the analyzes are given in Table 3. According to the IC_{50} values obtained in the results of both DPPH and ABTS tests, the methanol extract of the plant showed higher antioxidant capacities (DPPH: $0.18 \pm 0.001 \mu\text{g}/\mu\text{L}$, ABTS: $0.45 \pm 0.004 \mu\text{g}/\mu\text{L}$) than other extracts (Ethanol extract: $0.28 \pm 0.002 \mu\text{g}/\mu\text{L}$ for DPPH and $0.68 \pm 0.009 \mu\text{g}/\mu\text{L}$ for ABTS; Water extract: $0.77 \pm 0.009 \mu\text{g}/\mu\text{L}$ for DPPH and $1.03 \pm 0.031 \mu\text{g}/\mu\text{L}$ for ABTS; Table 3) and these differences were statistically significant ($p < 0.05$). FRAP data also showed similar results (Table 3). Methanol extract of *P. pungens* showed the highest metal chelating capacity ($92.58 \pm 0.005 \mu\text{g}/\text{g}$), while ethanol extract ($79.74 \pm 0.007 \mu\text{g}/\text{g}$) and water extract ($30.71 \pm 0.007 \mu\text{g}/\text{g}$) showed lower chelating capacities ($p < 0.05$). When the previous studies with different species belong to the *Phlomis* genus are examined, it can be seen that similar antioxidant activity results were obtained. For example, Firuzi et al. used DPPH and FRAP methods to determine the antioxidant and metal chelation capacity of methanol extracts of *Phlomis elliptica*, *Phlomis olivieri*, *Phlomis persica* and *Phlomis bruguieri* species and found that *Phlomis olivieri* had the highest radical scavenging activity ($0.42 \mu\text{g}/\mu\text{L}$), while *Phlomis persica* showed the lowest radical scavenging activity ($1.19 \mu\text{g}/\mu\text{L}$) (Firuzi et al. 2010). The researchers also determined that *Phlomis elliptica* had the highest metal chelating capacity ($23.1 \mu\text{M}/\text{g DW}$), while *Phlomis bruguieri* had the lowest ($11.0 \mu\text{M}/\text{g DW}$) according to the FRAP results (Firuzi et al. 2010). In another study, it was observed that ethanol extract of *P. pungens* L. started to scavenge DPPH radical at the lowest concentration studied ($10 \mu\text{g}/\text{L}$) (Isik et al. 2017). Taskin et al. also used DPPH, ABTS and FRAP tests to investigate the antioxidant activities of the methanol extract of *P. pungens* and IC_{50} values were observed as $0.064 \pm 0.005 \text{ mg}/\text{mL}$, $23.36 \pm 0.005 \text{ mM trolox}/\text{mg}$ and $8.60 \pm 0.008 \text{ mM Fe}^{2+}/\text{mg}$, respectively (Taşkın et al. 2018). Researchers also revealed the IC_{50} values of *P. pungens* determined by DPPH and ABTS tests as $2.41 \text{ mg}/\text{mL}$ and $3.32 \text{ mg}/\text{mL}$, respectively (Okur et al. 2021). These results showed that our plant *P. pungens*, showed better antioxidant activity than the common form of the plant and other *Phlomis* species. In addition, *P. pungens* has very high antioxidant capacity similar to the famous antioxidant plant species like sage (*Salvia* sp.), lemon balm (*Melissa officinalis*), peppermint (*Mentha piperita*) and thyme (*Thymus serpyllum*, *Origanum vulgare*) reported before (Dogan et al. 2022, Mekinici et al. 2014). Considering the fact that flavonoids act as antioxidants, free radical scavengers and radio protectors, it is not surprising that the antioxidant activity of *P. pungens* was very high because of the generous concentrations of quercetin (a well-known flavonoid) observed in this plant (Table 1). Quercetin is known to function as a free radical quencher to prevent lipid peroxidation because of its diffuse ability into the membranes and scavenging the oxyradicals in lipid bilayer or it can act as a metal ion chelator by orthodihydroxyphenolic structure so it scavenges alkoxyl/peroxyl radicals (Muthukumaran et al. 2008).

Table 3
Antioxidant activities of *P. pungens* extracts (Mean $\pm$ SE) .

	ABTS ($\mu\text{g}/\mu\text{L}$)	DPPH ($\mu\text{g}/\mu\text{L}$)	FRAP ($\mu\text{g}/\text{g}$ Trolox)
Water Extract	$1.03 \pm 0.031^{a,c}$	$0.77 \pm 0.009^{a,c}$	$30.71 \pm 0.007^{a,c}$
Methanol Extract	$0.45 \pm 0.004^{a,b,c}$	$0.18 \pm 0.001^{a,b,c}$	$92.58 \pm 0.005^{a,b,c}$
Ethanol Extract	$0.68 \pm 0.009^{a,b}$	$0.28 \pm 0.002^{a,b}$	$79.74 \pm 0.007^{a,b}$
Gallic Acid (Positive Control)	$0.001885 \pm 0.000004^{b,c}$	$0.005 \pm 0.000005^{b,c}$	-
Trolox (Positive Control)	-	-	$1.25 \pm 0.007^{b,c}$
^a statistically significant ($p < 0.05$) according to positive control (gallic acid for ABTS and DPPH tests, trolox for FRAP test), ^b statistically significant ($p < 0.05$) according to water extract, ^c statistically significant ($p < 0.05$) according to ethanol extract			

3.4 Antibacterial Activity Results

Drug resistance has been accelerated because of the extensive use of antibiotics and it is possible to discover new drugs with potential antibacterial activities using plants extracts which are known to have various secondary metabolites (Bhatia et al. 2021). In this study, the antibacterial activity of *P. pungens* was investigated (Fig. 1). The largest inhibition zones were observed against *Aeromonas* sp. (12.67 ± 0.32 mm) and *E. aerogenes* (12.26 ± 0.47 mm). The plant also caused inhibition zones against *E. coli*, *P. aeruginosa* and *S. aureus* as 10.97 ± 0.12 , 11.14 ± 0.33 and 11.70 ± 0.38 mm, respectively (Fig. 1). There are similar results in previous studies. For example, Alpay et al. investigated the antibacterial activities of the ethanol extract of *Phlomis russliana* species against *E. coli*, *P. aeruginosa* and *S. aureus* and the inhibition zones were 10 mm, 11.2 mm and 12.4 mm, respectively (Alpay et al. 2019). The antibacterial activities of hexane, acetone and methanol extracts of *P. pungens* were also studied before and it was determined that acetone and methanol extracts of the plant did not show antibacterial activity, but hexane extract (1000 μg per disc) showed the highest antibacterial activity against meat-isolated *S. aureus*, *S. aureus* ATTC 6538 and *S. epidermidis* strains (diameter of inhibition: 10 mm) (Ulukanli & Akkaya 2011). Özcelik et al. investigated the antimicrobial activity of petroleum ether and methanol extracts of *P. pungens* and found that the extracts had antibacterial and antifungal activities but they didn't show any antiviral activity (Özcelik et al. 2010). In another study, methanol extract of *P. pungens* was shown to have an antibacterial effect against *Pseudomonas aeruginosa* and *Bacillus subtilis* (Özkan et al. 2009). The emergence and spread of multidrug-resistant bacterial strains has become a significant public health threat due to the availability of fewer or sometimes no effective antimicrobial agents for infection by pathogenic bacteria. Therefore, the discovery of new antimicrobial agents is of great importance. Many plants offer an effective alternative in the treatment of these problematic bacterial infections, as they can be natural antimicrobial compounds (Manandhar et al. 2019). One of these plants, *P. pungens*, has proven to be an important candidate for drug studies or biotechnological applications with its high antibacterial activity capacity.

Figure 1 Antibacterial activity results of *Phlomis pungens* shown as inhibition zones (mm $\pm$ SE). Inhibition zones observed with gentamicin were 21.97 ± 0.28 , 22.60 ± 0.22 , 22.68 ± 0.09 , 24.52 ± 0.15 and 24.71 ± 0.21 mm for

Aeromonas sp., *E. coli*, *E. aerogenes*, *P. aeruginosa* and *S. aureus*, respectively. * is statistically significant according to gentamicin.

Figure 2 MTS results of healthy human lymphocyte cells treated with (a) water, (b) methanol and (c) ethanol extracts of *Phlomis pungens*. * indicates significance compared to negative control ($p < 0.05$).

3.5 MTS Assay Results

Cytotoxicity studies are known to be a useful first step to determine the potential toxicity of a test substance like plant extract and minimal toxicity is needed for the development of a pharmaceutical or cosmetic product (McGaw et al. 2014). In order to test the cytotoxicity of *P. pungens* extracts, MTS assay was used in this study and the results are given in Fig. 2. After 24 h of incubation, none of the plant extracts caused any cytotoxicity and there were statistically significant increases in ethanol and methanol groups (50 and 100 mg/L, $p < 0.05$, Fig. 2). When the cells were treated with the samples for 48 h, the absorbances started to decrease and these decreases were statistically significant for water extracts (50 and 100 mg L⁻¹), methanol extract (100 mg/ L) and ethanol extract (100 mg/ L) but the 50 mg/L doses of methanol and ethanol extracts didn't cause any significant decrease ($p > 0.05$, Fig. 2). It is seen that the growth of the lymphocyte cells were supported in all of the extract groups (Fig. 2), but the increased incubation time for the samples treated with the higher dose of the extracts (100 mg/ L) negatively affected the viability of the cells which is an expected situation as primary cells have limited life spans (Sultan & Haagsman 2009). There isn't any study in literature showing the cytotoxicity of *P. pungens* but there are some cytotoxicity studies performed with other *Phlomis* species. Mamadalieva *et al.* investigated the cytotoxic effect of *P. bucharica* on HeLa and HL-60 leukemia cell lines using the MTT method and it was observed that methanol, hexane, chloroform and water extracts of *P. bucharica* showed cytotoxic effects on cells due to increased concentrations of the extracts (Mamadalieva et al. 2015). It was also shown that methanol, hexane and water extracts of *P. bucharica* showed lower cytotoxic activity against HL-60 and HeLa cells than the chloroform extract (Mamadalieva et al. 2015). Sarkhail et al. investigated the cytotoxic effects of the methanol extracts of *P. anisodonteia*, *P. bruguieri*, *P. caucasica*, *P. olivieri*, *P. persica* and *P. kurdica* against tumor and normal cell lines using the MTT test and all of the plants were shown to have cytotoxic activity at the concentrations above 309.15 mg L⁻¹ (Sarkhail et al. 2017). Thoppil et al. also showed that *P. platystedia* was cytotoxic on human hepatocellular carcinoma cells after a 48-hour incubation period (Thoppil et al. 2013). Plant extract and phytochemical usage is very important in therapeutic treatments but medicinal plants in nature are known to synthesize toxic substances as a defense mechanism against insects, herbivores and infections which affects all of the organisms eating them. Therefore, evaluation of the cytotoxicity is necessary for safe use of the medicinal plants (Varalakshmi et al. 2011). In conclusion, *P. pungens* supported the healthy human cells in a dose dependent manner and can be considered as a potential candidate for therapeutic biomedical applications.

3.6 Micronucleus Assay Results

In order to analyze chromosomal damage resulted from the exposure of mutagens, the MN assay is a common technique and it detects micronuclei structures which are the chromosomal fragments separated during mitosis (Cik & Jurzak 2007). At the same time, the MN assay performed with peripheral blood lymphocytes is considered as a standard method to monitor chromosome damage in human populations (Bonassi et al. 2001). In this study, MN test with the healthy human lymphocyte cells treated with *P. pungens* extracts was used and the results are given in Table 4. According to the results, none of the extracts showed a significant change

compared to the negative control ($p > 0.05$, Table 3). On the contrary, when MN ‰ of the extracts were compared with the cells treated with H_2O_2 (6.5 $\mu\text{g/mL}$), all of the extracts showed significant decreases ($p < 0.05$). The water extract of *P. pungens* caused approximately 6 fold lower MN ‰ (5.18 ± 0.33) than the one that H_2O_2 caused ($p < 0.05$) but it was similar to the one observed with the water solvent control ($p > 0.05$). In addition, methanol and ethanol extracts of the plant caused 2.15 and 2.58 fold lower MN ‰ than the one that H_2O_2 caused ($p < 0.05$). The MN ‰ of the methanol and ethanol solvent controls were also similar to the *P. pungens* methanol extract and *P. pungens* ethanol extract samples ($p > 0.05$, Table 3). These results showed that none of the extracts was genotoxic against healthy human cells and this effect can't be attributed to the solvents used for the extractions. In addition, the nuclear division index (NDI) values were determined in this study (Table 3). NDI value is known to increase or decrease abnormally as it shows the cellular proliferative capability (Ipek et al. 2017). According to the results, there wasn't any significant difference in NDI values between samples showing the fact that the micronucleus test in this study was conducted using the cells dividing in similar rhythm (Table 4). To our best knowledge, there isn't any study in literature about the genotoxicity of *P. pungens*. However, there are some studies about other members of the Lamiaceae family. For example, Dirican et al. investigated the antigenotoxic effects of water, methanol and ethanol extracts of *Thymbra spicata* L. species belonging to Lamiaceae plant family on mercury-induced human lymphocyte cells by micronucleus method and they found that *T. spicata* extracts were able to protect the mercury-induced micronuclei formation (Dirican et al. 2012). In another study, the antigenotoxic activity of the essential oil isolated from *Origanum vulgare* L. (Lamiaceae) against the genotoxic effect of aflatoxin B1 on human lymphocyte cells was investigated by micronucleus test and the essential oils in 1 μL , 1.5 μL and 2.0 μL concentrations showed the highest antigenotoxic effects against aflatoxin B1 (Ceker et al. 2012b).

Table 4
Micronucleus assay results of *P. pungens* extracts.

Treatments	MN ‰ $\pm$ SE	NDI $\pm$ SE
Negative Control	11.44 ± 2.88^b	1.29 ± 0.04
H_2O_2 (6.5 $\mu\text{g/mL}$)	30.12 ± 1.63^a	1.32 ± 0.01
Water Solvent Control (5% v/v)	4.87 ± 0.69^b	1.32 ± 0.005
Methanol Solvent Control (5% v/v)	15.48 ± 0.56^b	1.35 ± 0.003
Ethanol Solvent Control (5% v/v)	14.01 ± 0.71^b	1.35 ± 0.001
<i>P. pungens</i> Water Extract (50 mg/L)	5.18 ± 0.33^b	1.33 ± 0.0002
<i>P. pungens</i> Methanol Extract (50 mg/ L)	14.00 ± 2.32^b	1.41 ± 0.1
<i>P. pungens</i> Ethanol Extract (50 mg/ L)	11.66 ± 2.78^b	1.19 ± 0.01
^a statistically significant ($p < 0.05$) according to negative control, ^b statistically significant ($p < 0.05$) according to H_2O_2 (6.5 $\mu\text{g/ mL}$)		

3.7 8-OHdG Assay Results

ROS are known to have an ability to attack DNA and as a result of this attack, the guanine (G) base is modified into 8-OHdG which lead to mutations during DNA replication as polymerases can't identify it as guanine (Emam et al. 2014). Thus, 8-OHdG was used as an indicator of oxidative DNA damage in this study and the results of 8-OHdG ELISA test are given in Fig. 3. When the 8-OHdG amounts (ng/mL) of the samples treated with plant extracts were compared to the ones treated with an oxidative damage source, H_2O_2 , there were dramatic decreases (Fig. 3). Although the water extract of *P. pungens* showed only 17% decrease, the methanol and ethanol extracts showed 41 and 50% decreases, respectively and all of those decreases were statistically significant ($p < 0.05$). When the solvent controls were examined, the water solvent control (Fig. 3a) caused the similar amount of 8-OHdG compared to negative control ($p > 0.05$) but it was lower than the one observed in the cells treated with H_2O_2 ($p < 0.05$). On the other hand, the methanol and ethanol solvent controls (Fig. 3b & Fig. 3c) caused statistically significant increases in 8-OHdG level compared to negative control ($p < 0.05$), while the values were similar to the one observed with H_2O_2 ($p > 0.05$). These results showed that methanol and ethanol extracts of *P. pungens* were able to decrease the oxidative DNA damage (41 and 50%, respectively) and this effect can't be attributed to the solvents used for the extractions which couldn't decrease the 8-OHdG levels when applied alone. Although there isn't any study in literature showing the preventive effect of *P. pungens* against 8-OHdG but various studies with different *Phlomis* species showed that this genus generally has antigenotoxic and antimutagenic properties. For example, Uysal et al. showed that methanol, ethyl acetate and water extracts of *P. nissoli*, *P. pungens* var. *pungens* and *P. armeniaca* had significant antigenotoxic effects according to the Ames test results (Uysal et al. 2016). Dellai et al. also demonstrated the antigenotoxic effects of ethyl acetate, chloroform and methanol extracts of *P. crinita* Cav. by Ames test (Dellai et al. 2009). In another study, researchers used Ames test to show that methanol and ethyl acetate extracts of *P. mauritanica* had the capacity to inhibit mutations (Limem et al. 2010). It is known that unrepaired DNA lesions can inhibit replication and transcription processes which potentially lead to mutations and/or cell death (Knezevic-Vukcevic et al. 2007). Therefore, the DNA damage prevention capacity of *P. pungens* shown in this study revealed significant potential for its use in the development of therapeutic products and applications.

3.8 SMART Results

SMART is an assay used for the detection of mutagenic and recombinogenic activities in somatic cells induced by xenobiotics (Yilmaz Cetinkaya & Yurtsever 2021). It is based on the genetic damage induction in dividing wing disc cells of *Drosophila melanogaster* larvae which results in heterozygosity loss during development and this can be observed on the adult wings as mutant wing spots (Pitchakarn et al. 2021). SMART is considered as a very sensitive genotoxicity test because the imaginal disc cells are known to duplicate every 10 hour during larval development so the genotoxins have a bigger chance of interacting with the larval genome (Pitchakarn et al. 2021). Therefore, in this study SMART was used for the detection of *in vivo* antigenotoxic effects of *P. pungens* extracts and the results are given in Table 4. As a genotoxic agent H_2O_2 caused significant increases in small single, large single and twin spots ($p < 0.05$). On the other hand, water, ethanol and methanol extracts of *P. pungens* showed similar small single spot frequencies like negative control ($p > 0.05$) and there weren't any large single or twin spots. Therefore, it can be concluded that the *P. pungens* extracts weren't genotoxic. In order to evaluate the influence of the solvents used to prepare the extracts, solvent control groups were also investigated (Table 4). The results showed that, ethanol and methanol solvents caused higher frequencies of large single spots compared to negative control and the small single spot frequencies were similar to the ones observed with H_2O_2 , while the water solvent control was non-genotoxic. Thus, the solvents are not responsible

for the antigenotoxic effects of the *P. pungens* extracts. When the extracts were coadministered with H₂O₂, all of the extracts caused inhibitions in frequencies of different spot types (Table 4). In fact, ethanol and methanol extracts of *P. pungens* caused more than 74% of inhibitions. Therefore, *P. pungens* extracts can be considered as the antigenotoxic substances with an ability to suppress the genotoxic effects of H₂O₂. There isn't any SMART data observed with *P. pungens* in literature. However, there are many studies showing the protective characteristics of the members of Lamiaceae family against the genotoxic agents shown by SMART (Guterres et al. 2022, Magombe et al. 2022, Yilmaz Kardas et al. 2023). To sum up, it can be said that mutations and mitotic recombination, which is known to be stimulated by H₂O₂ induced DNA breaks, were inhibited by the *P. pungens* extracts used in this study (Qi et al. 2019).

P. pungens is one of the medicinal plants that has been used by local people to treat diabetes, ulcer and infections. However, there are limited number of studies in literature about this plant and cyto/genotoxicity analyses have not been done before. Therefore, this study aimed to find out cell protective activity, DNA damage prevention capacity and antioxidant/antibacterial properties of the water, methanol and ethanol extracts of *P. pungens*. According to the LC results of this study, rutin hydrate ($560.27 \pm 6.18 \mu\text{g g}^{-1}$) and quercetin ($110.22 \pm 63.14 \mu\text{g g}^{-1}$) were found in highest concentrations. Antioxidant activity results showed that the methanol extract of the plant showed higher antioxidant capacities than other extracts ($p < 0.05$). The plant was found as antibacterial against all of the strains tested but the largest inhibition zones were observed against *Aeromonas* sp. ($12.67 \pm 0.32 \text{ mm}$) and *Enterobacter aerogenes* ($12.26 \pm 0.47 \text{ mm}$). Although the increased incubation time for the samples treated with the higher dose of the extracts negatively affected the viability of the cells, it was clearly seen that the growth of healthy human lymphocyte cells were supported by all of the extracts. Micronucleus assay showed that none of the extracts was genotoxic against healthy human cells. 8-OHdG ELISA test also showed that methanol and ethanol extracts of *P. pungens* were able to decrease the oxidative DNA damage (41 and 50%, respectively). According to the SMART results, *P. pungens* extracts were shown as the antigenotoxic substances with an ability to suppress the genotoxic effects of H₂O₂. As a result of the *in vitro* analyses, the biological activity profiles and toxicity of routine hydrate and quercetin, which discovered to be the main constituents of the plant, were investigated *in silico* analyses, and the obtained prediction results support our experimental findings. To conclude, this is the first study which demonstrated the *in vitro* cell protective activity and DNA damage prevention capacity of *P. pungens* which should be regarded as an important medicinal plant that can be used for nutritional supplements and therapeutic-products.

Table 4
Results of the somatic mutation recombination test (SMART).

Treatments	Average Number of wings (N)	Small single spots (S)			Large single spots (L)			Twin spots (T)			Inhibition % (S,L,T, Total)
		n	Fr.	SE	n	Fr.	SE	n	Fr.	SE	
Negative Control	70	2	0.03 _b	0	0	0 _b	0	0	0 _b	0	
H ₂ O ₂	68	3.7	0.05 _a	0.005	4.7	0.069 _a	0.005	15.7	0.23 _a	0.02	
Water Solvent Control	70	2	0.03 _b	0	1	0.014 _b	0	0	0 _b	0	
Ethanol Solvent Control	69.3	2.7	0.04	0.005	4.7	0.067 _a	0.005	7.3	0.11 _{ab}	0.01	
Methanol Solvent Control	64	3.7	0.06 _a	0.005	9	0.141 _b	0	7.3	0.11 _{ab}	0.01	
<i>P. pungens</i> Water	70	2	0.03 _b	0	0	0 _b	0	0	0 _b	0	
<i>P. pungens</i> Ethanol	75.3	2	0.03 _b	0.0002	0	0 _b	0	0	0 _b	0	
<i>P. pungens</i> Methanol	68	1.7	0.02 _b	0.005	0	0 _b	0	0	0 _b	0	
<i>P. pungens</i> Water + H ₂ O ₂	67.3	2	0.03 _b	0.001	0	0 _b	0	0	0 _b	0	44.7, 100, 100, 91.6
<i>P. pungens</i> Ethanol + H ₂ O ₂	73.3	1	0.01 _{ab}	0.0001	0.7	0.009 _b	0.005	0	0 _b	0	74.7, 86.7, 100, 93.5
<i>P. pungens</i> Methanol + H ₂ O ₂	72	1	0.01 _b	0	0.7	0.009 _b	0.005	0	0 _b	0	74.2, 86.5, 100, 93.4
^a statistically significant (p < 0.05), according to negative control, ^b statistically significant (p < 0.05), according to H ₂ O ₂ (6.5 mg/L), Fr.: Frequency, n: Average spot											

Declarations

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Ethical Approval

Ethical approval for this study was obtained from Balikesir University Faculty of Medicine Clinical Research Ethics Committee (2021/132).

Consent to Participate

All authors approved to participate in this research.

Consent for publication

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Competing interests

Authors declare that, there is no conflict of interest.

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Figures

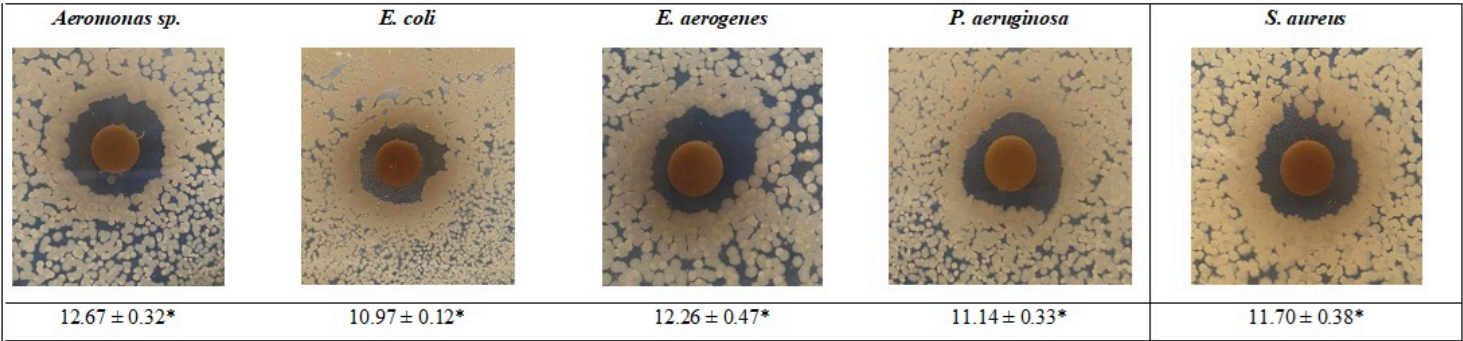


Figure 1

Antibacterial activity results of *Phlomis pungens* shown as inhibition zones (mm ± SE). Inhibition zones observed with gentamicin were 21.97 ± 0.28, 22.60 ± 0.22, 22.68 ± 0.09, 24.52 ± 0.15 and 24.71 ± 0.21 mm for *Aeromonas sp.*, *E. coli*, *E. aerogenes*, *P. aeruginosa* and *S. aureus*, respectively. * is statistically significant according to gentamicin.

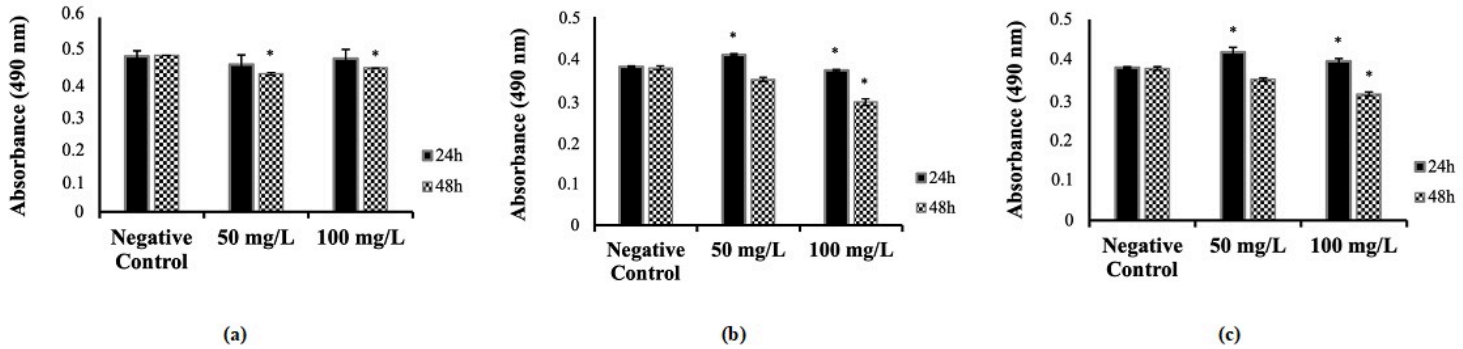


Figure 2

MTS results of healthy human lymphocyte cells treated with (a) water, (b) methanol and (c) ethanol extracts of *Phlomis pungens*. * indicates significance compared to negative control (p<0.05).

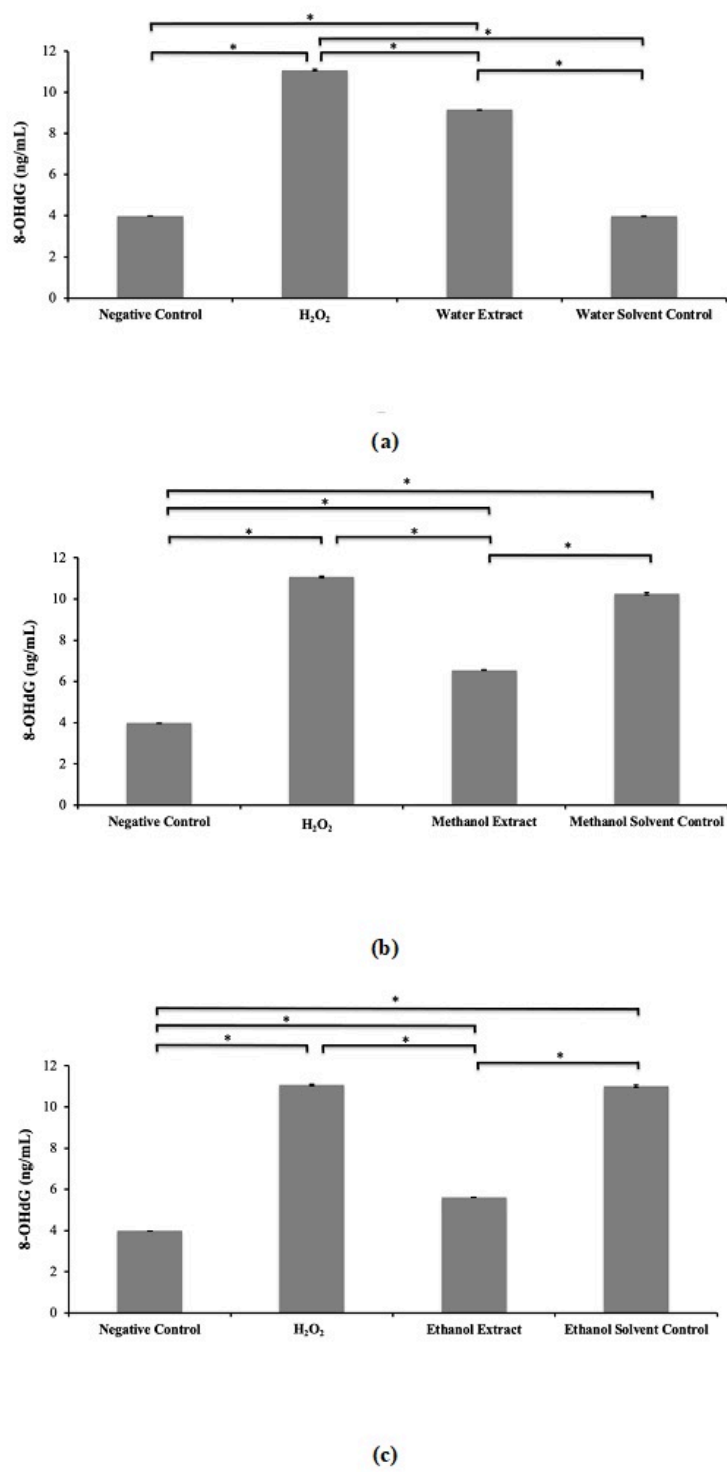


Figure 3

8-OHdG (ng/mL) levels observed in cells treated with (a) water, (b) methanol and (c) ethanol extracts of *P. pungens** indicates statistical significance (p<0.05).

