# Antioxidant properties and prevention of cell cytotoxicity of *Phlomis persica* Boiss.

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**ABSTRACT**

The free radical scavenging capacity, reducing power and inhibition of *β*–carotene peroxidation of *Phlomis persica (PP*) extracts was investigated. In addition, the effect of this extract on reduction of the hydrogen peroxide-induced cytotoxicity in non-immortalized fibroblast was examined. The extracts showed free radical scavenging capacity, and the ethyl acetate extract showed marked effect on inhibition of lipid peroxidation similar to that of gallic acid. These results were further supported by a protective effect of *Phlomis* crude extract on $H_2O_2$-induced cytotoxicity in human non-immortalized fibroblasts.

**Keywords:** Antioxidant activity; *Phlomis persica;* DPPH scavenging; *β*-Carotene bleaching test; Cell cytotoxicity.

## INTRODUCTION

Free radicals are electrically charged molecules which have unpaired electron and tendency to capture electrons from other substances in order to be neutralized. Although the initial reactions result in neutralization of the free radical, but another free radical is formed
in the process, which by itself causes a chain of reactions to occur and as a result, thousands of free radicals reactions can occur within seconds of the initial reaction (1).

Cell damages caused by free radicals appear to be a major contributor to aging cancer, cardio-vascular disease, cataracts, immune system decline, and brain dysfunction (2). Fortunately, free radicals formation is controlled naturally by various beneficial compounds known as antioxidants (3, 4).

Antioxidants are a group of substances when present at low concentrations compared to oxidized substrates significantly inhibit or delay oxidative processes, while being oxidized themselves (5). Phenolic compounds such as flavonoids are ubiquitous in the plant kingdom and approximately 3000 flavonoid substances have been described (6). In addition to an antioxidant effect, flavonoids may exert protection against heart disease through inhibition of lipooxygenase and cycloxygenase activities in platelets and macrophages (7). Flavonoids have also been demonstrated to have anti-inflammatory, anti-allergenic, anti-viral, anti-aging and anti-carcinogenic activity (6-9).

The plants of Labiatae's family contain flavonoid compounds (10).One of the labiatae family species is *Phlomis persica*, and in this study the antioxidant activity of its extracts and prevention of cell cytotoxicity of crude extract was investigated.

## MATHERIALS AND METHODS

*Chemicals*

Butylated Hydroxy Toluene (BHT), 1, 1 Diphenyl -2-Picryl Hydrazyl radical (DPPH), Gallic acid, and 3-(4, 5-dimethyl-iazol-2yl), 2.5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. All other reagents were from Merck Chem. Co. Human non-immortalized fibroblasts (HFFF- P16) were obtained from Pasteur Institute of Iran.

*Plant materials*

Aerial parts of *Phlomis persica* were collected in March 2005, from the area around Shahre- Kord, Iran, identified and authenticated (Voucher No 125) in the Department of Pharmacognosy, Shaheed Beheshti University of Medical Sciences, Tehran, Iran. The leaves were separated, dried at room temperature and grounded into powder.

*Extraction and fractionation*

Aerial parts of *Phlomis persica* (3000g) were extracted with ethanol (38.4 L) to give 618.9 g of concentrated crude extract. Four hundreds and thirty grams of the crude extract was then

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suspended in 400 ml of water and filtered. A 250 ml of the aqueous solution was extracted concecutively with 250 ml portions of petroleum ether, chloroform, ethyl acetate and n-butanol.

*Measurement of reducing power*

The reducing power of extracts of *Phlomis persica* was determined using the method described by Hartwell (11). A series of concentrated extract of *Phlomis persica* (12.5, 25, 50, 100 and 200 µg/ ml) in methanol were made and mixed with 0.2 M phosphate buffer of pH, 6.6 containing 1% ferrocyanate. The mixture was incubated at 50 ºC for 20 minutes. To 5 ml of this mixture was added 2.5 ml of 10% trichloroacetic acid and the mixture was centrifuged at 3000 g for 10 minutes. The supernatant was separated and mixed with 2.5 ml of distilled water containing 0.5 ml of 1% ferric chloride. The absorbance of this mixture which could be a measure of antioxidant activity of the extract was determined at 700 nm (11).

*Determination of antioxidant using DPPH*

The antioxidant activity of plant extract and the standard antioxidants were assessed on the basis of radical scavenging effect of the stable DPPH free radical. In a modified assay (12), 200 µl of a 100 mM solution of DPPH radical in methanol was mixed with 20 µl of 12.5-400 µg/ml extracts, BHT and gallic acid respectively and solutions were left at room temperature for 30 minutes. The DPPH radical inhibition was measured at 490 nm by using a micro-plate reader model Panasonic KX-P108 athos 2020.

The $IC_{50}$ of each sample (concentration in µg/ml required to inhibit DPPH radical formation by 50%) was calculated by Matlab software. Tests were carried out in triplicates.

The antioxidant activity (AOA) is given by:

100- [(A) sample-(A) blank) × 100/(A) control]

Where "A" is the absorbance of the samples in wells. DPPH (without plant extract) and methanol were used as control and blank respectively.

*β- Carotene bleaching test*

For this assay modification of a reported method (13) was employed. One miligram of *β*- carotene was dissolved in 5 ml of chloroform and to 1 ml of this solution was added 25 µl of linoleic acid and 200 mg of tween 40. Following evaporation of chloroform under vacuum at 40˚C, the residue was treated with 50 ml of oxygenated pure water (oxygenation was performed by bubbling air through water for 15 min) and the mixture was vigorously shaken. This emulsion was prepared freshly before each experiment. Stock solution of extract (0.1%) and antioxidants as standards (0.01%) were prepared in methanol .To each well of 96 well microliter was added 250 µl of *β*-carotene emulsion in linoleic acid, and 30µl of solution of extracts in methanol. An equal amount (30 µl) of methanol was used as the blank. The microtiter plates were incubated at 55°C for 105 min and the absorbance of samples were measured by a micro plate reader (Panasonic Kx-P108 athos 2020) at 492 nm (14). The antioxidant activity coefficient (ACC) was given by a modified version of the formula of Chevolleau et.al (15)

$$ACC = [(A_A105-A_B105)/(A_B0 -A_B105)]$$

In which $A_A105$ and $A_B105$ are the absorbencies of the test and blank samples at zero and 105 min respectively and $A_B0$ is the absorbance of the blank sample at t=0 min.

*Determination of the phenolic compounds*

The content of total phenolic compounds in methanolic solutions of the crude extract was determined by a described method (16). For the preparation of calibration curve, 1ml aliquots of 0.024, 0.075, 0.105 and 0.3mg/ml gallic acid solutions in methanol were mixed with 5ml of folin-ciocalteu reagent (diluted ten fold) and 4ml (75g/l) of sodium carbonate. The absorption was read after 30 min at 20 °C at 765 nm and the calibration curve was constructed. One ml of methanolic solution of the crude extract (10 g/l) was mixed with the same reagents as described above, and after 1 hour the absorption was measured for determination of plant phenolic componds. All determinations were performed in triplicates. Total content of the phenolic compounds in methanolic solution of the crude extract equivalent to gallic acid (GAE) was calculated from the formula:

$$C = c.V/m´$$

Where C is total content of phenolic compounds in mg/g of the extract, equivalent to GAE; c is the concentration of gallic acid which is found from the calibration curve in mg /ml; V is the volume of extract in ml; and m is the weight of the plant crude extract.

*Cell culture and treatments*

Human non –immortalized fibroblast (HFFF-P16) cell were obtained from Pasteur institute. The cells were grown at 37º C in humidified 5% $CO_2$ 95% air mixture in DMEM ( Dulbecco's modified Eagle medium) supplemented with 15% fetal calf serum(FCS), 1m of L-glutamine, 100U/ml of penicillin and streptomycin. The medium was replaced 3 times in a week until the cells were near confluency. The crude extract of *Phlomis persica* was dissolved in the minimum amount of

dimethyl sulphoxide (DMSO) in a way that the final DMSO concentration was never higher than 0.05% when mixed with culture medium. DMSO at this concentration has not been reported toxic for the cells. The solution of crude extracts in DMSO (12.5-25 µg/ml) were added to the culture medium and kept at incubator for 24 hours, solution of gallic acid in DMSO (6.25µg/ml) was used as a standard. After 24 hours the cells in plates ($32\times10^4$/ml) were treated with 0.1 mM $H_2O_2$ for 30 minutes and then washed with buffer phosphate.

*MTT bioassay*

MTT bioassay measures the conversion of tetrazolium salt to the colored product, the concentration of which can be measured spectrophotometrically (17). Twenty µl of 0.5% MTT in PBS was added to each micro well. The microplate was put in incubator (37ºC) for 6 hours, the supernatant was removed and replaced with 200µL of acidified isopropranol and 20µL of 3% (W/V) dodecyl sulphate in water.

The optical density of each sample was measured with a micro plate spectrophotometer reader (Panasonic Kx-P108 athos 2020) at 570 nm. Five replicates were performed for each sample. Reduction in $H_2O_2$ cytotoxicity were determined and expressed in percent by crude extract and gallic acid as standard.

*Statistical evaluation*

Mean ±SD are given. The data were analyzed for statistical significance using one way ANOVA followed by Tukey or Dunnett post test. P values less than 0.05 were considered significant.

## RESULTS

*Reducing power capacity*

Fig (1) shows the reducing power of the crude, butanolic and ethyl acetate extracts of *Phlomis persica*, and BHT and gallic acid as standards at equal concentrations of 12.5 µg /ml to 200 µg /ml.

*Radical scavenging activity*

Fig (2) depicts radical scavenging effect of the crude, buthanolic and ethyl acetate extracts of *Phlomis persica* (PP) and BHT and gallic acid as standards in concentrations of 12.5 to 400 µg/ml. The $IC_{50}$ of BHT and gallic acid were found to be 68 and 2.49 µg/ml respectively. The $IC_{50}$ of the ethyl acetate extract (116.2±1.5 µg/ml) was less than butanolic extract (148.1±3.5 µg/ml).

*β-carotene bleaching test*

The results of *β*- carotene bleaching test are illustrated in table 2. The ACC of ethyl acetate extract of *Phlomis* was more than *Phlomis* crude extract and butanolic fraction ($P < 0.001$).

**Table 1.** $IC_{50}$ of antioxidant compounds and *Phlomis* extracts compared with BHT and Gallic acid

| Antioxidant compounds | $IC_{50}$ µg/ml [a] |
|---|---|
| Gallic acid | 2.49±.6 |
| BHT | 63.17± .9 |
| *Phlomis* ethyl acetate extract | 116.2±1.5 |
| *Phlomis* butanolic extract | 148.1± 3.5 |
| *Phlomis* crude extract | 169.8±5 |

a: Each value reperesents the mean ± SD

The $IC_{50}$ of ethyl acetate extract was less than butanolic extract and crude extract ( $P < 0.05$).

**Table 2.** Antioxidant activity of *Phlomis* extracts in comparison with BHT and gallic acid as measured by the BCBT[a] method.

| Antioxidant compound | ACC[b] |
|---|---|
| BHT | 866.6±15.7 |
| gallic acid | 754.03±44.28 |
| *Phlomis* crude extract | 218.27±26.5 |
| *Phlomis* butanolic extract | 350.6±24.4 |
| *Phlomis* ethyl acetate extract | 656.54±30.56 |

a) BCBT ( *β* –Carotene Bleaching Test).

b) Antioxidant activity coefficient by BCBT method given as ACC.

**Table 3.** Effects of *Phlomis* extracts on $H_2O_2$ induced cytotoxicity in non immortalized fibroblasts. MTT (% of control) % Inhibition of $H_2O_2$ cytotoxicity

| Control | 100 |
|---|---|
| $H_2O_2$ (0.1mM) | $45.2 \pm 2.9^a$ |
| Gallic acid 6.25 µg/ml | $92.4 \pm 2.8^b$ |
| *Phlomis* extract | |
| 12.5 µg/ml | $72.8 \pm 3.3^b$ |
| 25.0 µg/ml | $80.2 \pm 4.5^b$ |

[a] Each value represents the mean ± SD of five experiments significant vs control untreated fibroblasts and [b] vs $H_2O_2$ treated cell ($P < 0.01$).

*Cell culture*

The results of $H_2O_2$ cytotoxicity in fibroblasts cells was evaluated on the basis of its effects on cell growth factor (MTT test) and are listed in Table 3. Cell growth was significantly suppressed in the $H_2O_2$ treated cells. Addition of the crude extract (12.5 and 25µg) before exposure to peroxide mitigated the cytotoxic response and this effect was concentration dependent. Under the same conditions gallic acid (6.25 µg/ml) reduced the cytotoxicity of $H_2O_2$. The extract of *Phlomis persica* at concentrations of 12.5, 25, µg/ml, were not cytotoxic.

## DISCUSSION

Species of labiatae's due to their distribution and diversity in the Mediterranean region and their essential oils are a point of interest from the botanic, taxonomic, chemical composition and ecological aspects (18).

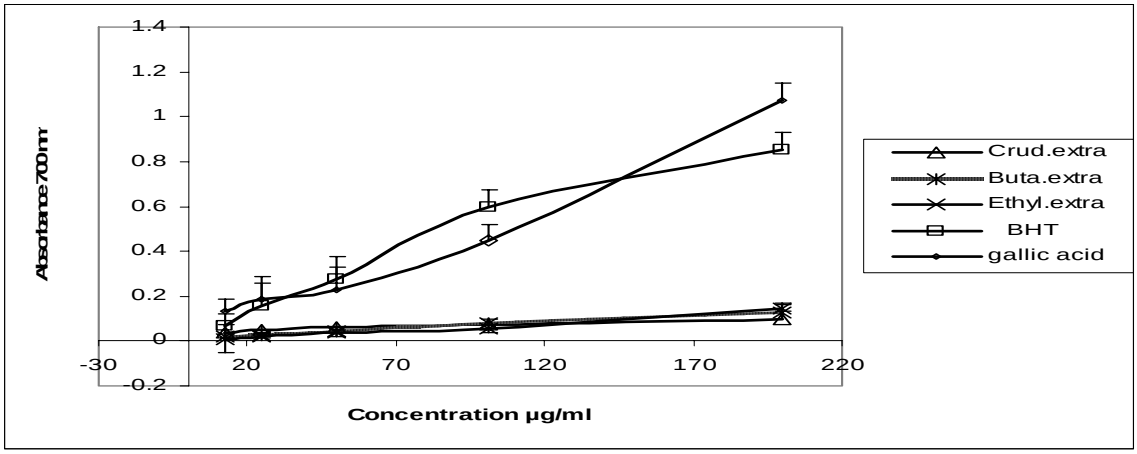


**Figure 1.** Reducing power of *Phlomis persica* compared with antioxidant standards BHT and gallic acid (gallic acid is diluted 1:10).

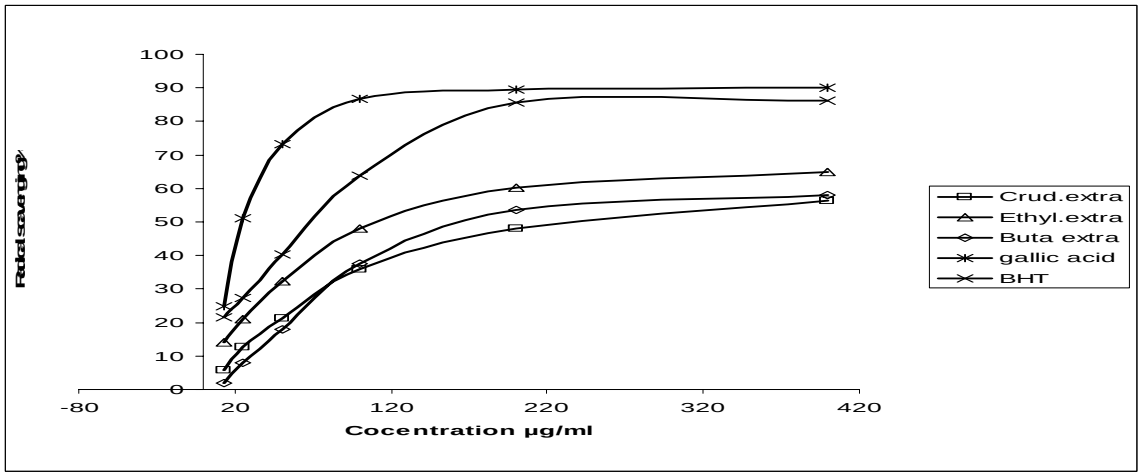


**Figure 2.** Radical scavenging activity of *Phlomis* extracts compared with BHT and gallic acid (gallic acid is diluted 1:10). Radical scavenging activity of *Phlomis* ethyl acetate extract is more than its butanolic extract ($P < 0.05$).

*Phlomis* is one of the labiatae species and the genus *Phlomis* is composed of about 100 species, of which 17 species are described in flora of Iran, among them 10 are endemic (19, 20). Recently isolation of irridiosl (21) and phenyl propanoid glycoside (22) from the oil of *Phlomis fruticosa* (21), have been reported. Of these compounds Phenyl propanoid glycoside has shown antioxidant activity (25).

In other investigations, 4 flavonol glycosides were isolated from a methanolic extract of *Phlomis spectabilis* (26). In this investigation the antioxidant activity of *Phlomis persica* was investigated.

The antioxidant potential of a compound can be attributed to its reducing power, radical scavenging ability and prevention of *β*-carotene bleaching. These properties may serve as significant indicators of its potential as antioxidant. However the activity of putative antioxidants has been attributed to various mechanisms, among which are prevention of chain initiation, binding to transition metal catalysis, decomposition of peroxides (27). Our experiments using DPPH and reducing power tests showed that there is a strict proportionality between the concentration of extracts as antioxidant and BHT and gallic acid as standards.

The reducing power of *Phlomis persica* and its fractions increased by increase in their concentrations (Fig.1). However their reducing powers were lower than BHT and gallic acid.

Table 1 shows that $IC_{50}$ of the ethyl acetate extract was less than butanolic and crude extracts. It is generally accepted that phenolic compounds which are present in fractions of plant extract

inhibit the oxidation by donation of hydrogen atoms to scavenge free radicals (28).Furthermore, it is reported that the ethyl acetate extract is more active than other fractions in radical scavenging properties (28). Crude extract of *Phlomis* and its fractions (containing phenolic compounds) can inhibit *β*- carotene bleaching and there is a significant difference in ACC between standards and butanolic extract (P<0.001, F=424.06). Also a significant difference in ACC between BHT and ethyl acetate extract was observed (P<0.001, F=424.06).

The amount of phenolic compounds of *Phlomis* crude extract and its ethyl acetate extract were 28.8±0.8 and 40±0.1mg/g respectively. The higher antioxidant activity of the ethyl acetate extract may be related to its higher phenolic compounds.

In this study, the cytotoxic effects of $H_2O_2$ on non -immortalized fibroblast cells were evaluated by inhibition of the cell growth which was significantly suppressed in the $H_2O_2$-treated cells (Table-3). Addition of *Phlomis persica* extract before exposure to $H_2O_2$ mitigated the cytotoxic response and it was observed that this effect was concentration dependent. In other words a significant difference (P<0. 01, F=168.89) was observed between non-immortalized fibroblasts which were treated only with $H_2O_2$ and the cells which were treated with crude extract or gallic acid before exposure to $H_2O_2$.

In conclusion, the results of this study indicate that this extract exhibits interesting antioxidant properties expressed by its capacity to scavenge free radicals (DPPH), prevention of lipid peroxidation, reducing ability and reduction of $H_2O_2$ –induced cytotoxicity in human non immortalized fibroblast cells.

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