Antioxidant Properties and Protective Effect of Turkish Propolis on t-BHP-Induced Oxidative Stress in Foreskin Fibroblast Cells

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ABSTRACT

Objective: Propolis is an important bee product, rich in polyphenolic compounds. It has antitumoral, antioxidant, antimutagenic, and other useful activities. Biological activities of propolis are generally attributed to its substance of polyphenolic compounds. The aim of this study was to investigate the in vitro antioxidant properties and reduction amount of intracellular reactive oxygen species (ROS) in human normal foreskin fibroblast cells by Turkish propolis ethanolic extract (EEP).

Method: Total phenolic and flavonoid contents, reducing power, radical scavenging capacity, and iron chelating activity of EEP were determined using spectrophotometric methods. Intracellular ROS levels were determined by spectrofluorometric analysis with CM-H₂DCFDA.

Results: Our results indicated that antioxidant activity was correlated with the higher total phenolic and flavonoid contents of EEP. The generated ROS levels were significantly decreased by EEP compared to only t-BHP group.

Conclusion: Ethanolic propolis extract may be evaluated as a potency antioxidant resource and/or a novel natural agent in food and drug industries.

Key words: Antioxidant activity, CM-H₂DCFDA, Fibroblast cells, Polyphenols, Propolis.

INTRODUCTION

Human beings have recognized and have benefited from the therapeutic aspect of natural products thousands years ago. In recent years, the emergence of the side effects of the drugs has led people to the consumption of natural products known as medicine. One of the most widely used products is propolis, which is a bee product.¹ Propolis is quite rich in polyphenolic compounds (caffeic acid, ferulic acid, etc.), flavonoids (quercetin, rutin, pinocembrin, naringenin, crysin, etc.) and contains many vitamins, minerals, and trace elements.²,³,⁴ Propolis has been used in traditional medicine for many years for a variety of purposes.⁵ Bee products are used to prevent the progression of the disorders, to reduce pain and to treat the disease.¹,⁵ Valuable information have been revealed about antioxidant, antibacterial, antiviral, anti-inflammatory, anticancer, antimutagenic, radioprotective, and other beneficial biological effects and chemical structure of propolis.⁶,⁷,⁸,⁹,¹⁰ In recent years, propolis has become quite popular in pharmaceutical, cosmetic, and food industries because of above-mentioned properties that it possesses.⁶,¹¹ Flavonoids are natural polyphenolic compounds that can be found in fruits, vegetables,
and beverages, such as tea, wine, and fruit juice. Since these compounds are secondary plant metabolites, they cannot be synthesized by humans and so they are forming a significant part of human diet. Flavonoids display their biological activities by binding heavy metal ions and biological polymers, by catalyzing electron transport and scavenging free radicals. Recent studies have showed that flavonoids protect DNA and other components of the cell from oxidative damage. Free radicals are defined as atoms or groups of atoms that includes an unpaired electrons. Reactive oxygen species (ROS) are described as chemically reactive molecules that include one or more oxygen atoms, which contain free radicals and non-radical reactive compounds that can oxide biological molecules. Free radicals give rise to many damages in cells and tissues. Numerous studies have evaluated the antioxidant activity and beneficial biological effects of propolis. Propolis shows antioxidant properties because of its free radical scavenging capacity, which aroused the need of conducting further researches about them in many areas.

In our previous study, we determined that ethanolic extract of Turkish propolis (EEP) (200 µg/mL) reduces the damage induced by t-BHP in fibroblast cells after 4 h recovery period. EEP reduce that the amount of intracellular ROS has not been shown any study revealed in fibroblast cells. The purpose of this study was to investigate in vitro antioxidant properties and also for the first time the protecting effect of Turkish propolis on t-BHP-induced oxidative stress in fibroblast cells was evaluated.

MATERIALS AND METHODS

Chemicals

All chemicals used for antioxidant activity analysis were analytical grade and purchased from Sigma (St. Louis, MO, USA). All chemicals used for cell culture studies were obtained from Lonza (Verviers, Belgium) and Biological Industries (Kibbutz Beit Haemek, Israel). 5-((and)-6)-chloromethyl-2',7'-dichlorodihydrofluoresce-indiacetate acetyl ester (CM-H$_2$DCFDA) was purchased from Invitrogen (Paisley, UK).

Preparation of EEP

First, 0.5 g of propolis powder, which has been harvested from Trabzon and its surrounding, was solved into 20 mL pure ethanol, after vortexing, they were held in incubation, at 60°C, 150 rpm in a mechanical shaker for 24 h. EEP was filtered by a filter of 0.2 µm and stored at -20°C until used.

Total Polyphenolic Content (TPC)

Content of total polyphenols were measured with Folin-Ciocalteu method adapted to microscale, as described by Slinkard and Singleton. The method of based on phosphotungstic acid reduction reaction in the basic solution. 12.5 µL EEP, 62.5 µL Folin-Ciocalteu reagent, and 125 µL 20% aqueous sodium carbonate were mixed. The final mixtures were incubated for 30 min at 25°C. The results were expressed as mg gallic acid equivalents (GAE)/g sample.

Total Flavonoid Content (TFC)

Content of total flavonoids were determined by colorimetric method in a 96-well microplate, as described by Chang et al. 20 µL EEP, 172 µL 80% ethanol, 4 µL 10% aqueous Al(NO$_3$)$_3$, 4 µL 1 M KCH$_3$COO were mixed and incubated for 40 min. The values were expressed as mg quercetin equivalents (QE)/g sample.

Reducing Power

The reducing antioxidant power were evaluated by spectrophotometric method. Briefly, 40 µL EEP, 100 µL 0.2 M phosphate buffer, 100 µL 1% K$_2$Fe(CN)$_6$ were mixed and incubated for 20 min at 50°C. Then, 100 µL 10% TCA was added to the tubes. Tubes were centrifuged at 3000 g for 10 min. 100 µL supernatants, 100 µL distilled water, 20 µL 0.1% FeCl$_3$ were mixed and incubated for 5 min. The results were expressed as mg trolox equivalents (TE)/g sample.

Determination of Radical Scavenging Activity

For the scavenging activity of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical by EEP, the method developed by Yu et al. was used. When treated by an antioxidant substance or substances, the intensity of the purple color originated from DPPH is reduced and absorbance values decrease. In this study, 0.1 mM ethanolic DPPH solution was used. EEP were prepared in different concentrations. Briefly, equal volumes (750 µL) of DPPH and sample solutions were mixed and incubated for 50 min. To compare the outcomes, a natural antioxidant ascorbic acid was used. Radical scavenging activity (RSA) of the samples and the standard were given as their percentage (%) to negative control (DPPH alone) and they were calculated using the Equation 1.

$$RSA(\%) = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

Determination of Metal Chelating Activity

For ferrous ions (Fe$^{2+}$) chelating activity of EEP, a modified version of the method developed by Dinis et al. was used. Metal chelating activity was assessed by comparing its ability to bind Fe$^{2+}$ ions in the solution with ferrozine.
Briefly, 50 μL EEP, 50 μL 0.2 mM FeCl$_2$ solution, 200 μL 5 mM ferrozine solution, and 1.7 mL distilled water were mixed and incubated for 10 min. EDTA, which is a good metal chelator, was selected as the standard. The pure solvent of the sample was used as the negative control. Iron binding capacity of the sample and the standards were given as their percentage (%) to negative control and control and they were calculated using the Equation 2.

\[
\text{Inhibition} \, (\%) = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right) \times 100
\]

**Cell Culture**

Human foreskin fibroblast cells (CRL-2522) were obtained from America Type Culture Collection (Manassas, VA). Cells were cultured in DMEM containing 10% FBS, 1% antibiotic solution in an incubator at 37°C.

**Spectrofluorometric Analysis of Intracellular Oxidative Stress with CM-H$_2$DCFDA**

5- (and-6) -chloromethyl-2',7'dichlorodihydrofluorescein diacetate, acetyl ester that was oxidized radicals was used to measure ROS generation occurred in our cell samples. 2',7'- dichlorodihydrofluorescein diacetate (DCFH-DA) is deacetylated by esterase and it is transformed into a dichlorofluorescein (2',7'-DCF) product that doesn’t have fluorescent property. DCFH is a probe that is transformed to DCF (dichlorofluorescein) by ROS in the media giving a strong fluorometric emission. Fluorescence intensity can be identified spectrofluorometrically.

5000 cells were plated in each well of 96 wells cell culture plate in 200 μL media. After 24 h, the media of the plate were removed and cells were treated with 200 μg/mL EEP, 15 mM deferoxamine mesylate (DFO), and 200 μM ascorbic acid for 1 h at 37°C in the new media. Next, the media of the plate was removed and new media was added to the plate. 300 μM t-BHP was added and incubated at 37°C for 1 h. The media of the plate was removed and new media was added to the wells and incubated at 37°C for 4 h, CM-H$_2$DCFDA dye was added to the plate, in a way to adjust the final concentration of the media to 10 μM, incubated at 37°C for 10 min. Fluorescence intensity was recorded at λ$_{\text{em}}$: 527 nm, λ$_{\text{ex}}$: 492 nm using a microplate reader fluorometer (Molecular Devices, Sunnyvale, CA). The results were given as relative fluorescence intensity according to negative control sample.

**Statistical Analysis**

All experiments were studied at triplicate. Descriptive statistical analysis was performed for all the studied variables. Data were given as mean±SD for normally distributed variables. ANOVA was used to compare parameters among groups and Tukey's test was performed for post-hoc comparisons. Statistical significance was set at p<0.05.

**RESULTS**

The value for the results of TPC, TFC, and reducing power of EEP are given in Table 1. Radical scavenging activities of EEP were studied through DPPH determination. % inhibition graphic of EEP which are radical scavenging activity are shown in Figure 1. The highest RSA of EEP was observed at the concentration of 125 μg/mL. Concentration % inhibition graphic showing metal chelating activity of EEP and EDTA were presented in Figure 2. The highest metal chelating activity of EEP was observed at the concentration of 25 mg/mL. Relative density percentage of ROS production is given in Table 2. At the end of 4 h treatment performed on fibroblast cells, which were damaged with 300 μM t-BHP, it was observed that 200 μg/mL EEP, 15 mM DFO, and 200 μM ascorbic acid significantly reduced the amount of generated ROS compared to the positive control (p values; 0.0001, 0.0001, and 0.001, respectively).

**DISCUSSION**

Many studies discuss the biological properties and the composition of propolis have raised the interest of the researchers towards this bee product. Also in our study, we used total propolis extract instead of isolating one of the flavonoids included in the propolis. Biological activity of propolis mainly ascribed to its content of flavonoids and various phenolic compounds. There are many methods for extraction of phenolic components from plant samples. Maceration is well known that the methods is used for extraction of the bioactive components. In this method different solvents (ethanol, methanol, DMSO, etc.) are used to extract the components.

<table>
<thead>
<tr>
<th>Table 1: Antioxidant properties of EEP.</th>
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<tr>
<td><strong>Total Phenolic Content</strong></td>
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<td>mg GAE/g sample</td>
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<td>Ethanolic Extract</td>
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from plant samples directly without compromising the structure. It is known that ethanolic propolis extracts are one of the richest sources of phenolic and flavonoid compounds. In this study, the ethanolic extract of Turkish propolis was prepared by the maceration methods. Various in vitro assay can be used to determine antioxidant activity of natural product extracts. In our study the values of TPC and TFC of EEP were found 114.7 mg GAE/g powder and 36.02 mg QE/g powder should be changed with GAE/g powder and 36.02 mg QE/g powder, respectively. Mihai et al. investigated TPC values of various propolis samples from Transylvania and these values were changed between 24.46 and 62.39 g standards mixture/100 g of propolis. In another study, Can et al. were analyzed of propolis from 15 different locations in Azerbaijan. TPC values of these samples was between 10.94 and 79.23 mg GAE/g propolis. TPC and TFC values of propolis extracts have been reported that as 174.7 mg GAE/g dry sample from China, between 150-197 mg GAE/g dry sample, 35.64-62.04 mg QE/g dry sample from Poland, and 31.2-299 mg GAE/g dry sample, 2.5-176 mg QE/g dry sample from other parts of the world, respectively. Reducing antioxidant potential was used to determine antioxidant activity of the propolis. It was found that reducing power values of EEP was 246.8 mg trolox equivalents. FRAP values of propolis have been reported at between 170-438 µM trolox/g dry sample from 15 different locations in Azerbaijan, between 0.74-2.54 mmol FeSO₄/g propolis from Transylvania.

Free radical scavenging activity of propolis was tested through DPPH, which is a stable radical. It is known that the solvents used for extraction are also effective in the radical scavenging activity. It was observed that EEP were quite effective, even in low concentrations, such as 125 µg/mL. Geckil et al. showed that radical scavenging activity of ethanolic propolis extracts are over 90% for the concentration of 200 µg/mL and over. Siripatrawan et al. evaluated that free radical scavenging activity of ethanolic propolis extracts are 94.2% for the concentration of 30 mg/mL. It’s clear that results of TPC, TFC, radical scavenging activity, and reducing power analysis were generally similar to those of other studies in literature.

It was found that metal chelating activity of EEP was around 90% for the concentration of 25 mg/mL and more. Metal chelating activity of different propolis extracts have been reported as 2 mg/mL. It has showed around 50% inhibition from Brazilian, 70% inhibition for the concentration of 2 mg/mL from Malaty, Turkey and 2 mg/mL has showed around 55.9% inhibition from Slovenian. Study of Miguel et al. reported that chelating Fe³⁺ ions activity of methanolic propolis extract has...
between 4.33-29.68% for the concentration of 100 µg/mL from Algarve, South Portugal. Our metal chelating activity results were different from the previous reports. This situation may arise from harvested region, type of solvents, and extraction methods.

Various chemicals, such as hydrogen peroxide (H₂O₂), t-BHP, methyl methanesulfonate, and ferrous sulfate are used in cells to generate ROS in vitro. t-BHP was selected as the chemical to be used for generating ROS. To measure the production of ROS in cells, CM-H₂DCFDA was used. The ROS levels were significantly reduced with EEP pre-treatment compared to positive control (p=0.0001). DFO and ascorbic acid were used in ROS studies as controls due to their scavenging and metal chelating activities. ROS amounts were significantly reduced with both DFO and ascorbic acid compared to positive control. In the literature, there are many studies showing that both extract of natural compounds and single polyphenolic compounds decrease intracellular ROS amount and protect biomolecules from oxidative stress. Sharon et al. reported that quercetin and rutin prevent t-BHP induced oxidative damage in Caco-2 cells. Kang et al. demonstrated that caffeic acid prevents H₂O₂-induced damage in WI-38 cells due to decreasing intracellular ROS and increasing catalase activity with its radical scavenging activity. Yen et al. reported that ascorbic and gallic acid prevents H₂O₂-induced oxidative damage in lymphocytes through decreasing DNA damage with their radical scavenging activity. Wang et al. reported that caffeic acid phenethyl ester (CAPE) and its derivatives prevent endothelial cells against menadione-induced oxidative damage. In another study, Song et al. demonstrated that CAPE prevent H₂O₂-induced oxidative stress in human middle ear epithelial cells due to decreasing intracellular ROS. Dhanya et al. found that quercetin prevents t-BHP induced oxidative stress in L6 myoblasts, while Lee et al. demonstrated that CAPE protects human liver (HepG2) cells against t-BHP induced oxidative stress through decreasing amount of intracellular ROS.

Mentioned above in literature showed that reduction intracellular ROS in different cell lines both by each extract components of propolis and total propolis extract were reported and similar results was found for EEP effect in this particular study. The reduction in intracellular ROS amount by propolis extracts may be because of the flavonoids and phenolics that it contains, which is unique to the region. Polyphenols show their antimutagenic and anticarcinogenic effects not only that by preventing the spread of free radicals, but also by chelating the transition metals in the environment and inhibiting radical generating reactions, therefore by preventing radical generation.

**CONCLUSION**

Consequently, our results demonstrated that EEP reduced the amount of intracellular ROS due to its phenolic content, radical scavenging, and metal chelating activity. Thus, EEP might be evaluated a novel potential antioxidant source and a therapeutic agent for drug industries.

**ACKNOWLEDGEMENT**

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**CONFLICTS OF INTEREST**

The authors declare that there are no conflict of interest.

**ABBREVIATIONS USED**

ROS: Reactive Oxygen Species; EEP: Turkish Propolis Ethanolic Extract; DPPH: 2, 2-diphenyl-1-picrylhydrazyl; CM-H₂DCFDA: 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester; TPC: Total Phenolic Content; TFC: Total Flavonoid Content; FRAP: Ferric Reducing Antioxidant Potential.

**REFERENCES**

Our results suggest that EEP may be a novel candidate for the development of new natural product based therapeutic agents.

SUMMARY

- Propolis is a natural resinous material with antioxidative, antitumoral, antimutagenic, and other beneficial properties.
- EEP has good antioxidant properties, rich in polyphenolic and flavonoids compounds.
- EEP reduced the amount of intracellular ROS on t-BHP induced oxidative stress in foreskin fibroblast cells.
- Our results suggest that EEP may be a novel candidate for the development of new natural product based therapeutic agents.
Misir et al.: The antioxidant activity of Turkish propolis

PICTORIAL ABSTRACT

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