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Investigation of Inhibition Effects of Honey, Pollen, Propolis and Royal Jelly Extracts on Thioredoxin Reductase Enzyme Activity

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ABSTRACT

The thioredoxin reductase enzyme is an enzyme that prevents the mechanism of apoptosis from working and thus triggers the formation of cancer. Therefore, the inhibition of the thioredoxin reductase enzyme is thought to prevent or inhibit cancer. In this study, the effects of extracts of plateau honey, pine honey, chestnut honey, mad or wild honey, pollen, propolis and royal jelly on thioredoxin reductase enzyme activity were investigated. Enzyme activities were measured at constant substrate and different inhibitor concentrations to calculate IC_{50} values. Total antioxidant activity were investigated in order to compare the extracts used in the inhibition study. The strongest inhibitory effect was seen in the pollen methanol extract (IC_{50} = 2.44µg /mL)

Keywords: cancer, bee products, thioredoxin reductase

1. INTRODUCTION

In today's world, it is commonly recognized that cancer is one of the biggest global public health issues that we face. The high cost of drugs used in cancer treatment and the significant side effects of these drugs have been the driving force behind the search for new drugs, the results of which have led to many candidate drugs being synthesized. In order to evaluate the synthesized substances as medicines, these substances must undergo many long, demanding tests. Data from 2012 show that there were 14.1 million cancer cases worldwide. This number is expected to rise to 19.3 million by 2025 [1].

Thioredoxin redutase (TrxR; EC 1.6.4.5), which is a member of the Flavo Enzyme class and has a homodimeric structure, catalyzes the reduction of thioredoxin. Thioredoxin is known to be present in all organisms [2]. The TrxR/Trx system also plays a functional role in the construction of deoxyribonucleotides, where Trx protein acts as an electron provider [3], [4]. TrxR plays an important role in DNA synthesis, redox signaling, antioxidant defense, selenium metabolism and regulation of apoptosis [2]. Owing to these stated functions, thioredoxin reductase has become the focus of researchers. AIDS, cancer and autoimmune disease-related studies have shown that TrxR may be associated with many human diseases [4]. TrxR is the target enzyme in cancer research, particularly because of its relationship with apoptosis [3], [5], [6].

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Honey, pollen, propolis and royal jelly, all of which are recognized as major bee (Apis mellifera) products, have many phenolic compounds [7]. Throughout human history, bee products, in addition to serving as a source of food, have been used for therapeutic purposes, including the treatment of burns, gastrointestinal disorders, asthma, infected wounds, and skin ulcers [7-9]. The therapeutic effects of bee products continue to be investigated to this day. As a source of food, bee products have been reported to contain about 150–200 ppm compounds, such as polyphenols (phenolic acids, flavonoids, and their derivatives), terpenes, steroids, and amino acids [7], [8], [10], [11]. The content of these products depends on many factors, like plant type, climate and environmental conditions [11].

The main reason for the different colors, tastes and compositions of the honeys is that they are obtained from different botanical [11]. Four distinct types of honey (three floral type and one secretion type) were used in our study: plateau honey, a multiflora honey, which has a sharp taste and is very nutritious [12]; chestnut honey, which is made from the nectar or pollen of a chestnut flower and has a dark brown color and a bitter taste [13]; “mad” or "wild" honey, which it is known as locally, is obtained from Rhododendron ponticum, an endemic species that grows only in the Black Sea region (this honey contains grayanotoxins, which are polyhydroxylated cyclic diterpenes possessing structures, and it has been reported that excessive consumption of mad honey causes hypotension, bradycardia, and vertigo [14]; and pine honey, which is produced from the secretion of a bug known as the "Basra bug", or "Marchalina hellenica", instead of flower nectar [15].

Propolis is a resinous substance with a dark brown color and is produced to protect hives from bacterial/fungal infections from bees. In addition, propolis is used for cleaning and sealing the hives. Propolis has antibacterial, antiviral, antioxidant, anticancer, and anti-inflammatory activities [16], [17]. Pollen is the reproductive cells of plants. Bees consume pollen as part of their diet and use it to feed the larvae [15]. Royal jelly is a natural product produced by the main worker bees for the purpose of feeding the bee embryos. Royal jelly is a very rich product that includes protein, carbohydrates, various fatty acids (short chain) and mineral substances [18].

Bee products are known to have anticancer properties. However, the mechanism governing its anticancer activities remains unclear. Taking into account the complex nature of cancer, in this study, we aimed to determine which of the bee products studied has better effectiveness on thioredoxin reductase enzyme activity and the impact of the products on cancer mechanisms.

2. MATERIALS AND METHODS

2.1. Chemicals and instruments

Analytical grade solvents (methanol, ethanol, and dimethyl sulfoxide (DMSO)), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), nicotinamide adenine dinucleotide zhosphate (NADPH), and other reagents were obtained from Sigma-Aldrich (Milan, Italy). Thioredoxin reductase recombinant from rat liver was also obtained from Sigma-Aldrich (Milan, Italy). Honeys, pollen, royal jelly and propolis were procured commercially on the Turkish market. In this study, Evolution 201 UV-Visible Spectrophotometer (Thermo Scientific) was used in kinetic study.

2.2. Samples and preparation of extracts

A modification of the procedure described by Şahin et al. was used to perform extractions [19]. Approximately 5 g of the samples were placed in 100 mL of solvent (methanol, ethanol, DMSO and water) medium. Each sample was then stirred at room temperature for 24 hours using a shaker. The suspension was centrifuged at 10,000g for 15 min. The resulting supernatant (methanol and ethanol) was concentrated in a rotary evaporator under reduced pressure. The other supernatant (DMSO and water) was concentrated in a lyophilizator (-52ºC and 0,132 bar). The obtained residue was dissolved in a very small amount of the same solvent and held at 4°C until used.

2.1. Thioredoxin reductase catalytic activity and inhibition

The DTNB method was used to measure the activity of the thioredoxin reductase enzyme. This method catalyzes the reduction of disulfide bonds in DTNB by the NADPH-dependent thioredoxin reductase enzyme [20]. In a 1.0 ml reaction mix, the final concentrations were 20 mM potassium phosphate, 2 mM ethylene diamine tetra acetic acid, 0.02 mM b-nicotinamide adenine dinucleotide phosphate, reduced form, 0.02% (w/v) bovine serum albumin, 0.1% ethanol, 0.5

mM 5,5'-dithio-bis(2- nitrobenzoic acid) DTNB, and 0.15 unit thioredoxin reductase. Concentrations were immediately mixed by inversion, and the increase was recorded in a Spectrophotometer A412nm for approximately 3 minutes. One unit is based on the determination of TNB oxidation per minute. Enzyme activities were measured at constant substrate and different inhibitor concentrations to find IC_{50} value. In preliminary experiments, DMSO, ethanol, and methanol were found to have no significant inhibition effect on ThxR. The tube not containing inhibitor was used as control and its activity was considered as 100%. Each experiment was repeated 3 times. Activity-% [Inhibitor] plots were drawn for inhibitors.

2.4. Determination of total phenolic content

The content of total polyphenols was estimated according to the Folin-Ciocalteu method using gallic acid as a reference standard [21]. Using a standard graph, total phenolic content was expressed as mg of gallic acid equivalents per g of extract.

3. RESULT

Solvents with different polarities (methanol, ethanol, DMSO and water) were used to determine the differences created by the solvent environment. In methanol, the solubility of plateau honey, chestnut honey, pine honey, mad honey, pollen, propolis and royal jelly were determined to be 0.2909, 0.3071, 0.4188, 0.3036, 0.1797, 0.3643 and 0.1084 g/ml, respectively (Table 1); in ethanol, the solubility of plateau honey, chestnut honey, pine honey, mad honey, pollen, propolis and royal jelly were found to be 0.0263, 0.0313, 0.1289, 0.0240, 0.0445, 0.4104 and 0.0065 g/ml, respectively (Table 1); and in DMSO, the solubility of plateau honey, chestnut honey, pine honey, pollen, and royal jelly were calculated as 0.4582, 0.4708, 0.4580, 0.2064, and 0.0881 g/ml, respectively (Table 1). DMSO (mad honey and propolis) was not studied because it could not be evaporated in the last stage of extraction. Finally, in water, the solubility of plateau honey, chestnut honey, pine honey, mad honey, pollen, propolis and royal jelly were found to be 0.3668, 0.3782, 0.3395, 0.3400, 0.2025, 0,0152 and 0.0109 g/ml, respectively (Table 1).

Table 1. Inhibitory effects of bee products extracts against TrxR and the solubility

Samples	Solvent	Concentration of extracted sample (g/mL)	ThxR	
			IC_{50} (mg/mL)	R^2
Mad Honey	Ethanol	0,1289	5,350	0.8917
	Methanol	0,4188	4,260	0.9560
	DMSO	-	-	-
	Water	0,3395	Not inh	Not inh
Plateau Honey	Ethanol	0,0263	0,862	0.9140
	Methanol	0,2909	2,500	0.9752
	DMSO	0,4582	4,030	0.9766
	Water	0,3668	0,191	0.9729
Pine Honey	Ethanol	0,0240	Not inh	Not inh
	Methanol	0,3036	Not inh	Not inh
	DMSO	0,4580	36,58	0.9010
	Water	0,3400	Not inh	Not inh
Chestnut Honey	Ethanol	0,0313	2,768	0.9249
	Methanol	0,3071	151,1	0.8862
	DMSO	0,4708	4,200	0.9731
	Water	0,3782	25,24	0.9448
Pollen	Ethanol	0,0445	0,056	0.8260
	Methanol	0,1797	0,024	0.9726
	DMSO	0,2064	2,14	0.9608
	Water	0,2025	*	*
Propolis	Ethanol	0,4104	0,400	0.9832
	Methanol	0,3643	0,068	0.8534
	DMSO	-	-	-
	Water	0,0152	*	*
Royal Jelly	Ethanol	0,0065	0,060	0.9134
	Methanol	0,1084	3,204	0.9496
	DMSO	0,0881	10,69	0.9382
	Water	0,0109	*	*

-The solvent could not be evaporated
*Could not be determined

Enzyme activities were measured at constant substrate and different inhibitor concentrations to find IC_{50} value. The tube not containing inhibitor was used as control and its activity was considered as 100%. Activity-% [inhibitor] plots were drawn for inhibitors. According to the resulting plots, the IC_{50} values for each plateau honey extract (water, ethanol, methanol and DMSO) were 0.191, 0.862, 2.500 and 4.030 mg/mL for TrxR, respectively (Table 1, Figure 1); the IC_{50} values of each chestnut honey extract (water, ethanol, methanol and DMSO) were 25.24, 2.768, 151.1 and 4.200 mg/mL for TrxR, respectively (Table 1, Figure 1); the IC_{50} values of each mad honey extract (ethanol and methanol) were 5.350 and 4.260 mg/mL for TrxR, respectively (Table 1, Figure 1); the IC_{50} values of each pine honey extract (DMSO) were 36.58 mg/mL for TrxR (Table 1, Figure 1).

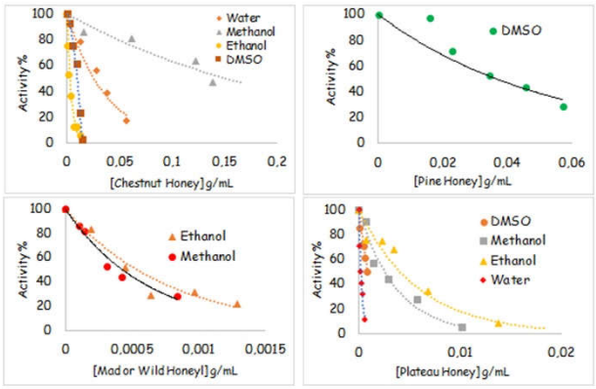

Figure 1. Inhibitory effects of honey extracts against TrxR

According to the resulting plots, the IC_{50} values of each pollen extract (ethanol, methanol and DMSO) were 0.056, 0.024 and 2.14 mg/mL for TrxR, respectively (Table 1, Figure 2); the IC_{50} values of each propolis extract (ethanol and methanol) were 0.400 and 0.068 mg/mL for TrxR, respectively (Table 1, Figure 2); and finally, the IC_{50} values of each royal jelly extract (ethanol, methanol and DMSO) were 0.060, 3.204 and 10.69 mg/mL for TrxR, respectively (Table 1, Figure 2).

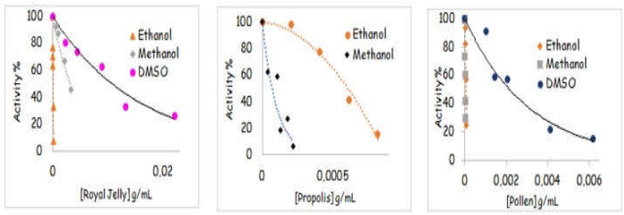

Figure 2. Inhibitory effects of bee products extracts against TrxR

In the investigation of different solvent extractions (methanol, ethanol, water, and DMSO) of plateau honey, chestnut honey, pine honey, pollen, royal jelly, and in the measurement of their total phenolic contents (Table 2), it was observed that for the honey samples, the total polyphenol content was between 28,067 and 116,91 mg GAE/g, whereas for the pollen (ethanol and DMSO) and royal jelly (ethanol, methanol and DMSO) the amounts were respectively between 471,567–1.151,0 GAE/g and between 0.060–10.69 GAE/g (Table 2). With the method that we applied, the total phenolic contents of pollen methanol and water extracts and all extracts of propolis were not able to be determined due to the occurrence of settling in the tube.

Table 2. Phenolic content of bee products

Samples	Solvent	Total polyphenol mg GAE/g sample
Mad Honey	Ethanol	31,733
	Methanol	54,733
	DMSO	-
	Water	48,900
Plateau Honey	Ethanol	28,067
	Methanol	68,733
	DMSO	84,400
	Water	53,233
Pine Honey	Ethanol	31,067
	Methanol	81,233
	DMSO	80,733
	Water	86,733
Chestnut Honey	Ethanol	30,733
	Methanol	85,900
	DMSO	116,91
	Water	87,233
Pollen	Ethanol	471,567
	Methanol	*
	DMSO	1.151,0
	Water	*
Propolis	Ethanol	*
	Methanol	*
	DMSO	-
	Water	*
Royal Jelly	Ethanol	48,233
	Methanol	81,567
	DMSO	113,40
	Water	*

-The solvent could not be evaporated/
*Could not be determined

4. DISCUSSION

Studies have shown that the human TrxR system is associated with cancer cell proliferation and anti-apoptosis processes [2], [22]. The thioredoxin system regulates the redox state of Trx (thioredoxin) and the transfer of apoptosis signals. For example, reduced Trx binds apoptosis signaling kinase-1 (ASK1) and stops apoptosis. Oxide Trx, however, cannot do that [22], [23]. TrxR is a unique enzyme present in all living cells, yet in tumor cells, the TrxR level is 10 times greater than that seen in normal cells, indicating that the active thioredoxin system is effective in proliferating tumor cells. Therefore, this protein has been proposed as a target for cancer treatment. In a study conducted for this purpose, it was reported that the interaction of TrxR1 with electrophiles results in two important biological consequences, namely p53 conformational degradation and apoptosis induction [22], [24],

[25]. There are many cancer drugs that inhibit TrxR [3], and many new drug candidates are regularly being synthesized. In order to evaluate the synthesized substances as medicines, these substances must undergo many long, demanding tests. As a result, many researchers have turned to natural products. For this reason, we preferred to focus on bee products in our work.

Many studies have reported that propolis, pollen, royal jelly and honey contain a variety of phenolic acids and flavonoids, which have a wide range of biological effects, including antioxidant, antibacterial, anti-inflammatory, and anticancer activities [16], [18], [26], [27], [28] . As mentioned earlier, bee products have been shown to elicit anti-cancer activity, yet precisely how this is done is unclear. Our study has aimed to identify which of the bee products has the best inhibitory activity on ThxR and to offer insight into how bee products prevent cancer. When we look at Table 1, it can be seen that plateau honey with an IC_{50} value of 0.191-4.030 mg/mL showed the best inhibition properties among all the honeys. In other words, multiflora honey has a better inhibitory effect on TrxR enzyme activity than uniflora honey. Looking again at Table 1, the floral honey can be seen to cause better inhibition than the secretion honey.

When examining the inhibition effects of pollen, propolis and royal jelly on thioredoxin reductase enzyme activities, pollen methanol extract was found to have the strongest inhibitory effect (Table 1). Overall, it was found that out of all the bee products, pollen, when included in the honey, had the strongest inhibitory effect. In terms of the total phenolic content (Table 1), pollen had the highest. We suspect that the inhibitory effect on the enzyme is high due to the phenolic content of its structure. With these conclusions we have determined that pollen, out of all the bee products, may be more effective on cancer and would be beneficial for cancer patients to use under the guidance of a doctor.

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