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Phenolic composition, antimicrobial and antioxidant activity of *Castanea sativa* Mill. pollen grains from Black Sea region of Turkey

*Avşar, C., Özler, H., Berber, İ. and Civek, S.

Department of Biology, Faculty of Science and Arts, Sinop University, Sinop, 57000, Turkey

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Abstract

Sinop is one of the chestnut growing regions of Turkey. The pollen of *Castanea sativa* is consisting of the main source of honeybee. In the present study were investigated to phenolic composition, antioxidant and antimicrobial efficiency of methanolic extracts of *C. sativa* pollen grains collected from 9 different populations during inflorescence period (July, 2013). The extracts were screened for their antimicrobial activities against to the 8 bacteria and 3 yeast species by using disc-diffusion procedure. The pollen extracts displayed more effective against to Gram-positive bacteria rather than Gram-negative, the extracts had also moderate anti-yeast activity. *In vitro* the antioxidant activity based on the 1,1-diphenly-2-picryl hydrazyl (DPPH) free radical was evaluated for all the extracts, and it was found that the extracts had good antioxidant activity in the range of the IC_{50}= 0.093-19.5 mg mL^{-1}. The total phenolic contents of the extracts were determined between 64.02 ± 0.26 mg GAE g^{-1} and 103.8 ± 6.72 mg GAE g^{-1}. In addition, we determined a negative linear correlation (r= - 0.59) between the total phenolic content and IC_{50} values. In this sense, our findings confirmed that the methanolic pollen extracts had good antioxidant activity. In conclusion, the result of the study suggested that *Castanea sativa* Mill. pollen grains can be a good natural product source of antioxidant and antimicrobial agents for potential health benefits.

Introduction

Castanea sativa Mill. is the only natural species in Europe and Turkey. This species has a distribution from East Black Sea to Mediterranean region (Ketenoğlu *et al*., 2010). The Aegean, Black Sea and Marmara are the leading chestnut-growing regions of Turkey (Ertan *et al*., 2007). It is a monoecious or deciduous tree and pollinated by bees or wind. Sinop province in Turkey is the one of the chestnut production. This species has economical important both edible fruits and good quality timber. The pollen grains of *C. sativa* also constitute content of honeys produced in this region.

Pollen is recognized as a folk medicine in China and Germany because of having several valuable phytochemicals such as carotenoids, steroids, terpenoids and flavonoids (Kao *et al.*, 2011). Phenolic compounds especially flavonoids display a wide range of biological effects including antioxidant activity, antiviral, anti-inflammatory, antiallergic, antithrombotic, vasodilatory actions and the ability to lower the risk of coronary heart diseases. Antioxidative activity of phenolic compounds is base on the principle giving hydrogen atoms to free radicals (Hafidh *et al*., 2009). According to Almaraz-Abarca *et al.* (2007), antioxidant capacity of pollen arises from phenolics and flavonoids. It is known that chestnut honey was effective against to β-hemolytic *Streptococcus* (Sorkun, 2008). Hand collected pollen is varying differences than compared to be collected from the same plant. This changes stems from occurring in partly the bee pollen and due to contamination with nectar and honey. Pollen stored in the beehive undergoes lactic acid fermentation (Johri and Vasil, 1961).

In recently years, many researchers have been focused on antimicrobial, antioxidant activities and phenolic contents of honey and bee pollen due to their good biological efficacy (Kolonkaya, 2001; Hegazi *et al*., 2001, 2002; Almaraz-Abarca *et al.*, 2004; Mercan *et al*., 2007; Çam *et al*., 2010; Graikou *et al*., 2011; Kao *et al*., 2011; Freire *et al.,* 2012; Basuny *et al.*, 2013; Fatrcová-Šramková *et al*., 2013; Salgajova *et al*., 2014). According to our knowledge in this field, there has been no enough data related to biological activity of chestnut pollen grain in literature. The aim of the present study was to determinate to the antimicrobial, the antioxidant activities and total phenolic composition of *C. sativa* pollen taken from 9 sites of Sinop province in Black Sea region of Turkey.

*Corresponding author.
Email: *cumhur.avsar@gmail.com*

Materials and Methods

Collection of plant samples and preparation of pollen extracts

Mature pollen grains were collected in stage of inflorescence (July) from 9 populations growing in Erfelek (4) and Ayancık (5) district of Sinop in 2013 (Table 1). Castanea inflorescences containing dehiscent anthers were vigorously shaken in white paper bags. Pollen grains were separated from debris by sieving the material through an appropriate mesh. Samples were stored at ±4°C until analysis.

To prepare pollen extracts about 10 g of pollen grains was extracted by 100 mL of methanol for 24 h in a shaking water bath. The combined extracts were filtered and concentrated under vacuum to obtain a crude extract.

Microorganisms

The test organisms 6 Gram-positive bacteria *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 51299, *Micrococcus luteus, Bacillus cereus* 7064, Vancomycin Resistant *Enterococcus* (VRE), Methicillin Resistant *Staphylococcus aureus* (MRSA); 2 Gram-negative bacteria *Escherichia coli* ATCC 11293, *Klebsiella pneumonia* and 3 yeast species *Candida albicans* ATCC 14053, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 collected as pure cultures from the Molecular Biology and Microbiology Laboratory, Department of Biology, Faculty of Arts and Science, Sinop University, Turkey. All the microorganisms were maintained at -70°C in Nutrient Agar (NA) for bacteria and Sabouraud Dextrose Agar (SDA) for fungus (Difco) containing 17% (v/v) glycerol.

Antimicrobial activity assay

The antimicrobial activity of the methanol extracts of each sample was evaluated by using disc diffusion method (Bauer *et al*., 1966). All the microorganisms were maintained at -20oC in Nutrient Agar (NA) for bacteria and Sabouraud Dextrose Agar (SDA) for fungus (Difco) containing 17% (v/v) glycerol. Before testing, the microorganisms were transferred to Nutrient Broth (NB) for bacteria and Sabouraud Dextrose Broth (SDB) for fungus (Difco) and cultured overnight at 37°C. Then, the turbidity was adjusted equivalent to 0.5 McFarland standards. Then, 100 µl of microorganisms was spread over the surface of an agar plate. The filter paper discs (6 mm) were loaded with methanol extracts (3 mg/disc) and were allowed to dry completely. Then, it was placed on the surface of the freshly inoculated medium. The media were incubated for 24 h at 37°C.

Table 1. Pollen grains and their stations

Stations	Symbol	Number
Ayancık (Abdülkadir Village)	A1	1
Ayancık (Gökçeber Village)	A2	2
Ayancık (Kirazlık Village)	A3	3
Ayancık (Sakarya Başı)	A4	4
Ayancık (Şerifiye Village)	A5	5
Erfelek (Bezirgen – Salı Village)	E1	6
Erfelek (Güven Village)	E2	7
Erfelek (Güven Village – Sökü Neighborhood)	E3	8
Erfelek (Soğucak Village)	E4	9

Antibiotic susceptibility discs including bacitracin (0.04 U), ceftazidime (30 µg), imipenem (10 µg), novobiocin (5 µg), polymyxin B (300 U), tetracycline (30 µg), ampicillin (10 µg) and cycloheximide were used as control, and negative control was to 12.5% DMSO. The antimicrobial activity was evaluated by measuring the diameter of inhibition zone.

In vitro *free radical scavenging activity assay*

The antioxidant potential (free radical scavenging activity) of the extracts on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by Blois (Blois, 1958) and Kumar *et al*. (2011) method. The pollen grains were dissolved as 1000 µg/mL in ethanol. The solution at different concentrations such as 1000, 500, 250, 125 and 62.5 µg/mL was obtained using serial dilution technique. 1 mL of an ethanol solution of the extract of each concentration was mixed with 4 mL of a DPPH-ethanol solution (0.1 mM). These samples were shaken well and kept in dark for 30 min at room temperature. The absorbance was measured at 517 nm. The scavenging activity on the DPPH radical was calculated by using the following equation:

$$\% \text{ inhibition} = [(A_B - A_S) / A_B] \times 100$$

The A_B is the absorbance of the control reaction and A_S is the absorbance of the test compound in this equation. Ascorbic acid was used as a standard or positive control. Not contained compound/standard was used as the negative control. Scavenging activity was expressed as IC_{50}, which represent the concentration of the extract (mg mL^{-1}) required to inhibit 50% of the free radical scavenging activity.

Determination of total phenolic composition

The total phenolic content of the 9 methanolic extracts (1000 µg/mL) of the pollen grains were estimated by the Folin-Ciocalteu method (Taga *et al.*, 1984). One hundred microliters of diluted sample was added to 2 mL of 2% Na_2CO_3 reagent.

After 2 min room temperature incubation, 100 µl of 50% Folin-Ciocalteu reagent was added. After 30 min of incubation at room temperature in the dark, the absorbance at 720 nm using spectrophotometer (Thermo Scientific, Helios-Alpha) was measured. Gallic acid (0.05 to 1 mg/mL) was used for the standard calibration curve. The results were expressed as Gallic acid equivalent (GAE)/g dry weight of extracts.

Statistical analysis

All the experiments were carried out in triplicates and values were expressed as mean ± standard deviation (SD). Graphics were made using MS Office Excel 2007. In addition, extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentration in Excel 2007. Pearson's correlation efficient was calculated using Excel 2007.

Results

Antioxidant activity

The percentage values of DPPH radical scavenging activity of methanolic extracts of pollen grains are shown in Figure 1. The IC_{50} values of the extracts are given in Figure 2. The IC_{50} values of the pollen extracts were found between 0.093 ± 0.06 mg/mL and 19.5 ± 2.05 mg/mL, and ascorbic acid (control) was determined as 0.74 ± 0.05 mg/mL.

According to the experimental data in terms of IC_{50}, the extract marked by symbol E1 showed strong antioxidant activity (IC_{50}, 0.093 ± 0.06 mg/mL) as compared to control and other pollen extracts. In addition, the pollen extracts shown with symbol A2, A3 and E4 presented moderate activities (Figure 2).

Antimicrobial activity

Antimicrobial activities of the methanolic pollen extracts against 8 bacteria and 3 yeasts by using disc diffusion method are shown in Table 2. According as antimicrobial assays, each of the tested pollen extracts inhibited at the different levels the growth of *B. cereus* ATCC 7064, *M. luteus,* MRSA, *S. aureus*, *C. krusei, C. albicans* and *C. parapsilosis*. On the other hand, it was determined that the pollen extracts had weak antibacterial efficiency against Gram-negative bacterial strains (*E. coli* ATCC 11293, *Enterococcus faecalis* ATCC 51299 and VRE), except for *K. pneumonia* (no activity). Our findings also showed that the E1 extract had activity only against to VRE (9 ± 0.2), while other pollen extracts were not antibacterial efficiency. Generally, the pollen extracts were more effective against Gram-positive bacteria rather than Gram-negative and yeast strains (Table 2). Additionally, we determined that some pollen extracts had higher level antimicrobial effective than certain commercial standard drugs (Table 2).

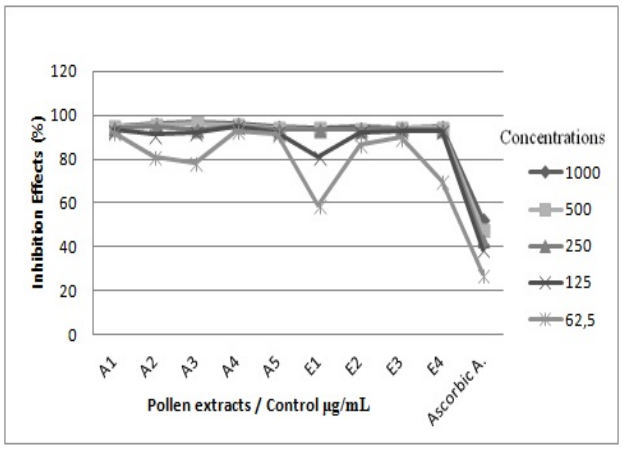

Figure 1. DPPH free radicals scavenging activity of the pollen extracts and control (ascorbic acid)

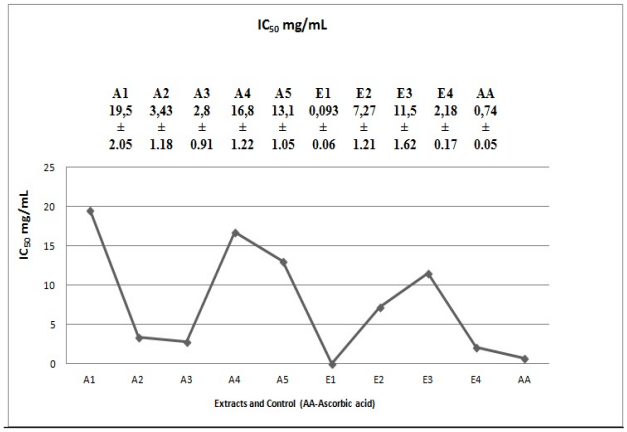

Figure 2. IC_{50} values of methanolic extracts of pollen grains

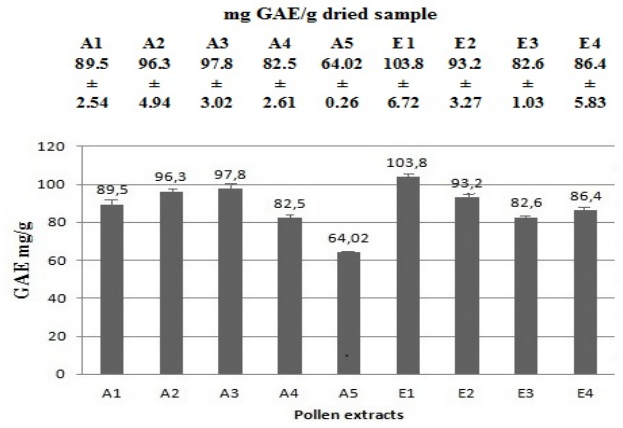

Figure 3. Total phenolic contents of *C. sativa* pollen grains collected from 9 different populations (mg GAE/g)

Total phenolic content

The total phenolic content of the methanol extracts were measured according to Folin-Ciocalteu

Table 2. Antimicrobial activities of pollen grain extracts against tested microorganisms by using disc diffusion method

Plant extracts	Inhibition zone diameters(mm) against different microorganisms										
	C. krusei ATCC 6258	*C. albicans* ATCC 14053	*C.parapsilosis* ATCC 22019	*B. cereus* ATCC 7064	*E. coli* ATCC 11293	*E. feacalis* ATCC 51299	*K. pneamonia* ESBL (+)	MRSA	*M.luteus*	*S.aureus* ATCC 6538	VRE
A1	-	10 ± 0.1	-	9 ± 0.3	-	13 ± 0.3	-	21 ± 0.8	20 ± 0.7	19 ± 0.5	-
A2	12 ± 0.5	-	-	-	9 ± 0.0	-	-	18 ± 0.4	17 ± 0.2	14 ± 0.1	-
A3	10 ± 0.4	-	-	-	9 ± 0.1	-	-	20 ± 0.2	18 ± 0.4	13 ± 0.05	-
A4	-	-	12 ± 0.5	9 ± 0.1	-	9 ± 0.2	-	21 ± 0.6	16 ± 0.3	17 ± 0.6	-
A5	-	-	-	-	9 ± 0.2	-	-	21 ± 0.5	20 ± 1.2	16 ± 0.2	-
E1	15 ± 0.8	-	18 ± 1.1	10 ± 0.5	-	-	-	23 ± 1.1	16 ± 0.09	20 ± 0.3	9 ± 0.2
E2	12 ± 0.0	15 ± 1.1	-	14 ± 0.5	9 ± 0.0	-	-	22 ± 0.2	22 ± 1.3	22 ± 1.2	-
E3	12 ± 0.1	-	-	-	-	-	-	21 ± 0.6	19 ± 0.2	19 ± 0.8	-
E4	18 ± 0.7	11 ± 0.2	12 ± 0.1	10 ± 0.7	-	-	-	21 ± 0.5	15 ± 0.1	18 ± 0.1	-
DMSO	-		-	-	-	-	-	-	-	-	-
Bac	*		*		-	-	-	-		-	-
Nov	*		*	10 ± 0.1	-	15 ± 0.2	9 ± 0.1	24 ± 0.7		29 ± 0.4	10 ± 0.5
Tet	*		*		26 ± 1.3	23 ± 0.5	24 ± 0.6	10 ± 0.2		40 ± 1.4	13 ± 0.9
Amp	*		*		-	35 ± 1.2	-	16 ± 0.5		42 ± 1.6	24 ± 0.5
Imp	*		*		28 ± 0.5	34 ± 1.2	26 ± 0.7	50 ± 2.3		50 ± 1.6	30 ± 1.3
Poly B	*		*		11 ± 0.4	-	15 ± 0.5	11 ± 0.2		11 ± 0.1	-
Cef	*		*		19 ± 0.2	20 ± 1.1	19 ± 0.4	23 ± 0.8		25 ± 0.6	8 ± 0.1
Cyc	43 ± 1.2		40 ± 1.1	*	*	*	*	*	*	*	*

(-) not effect, (*) not teste

method. TPC of the extracts were calculated from the regression equation of calibration curve (y= 0.0018x + 0.0159; R^2=0.9996) and expressed as mg GAE/g in dried weight. The results are summarized in Figure 3. The analysis of the total phenolic composition showed that the amounts of total phenolic of the pollen extracts were between 64.02 ± 0.26 mg GAE/g and 103.8 ± 6.72 mg GAE/g. The highest levels of the phenolic content was found in the E1 plant extract with value 103.8 ± 6.72 mg GAE/g dried sample. It was found that the there was a negative correlation (r= -0.59) between the total phenolic content and IC_{50} values. In this sense, our findings indicated that the methanolic extracts of the pollen grain had good antioxidant activity.

Discussion

A number of investigations suggested that natural phytochemical compounds isolated from some plants have been used to treating adverse effects of several bacterial, fungal and viral infectious because of high level of the antimicrobial and antioxidant properties (Kumazawa *et al*., 2004; Almeida-Muradian *et al.*, 2005; Basuny *et al*., 2013).

In the present work were evaluated the antioxidant property, antimicrobial activity and total phenolic contents of *Castanea sativa* Mill. pollen extracts. The samples were collected from 9 different points in Erfelek and Ayancık regions of Sinop province (Table 1). The results of the antimicrobial assay showed all of the extracts had high antibacterial activity especially against to *M. luteus*, MRSA and *S. aureus*. They were also effective moderate against to yeast. Although there was no effect of the extracts against to *K. pneumonia*, there was low against to *E. coli.*

Barbosa *et al*. (2006) reported that the *Cistus ladanifer, Rubus* sp. and *Castanea sativa* pollen lipophilic extracts had higher antibacterial activity against to *Paenibacillus larvae, B. subtilis* and *B. cereus,* but the extracts not exhibited activity against to *E. coli* and *Pseudomonas aeruginosa*. Our findings demonstrated that Gram-negative bacteria were more resistant to the pollen extract than Gram-positive bacteria and yeasts because of the differences in their cell wall structure. Because lipopolysaccharide (LPS) layer of Gram-negative bacteria in outer membrane have a strong permeability barrier to many phytochemical substances. Nevertheless, the some extracts showed slightly antibacterial activity against *E. coli* (Table 2). In a study done by Basim *et al.* (2006) reported that Turkish bee-pollen extracts had a strong antibacterial effect against plant pathogenic bacteria. According to Erkmen and Ozcan (2008), pollen extracts were very weaker against food-related microorganism. Graikou *et al.* (2011) explained that Greek bee-pollen extracts had antimicrobial and antioxidant effect. Our findings are good agreement with previous studies.

Freire *et al.* (2012) stated that the Brazil honeybee-pollen samples analyzed in their study showed to have considerable antioxidant activity, and they suggest that phenolic compounds contributed directly to antioxidant effect. Ulusoy and Kolaylı (2014) reported that total phenol contents of 13 Anzer pollens were range from 44.07 to 124.10 mg/g, and a correlation was found between phenolic contents and DPPH assay (r= -0.778). Morais *et al.* (2011) stated that total phenolic contents of 5 bee pollens

collected from Portuguese were between 10.5 and 16.8 mg GAE g^{-1}. In our present study, total phenolic content showed negative correlations (r= -0.59) with IC_{50} values of pollen extracts. This negative linear correlation proves that when the total phenolic content is higher, the IC_{50} will be lower. In this vain, our findings were coherent with as mentioned previous studies.

The results of our experimental data indicated that the E1 sample taken from Erfelek was to have higher antioxidant (IC_{50}, 0.093 ± 0.06 mg/mL), total phenol content (103.8 ± 6.72 mg GAE g^{-1}) and antimicrobial efficacy according to the other samples and control. Several researches proposed that there was a relationship between the antioxidant activity and total phenolic contents (Carpes *et al*., 2007; Moreira *et al*, 2008; Marghitaş *et al.*, 2009; Freire *et al*., 2012). Other some studies focused on the subjects also proposed that the different patterns of antimicrobial and antioxidant activities of the pollen samples collected from diverse regions are due to different phenolic compounds of pollen extracts (Bonvehi *et al*., 2001; Leja *et al*., 2007). In this context, we can say that this situation might be related to the high level total phenolic contents and the ecological differences of the plant sample taken the habitat.

Conclusions

The findings gathered from the study were revealed that *C. sativa* pollen collected from 9 sampling points of Sinop province in Black Sea region of Turkey had selective and effective antimicrobial activity against evaluated bacterial and yeast strains. In addition, the pollen extracts exhibited *in vitro* good free radical scavenging activity. Particularly, the extract signed by E1 showed excellent antioxidant activity with the value IC_{50}, 0.093 ± 0.06 mg/mL (Fig 2), comparing with control and other pollen extracts. In conclusion, we suggested that methanolic extracts of *C. sativa* pollens might be an alternative natural food resource due to the preventative properties. The extracts can also provide a natural solution for seeking an alternative to synthetic drugs.

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