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In Vitro Antioxidant and Enzyme Inhibitory Activities of Walnut Male Flowers

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ABSTRACT

Walnut (*Juglans regia* L.) male flowers are known for their high phenolic content and associated health benefits, including anti-hypoxic, antihaemolytic, anti-inflammatory, antidepressant, and antioxidant activities. This study represents the first investigation of the inhibitory effects of walnut male flower extract on α-amylase and α-glucosidase enzymes, employing HPAE-PAD (High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection). The inhibitory potential of the extract was compared to that of acarbose, a chemical drug commonly used for this purpose. Furthermore, the antioxidant activity of the extract was also evaluated. The extract demonstrated significant inhibition of α-amylase and α-glucosidase, with half maximal inhibitory concentration (IC_{50}) values of 1.507 mg/mL and 0.803 mg/mL, respectively. In contrast, acarbose exhibited IC_{50} values of 1.031 mg/mL and 0.985 mg/mL for α-amylase and α-glucosidase, respectively. Although the walnut male flower showed greater inhibition of α-glucosidase than acarbose, acarbose exhibited stronger inhibition of α-amylase activity than walnut male flowers. The extract exhibited a DPPH (1,1-Diphenyl-2-picrylhydrazyl radical) free radical scavenging activity, with an IC_{50} value of 19.51 µg/mL. Additionally, the total phenolic content of 277 mg GAE (gallic acid equivalent)/g dry weight (dw) was determined in the extract. These results may highlight the potential of walnut male flowers as a novel enzyme inhibitor for managing type 2 diabetes mellitus. The findings of this study could provide valuable insights for further investigation into the potential applications of walnut male flowers in the food and pharmaceutical industries.

Keywords: Walnut male flowers, α-Amylase, α-Glucosidase, Antioxidant, HPAE-PAD

Ceviz Erkek Çiçeklerinin *In Vitro* Antioksidan ve Enzim İnhibitör Aktiviteleri

ÖZ

Ceviz (*Juglans regia* L.) erkek çiçekleri, yüksek fenolik içeriğine sahip olup, antioksidan, antidepresan, antihipoksik, antienflamatuar ve antihemolitik aktiviteler dahil olmak üzere sağlık üzerine yararlı etkileri ile bilinmektedir. Bu çalışma literatürde ilk defa ceviz erkek çiçeği ekstraktının α-amilaz ve α-glukosidaz enzimleri üzerindeki inhibitör etkilerinin HPAE-PAD (Pulsed Amperometric Detection ile Yüksek Performanslı Anyon Değiştirme Kromatografisi) kullanılarak araştırılmasını temsil etmektedir. Ekstraktın inhibe edici potansiyeli, bu amaç için yaygın olarak kullanılan kimyasal bir ilaç olan akarbozunkiyle karşılaştırıldı. Ayrıca ekstraktın antioksidan aktivitesi de analiz edilmiştir. Ceviz erkek çiçeği ekstraktının IC_{50} değerleri sırasıyla α-amilaz ve α -glukosidaz için 1,507 mg/mL ve 0,803 mg/mL olarak belirlenmiştir. Buna karşılık akarboz için IC_{50} değerleri α -amilaz ve α -glukosidaz için sırasıyla 1.031 mg/mL ve 0.985 mg/mL olarak tespit edilmiştir. Ceviz erkek çiçeği, akarbozdan daha fazla α-glukosidazı durdurucu etki gösterse de, akarbozun ceviz erkek çiçeklerinden daha güçlü α-amilaz aktivitesine sahip olduğu belirlenmiştir. Ceviz erkek çiçeği ekstraktının DPPH serbest radikal yakalama aktivitesi (IC_{50}) 19.51 µg/mL olarak bulunmuştur. Ayrıca toplam fenolik madde miktarı ise 277 mg GAE (gallik asit eşdeğeri)/g kuru madde olarak belirlenmiştir. Bu sonuçlar, ceviz erkek çiçeklerinin tip 2 diyabet hastalığının tedavisinde alternatif bir enzim inhibitörü olarak kullanılabileceğini göstermiştir. Sonuç olarak,

ceviz erkek çiçeklerinin gıda ve ilaç endüstrilerinde potansiyel kullanımına yönelik daha detaylı çalışmalara ihtiyaç duyulmaktadır.

Anahtar Kelimeler: Ceviz erkek çiçeği, α-Amilaz, α-Glukozidaz, Antioksidant, HPAE-PAD

INTRODUCTION

The global diabetic population reached 536.6 million individuals in 2021, and it is expected to reach 783.2 million by 2045 [1]. In 2021, diabetes and its complications led to approximately 6.7 million reported deaths worldwide, with type 2 diabetes mellitus affecting males at a higher incidence [2]. The World Health Organization (WHO) reported in 2016 that one in eleven individuals currently suffers from diabetes, and this prevalence is anticipated to increase annually [3]. The development of technologies aimed at preventing high blood glucose levels holds promise for reducing these mortality rates. Therefore, there is a growing interest in alternative and natural treatment ways for the management of diabetes.

Juglans regia L., commonly known as walnut, is a tree species indigenous to North America, Europe, and Central Asia [4, 5]. According to the FAOSTAT, the walnut production in-shell is more than 3 million tonnes from a harvested area of 1,106,083 ha worldwide whereas Türkiye produced about 287k tonnes from 141,790 ha [6]. Due to the favourable climate conditions, walnut cultivation is widespread across all regions of Türkiye. The Aegean Region, along with the Mediterranean, Eastern Marmara, and Western Black Sea Regions, stands out as the main areas for walnut production [7]. Although walnut production in many countries focuses on nuts, kernels are widely consumed worldwide as a snack and incorporated into various culinary preparations such as baked goods, salads, breakfast cereals, pasta, and soups [2]. Walnut kernels are also used to extract walnut oil. Extensive research has been conducted on walnut kernels, revealing their high-fat content, including beneficial minerals, proteins, vitamins, phytochemicals, and polyunsaturated fatty acids such as phenolic acids and flavonoids [8-10]. These components offer potential health benefits, including antidiabetic, anti-aging, anti-cancer, anti-inflammatory, and neuroprotective properties. Additionally, various parts of the walnut tree, including leaves, bark, branches, immature green fruit, seed coat, and flowers, contain bioactive compounds with antibiotic, antioxidant, and medicinal properties [8-11]. On the other hand, there are only limited studies about walnut male flowers which is also known as catkins and it was reported that approximately 2000 male flowers per adult walnut tree are present. The major compounds of the walnut male flowers were reported as gallic acid, coumarin, quercetin, polyphenols, flavonoids, sterols, fat, protein, vitamin, and minerals [5, 9]. The phenolic composition of walnut male flowers was reported in different studies. Żurek et al. [10] detected the major phenolics as quercetin 3-*O*-glucoside, quercetin diglucoside and 5-*O*-caffeoylquinic acid. Another recent study also reported the major bioactive compounds of walnut male flowers as follows quercetin, hyperoside, quercitrin, and isoquercitrin [8]. In another study, vanillic acid was found as the main phenolic, and caffeic, ferulic, and chlorogenic acids were also detected [9]. The variation in composition mentioned may be influenced by factors such as the specific species, growing conditions (including soil type, environmental factors during growth, and geographical location), the stage of maturity at the time of harvest, and genetic variations [10, 12]. Walnut male flowers are accepted as a traditional vegetable consumed in specific regions of China and Poland that are employed to prepare infusions, tinctures, liquors, jams, and confectioneries [10]. In addition, in China, it is known as a longevity food due to its various health benefit such as anti-hypoxic, anti-inflammatory, antioxidant, antidepressant, and antihaemolytic activities [9, 10, 13]. Only one study analyzed its potential for regulating the insulin and blood glucose levels of STZ-induced rats [11] whereas there are no studies about the effect of walnut male flowers on digestive enzymes. Acarbose is a pharmaceutical drug prescribed for the treatment of type-2 diabetes and is known for its anti-diabetic properties [12]. It functions by inhibiting the activity of carbohydrate hydrolysis enzymes, specifically α-glucosidase and α-amylase. The phenolic extracts obtained from walnut male flowers have the potential to exhibit an acarbose-like effect.

This study aims to discover the effect of aqueous and methanolic extracts of walnut male flowers from Türkiye on α-glucosidase and human salivary α-amylase using a more sensitive method, HPAE-PAD. Analysis of carbohydrates is available with several methods in the literature. But the detection of carbohydrates with pulsed electrochemical detection at a gold working electrode is a reproducible and sensitive method. Besides, the effects of walnut male flowers extracts are aimed to compare with the chemical drug acarbose.

MATERIALS and METHODS

Plant Material Collection and Extract Preparation

The flowers were collected from trees in Odemis, Izmir, Türkiye in April. The collected material was placed into an ice box and carried to the laboratory within 4-5 h. After that, the walnut male flowers were freeze-dried to remove the moisture and following ground into a powdered form and stored at -20°C until further analysis. The extraction method developed by Żurek et al. [10] was adopted for the methanolic extraction of walnut male flower phenolics using ultrasound (Ivymen, Spain).

Total Phenolic Content (TPC)

The TPC of extract was determined by the Folin & Ciocalteau colorimetric method (T70+UV/VIS spectrophotometer, PG Instruments, UK) [13]. TPC was

expressed as mg of GAE per g of dw. Three replicates were performed for each sample.

DPPH Radical Scavenging Activity

The analysis was conducted using the method developed by Dorman et al. [14]. A mixture consisting of 50 µL of extract, 450 µL of Tris-HCl (hydrochloric acid) buffer (50 mM, pH 7.4), and 1.00 mL of fresh methanolic solution of DPPH (0.10 mM) was vigorously shaken and then placed in a dark place at room temperature (RT) for a duration of 30 minutes. The percentage of DPPH remaining was calculated using the following equation after measuring the absorbance of the samples at 517 nm, as described in equation (1) below.

$$\text{Inhibition \% (DPPH)} = [(Abs_{Control} - Abs_{Sample}) / Abs_{Control}] * 100 \quad (1)$$

The IC_{50} value, which represents the extract concentration required to inhibit 50% of the free radicals, was determined by plotting the DPPH (%) activity and was reported as IC_{50}=µg/mL. All analyses were conducted in triplicate.

Antidiabetic Activity

A buffer solution of 20 mM sodium phosphate and 6.7 mM sodium chloride (pH 6.9) were used to dissolve human salivary α-amylase (S/3160/53 from Fisher Scientific, Loughborough, UK). On the other hand, 10 mM sodium phosphate buffer with pH 7.0 was used to dissolve intestinal acetone rat powder (I1630). Following, both enzyme solutions were vortexed for 30 seconds and then centrifuged at 17,000 x g for 10 minutes. The supernatants were removed to conduct analysis, and these enzyme solutions were prepared freshly before each experiment [15].

Determination of α-Amylase Activity

The developed method by Nyambe-Silavwe et al. [18] was used. 12 g of sodium tartrate was added to 8 mL of 2 M sodium hydroxide and heated until dissolved. It was included to stabilize the colour and protect the product from oxidizing. The DNS (3,5-dinitrosalicylic acid) solution was produced by combining the DNS powder with deionized water (20 mL) before placing the mixture immediately on a heating plate to dissolve. The colorant employed in the α-amylase reaction was DNS. The reducing sugars are produced as a by-product of the hydrolysis of starch by human salivary α-amylase. In an alkaline environment, DNS reacts with the free carbonyl group of the reducing sugars to form 3-amino-5-nitrosalicylic acid, which can be detected at 540 nm. As the reducing sugars are released, DNS altered the colour. DNS and prepared sodium tartrate were combined with deionized water (40 mL) to create the colour reagent solution. 200 mL of the sucrose solution, 50 mL of buffer (pH 7.0-10 mM), 50 mL of plant material, and 200 mL of enzyme solution at various concentrations were combined for the experiment. Following, the mixture was vortexed (10 seconds) and incubated at 37°C (10 min). As it was mentioned by Nyambe-Silavwe and colleagues' study a cartridge (Waters Oasis MAX-003036349A) was applied to eliminate the polyphenol's potential to interfere with colour development, the samples were left for 10 minutes incubation in a boiling water bath to stop the enzyme activity before the addition of colour reagent solution. Following the incubation, to measure the production of reduced sugar amount, a colour reagent solution was added to each sample (1 mL). After that, the samples were immediately placed on ice for cooling down to RT after being placed in a boiling water bath (10 minutes) to prevent enzymic activity. Following this, the samples were transferred to a boiling water bath for 10 minutes again. Then they were placed in vials for electrochemical detection using HPAE-PAD.

Inhibition of α-Glucosidase Enzyme

A previously reported technique was modified to detect the activities of sucrase in an acetone extract of rat intestinal tissues [16] by analysing sugars [sucrose and its products (glucose and fructose)] via a Dionex system running Chromeleon 6.5. Ion-exchange- liquid chromatography (LC) combined with electrochemical detection permits the direct quantification of low-level (pM) carbohydrates below 10,000 Da without the need for derivatization or intensive sample preparation. To optimize the experimental conditions, we initially determined the Michaelis Constant (K_m/V_{max}), which yielded a K_m value of 18 mM and a V_{max} value of 0.09 µmoL sucrose hydrolysis/minute. Subsequently, varying amounts of the enzyme were tested at different concentrations, and the hydrolysis of sucrose was carried out over different time intervals at 37ºC to identify the optimal incubation time and enzyme quantity. For the control sample, a mixture of 18 mM sucrose (200 µL) and 15 mg/mL acetone rat intestinal powder (200 µL) was combined with 100 µL of sodium phosphate buffer, based on preliminary investigations. To evaluate the antidiabetic activity of the extract, the test sample consisted of 100 µL of the extract/solution instead of sodium phosphate buffer. Similarly, for the analysis of antidiabetic activity in walnut male flower extract and acarbose, the test sample contained 100 µL of the respective samples and sodium phosphate buffer. Following this, the samples were incubated at 37ºC for 15 minutes. To stop enzyme activity, 750 µL of acetone was added to the mixtures, which were then immediately vortexed for 10 seconds and cooled down on the ice at room temperature. The acetone was subsequently evaporated using nitrogen gas, and centrifugation was performed once again. Finally, the supernatant was filtered, and the amount of sucrose and its products (glucose and fructose) were determined using HPAE-PAD.

Detection of Carbohydrates with HPAE-PAD Chromatography System

Carbohydrate molecules are often separated using anion-exchange chromatography but considering they

are weak acids, it may be more sensitive to identify them using amperometric detection, due to its specialty to depend on the oxidation of carbohydrates in the presence of sodium hydroxide at the gold electrode. HPAE-PAD (Dionex DX500, Sunnyvale, CA) comprised of a GP40 (gradient pump), PAD (pulsed amperometric detector) system, an LC20 column oven, and electrochemical detectors (ED 40, e.g. gold working, titanium, and silver (reference) electrode). The analytical column for α-glucosidase assay was used as Carbopac PA20 [Dionex, 3×150mm and guard column (3×30mm)], and for human salivary α-amylase assay, Carbopac PA200 (Dionex, 3×250mm) with guard (3×50mm). In addition, the mobile phase was 200 mM sodium hydroxide (NaOH) (flow rate: 0.4 mL/minutes and injection volume: 10 µl). Finally, the elution was achieved using a gradient from 0-30% 200 mM NaOH in 10 minutes, 50% 200 mM NaOH from 10 to 15 minutes, and re-equilibration at 30%, 200 mM NaOH for 15 minutes.

Statistics

The data analysis process was carried out using IBM SPSS Statistics 22. The one-way ANOVA was followed by the Dunnett C test unless the condition was achieved, in which case the Tukey HSD post hoc test was used. If $p \leq 0.05$, differences were regarded as statistically significant unless otherwise stated.

RESULTS and DISCUSSION

TPC and DPPH Radical Scavenging Activity

In Table 1, the assessment of TPC and DPPH activity in walnut male flower extracts is presented, highlighting the relationship with the extraction method employed. In the current study, the TPC content of the walnut male flowers was found 277± 11.08 mg GAE/g dw. In a study, the methanolic extraction of walnut male flowers using ultrasound (UE) was analyzed for its TPC at four different flowering stages. It was detected that its early flowering stage (2.43 g GAE/100g dw) has higher TPC than the later pollen-scattering stage (2.13 g GAE/100g dw) [17]. Zhang et al. [21] also determined the TPC of male flowers using different extraction conditions for UE of methanolic extraction, enzymes assisted-methanolic extraction (EE), and methanolic condensation reflux extraction (ME) and found it as 1351, 1625, and 1236 mg GAE/g extract, respectively. A more recent study also reported the ethanolic extraction of walnut male flowers as 1351 mg GAE/g extract [18]. In another research, the phenolic content of the walnut male flowers was reported as 248 mg GAE/g dw [19]. This research extraction method was adopted for the current study and the results are shown to agree. Muzaffer and Paul [23], Nabavi et al. [24], and Pop et al. [8] conducted studies that yielded comparable results regarding the total phenolic content of walnut male flowers collected from India, Iran, and Romania, respectively, and the TPC of flowers from different countries were determined around 1.45 mg GAE/g dw. In addition, the TPC of various other aerial parts of the walnut tree such as walnut leaves were also examined with the range of 65 mg/g and 194 mg GAE/g dw [20], dry walnut seeds were reported to have a TPC between the range 8.2 to 20.9 mg GAE/g dw [21]. The TPC value reported for the various parts of walnut extract indicates that the male flowers may serve as a rich natural source of polyphenols in comparison to other parts of the walnut tree.

Table 1. Assessment of TPC and DPPH activity in walnut male flower extracts with respect to the extraction method utilized.

Area	Solvent type	Extraction Method	TPC (mg GAE/ g dw)	DPPH (IC_{50} µg/ mL)	References
Türkiye	Methanol	UE	277	19.51	Current study
		UE	1351	51	[22]
China	Methanol	EE	1625	59	[22]
		ME	1236	59	[22]
Poland	Methanol	UE	248	22.34	[19]
India	Methanol	Percolation	129.76	53.95	[23]
Iran	Methanol	Percolation	71.7	674	[24]
Romania	Methanol	UE	1.45	-	[8]

In this research, DPPH free radical scavenging activity demonstrated the lowest IC_{50} value (19.51 ± 1.08 µg/mL) in comparison to the results reported in earlier studies that assessed the antioxidant activity of flowers [10, 21, 24]. Nabavi et al. [24] found the IC_{50} for DPPH radical scavenging activity of extract as 674 µg/mL. While the DPPH radical scavenging capacity of the early flower stages was 84.33%, later pollen-scattering stage were reported as 79.26% [17]. Zhang et al. [21] analyzed the DPPH activity of methanolic extraction using EE, UE, and ME methods and found it as 59 µg/mL, 51 µg/mL, and 59 µg/mL, respectively. Another recent study reported the IC_{50} for DPPH radical scavenging activity of methanolic extraction (22.35 µg/mL) higher than the previously published studies [19]. Based on the available literature, the outcomes may show a strong correlation between the methanol concentration as Żurek et al. [10] used the higher concentration compared to other studies. Similar to TPC, the DPPH radical scavenging activity in the current study and Żurek et al [10] study results are in agreement. Therefore, the results obtained suggest that walnut male flowers possess a strong capacity to remove free radicals, potentially attributed to their rich concentration of bioactive compounds.

Antidiabetic Activity

The inhibition effect of walnut male flower extracts and the chemical drug acarbose, which is used to manage diabetes, on α-glucosidase and α-amylase activity was detected and is presented in Table 2.

According to the literature, the researchers were attracted to analysing the different parts of walnut for their anti-diabetic activity with both *in vitro* and *in vivo* methods. However, this is the first report known about the inhibition of α-glucosidase and human salivary α-amylase by walnut male flowers using a more sensitive method, HPAE-PAD. The results of the current study indicated that the walnut male flower has a higher inhibitory effect for α-glucosidase enzymes than acarbose (Table 1). In addition, the inhibitory effect on α-glucosidase activity was higher than human salivary α-amylase activity for both walnut male flower and acarbose. Also, acarbose indicated a significantly higher inhibitory effect on α-amylase compared to the walnut male flowers. There are several research where the inhibitory effect of bioactive compounds on α-glucosidase activity is higher than the α-amylase activity [12, 27-29]. The activity of polyphenols against α-amylase and α-glucosidase enzymes is influenced by their chemical structure. Changes in functional activity resulting from high binding efficiency may be directly associated with the potential biological activity of these molecules as anti-hyperglycaemic agents [27, 30]. In addition, Ćorković et al. [27] reported that the higher proanthocyanidins content of the extract may increase the inhibitory activity of α-amylase. In a scientific study conducted by Żurek et al. [10], the phenolic components of male flowers from walnut trees were identified using UPLC-PDA-MS/MS (ultra-performance liquid chromatography coupled to photodiode array detection and tandem mass spectrometry). The primary bioactive compounds observed were identified as *O*-flavonol glycosides, with quercetin and kaempferol serving as the aglycones. These compounds were found to be linked with oligosaccharide molecules at the 3- or 7-hydroxyl (OH) position. The higher inhibitory activity for α-glucosidase compared to α-amylase may be attributed to the phenolic content present in walnut male flowers. Based on the obtained results, the findings of the present study are consistent with the literature.

Table 2. Inhibition of digestive enzymes by walnut male flower phenolic extract and acarbose.

Product	IC_{50} (mg/mL)*	
	α-Glucosidase	Human salivary α-amylase
Walnut male flower	0.803 ± 0.01^{x}	1.507 ± 0.08^{x}
Acarbose	0.985 ± 0.01^{y}	1.031 ± 0.05^{y}

*Means that are shared by different superscripts within each column indicate a Tukey's test comparison between the extracts at $p < 0.05$.

In the literature, only one *in vivo* study investigating the anti-diabetic properties of a hydroalcoholic extract derived from walnut male flowers was found [11]. This study focused on streptozotocin (STZ)-induced mice and demonstrated a significant reduction in their blood glucose levels following the administration of the walnut male flowers extract. The antidiabetic activity of walnuts has been extensively investigated in both *in vitro* and *in vivo* studies, encompassing various parts of the walnut plant, including its leaves, fruit, and bark [31-34]. For instance, walnut leaves were extensively utilized in the studies, constituting around 79% of the plant material employed. These leaves were commonly prepared in powdered form or extracted through decoction, oil extraction, microwave, or Soxhlet extraction. In addition, it was also reported that the fruits (14.28%) and bark (7%) exhibited notable anti-diabetic effects when subjected to maceration or powdering [25]. In a clinical study conducted by Hosseini et al. [35] the use of walnut in patients with type 2 diabetes was shown to be both effective and safe. Administration of a daily dose of 200 mg over a period of 3 months resulted in reduced fasting blood glucose, HbA1c, cholesterol, and triglyceride levels. These positive effects can be attributed to the abundant presence of phenolic acids and flavonoids in the plant. However, further investigations are required to elucidate the precise mechanism underlying the antidiabetic activity of walnut male flowers. Additionally, *in vivo* analyses should be conducted to assess the potential toxicity associated with their use. Subsequently, clinical studies can be undertaken to comprehensively explore the therapeutic potential of walnut male flowers in the management of diabetes.

CONCLUSION

In conclusion, this study addresses a knowledge gap regarding the inhibitory activity of *Juglans regia* (walnut) male flowers on α-amylase and α-glucosidase enzymes. Results suggest that walnut male flowers could be utilized in diabetes management due to their abundant polyphenol content. The study demonstrated that the high phenolic content in walnut male flowers might results in greater inhibitory activity against α-glucosidase enzymes compared to the chemical drug acarbose. However, acarbose exhibited stronger inhibitory activity against α-amylase. Additionally, the walnut male flower extract exhibited antioxidant activity. Considering the global cultivation and accessibility of walnut species, combined with the notable health-enhancing attributes of its extract, these flowers could possess substantial potential as a valuable source of natural antioxidants and antidiabetic agents in various biotechnology products. However, further research is required, including *in vivo* analysis and clinical studies, to explore the potential of this valuable raw material. Ongoing scientific exploration holds the potential for the advancement of efficient therapeutic approaches through the utilization of isolated individual active

compounds or combinations derived from the walnut male flowers.

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