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Investigation of the Antioxidant Activity and Phenolic Compounds of *Andricus quercustozae* Gall and Host Plant (*Quercus infectoria*)

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Abstract: *Andricus quercustozae* (Bosc, 1792) is a cynipid gall wasp, which induces gall on oaks (*Quercus* spp.). It is known that both cynipid galls and oaks are used in traditional medicine. In this study, some biological characteristics of various extracts (acetone and ethanol) of *A. quercustozae* asexual gall and its host plant, *Quercus infectoria* Olivier, were investigated. The antioxidant capacities of the extracts were evaluated using radical scavenging activity (ABTS and DPPH assays), the β-carotene-linoleic acid method, the phosphomolybdenum method, and the reducing power (CUPRAC method). Total phenolics, flavonoid and tannin contents were measured in the gall and the oak leaf extracts. Moreover, ethanol extracts of the gall and the host plant were evaluated using HPLC for the composition of phenolics. Generally, the gall extracts (acetone and ethanol, respectively) exhibited the strongest radical scavenging (DPPH, IC_{50} value of acetone extract: 11.00 µg/mL and IC_{50} value of ethanol extract: 8.67 µg/mL; ABTS, 52.27 µg/mL and 44.97 µg/mL) and antioxidant activities with the highest level of phenolics. The antioxidant activity of the gall extracts was in the range of 80.74 to 87.49 % for β-carotene-linoleic acid method, while and it was ranged from 75.68 to 78.20 mgAEs/g for phosphomolybdenum method. In the results of some antioxidant methods (ABTS and β-carotene-linoleic acid), it is observed that the host plant extract has values close or high to the gall extract. In this context, our results suggested that the cynipid gall extracts could be used as a natural agent in food, medicinal and pharmaceutical applications.

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1. INTRODUCTION

Quercus infectoria belonging to the Fagaceae family is a small tree or a shrub widely grown in Turkey (Anatolia), Syria, Iran, and Greece. The oak is known as one of the medicinal plants, which has been traditionally used in oriental folks [1, 2]. The gall wasps or cynipids (Cynipidae), which are known as the gall inducers, is a large group with roughly 1400 species

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worldwide [3], and 151 cynipid species are also known from Turkey [4-6]. All cynipid species induce their galls on their host plant species, which are mostly the oaks (Fagaceae), Rosaceae, Lamiaceae, Asteraceae and Papaveraceae. The galls are structures formed by plants entirely for the benefit of the gall inducer. The gall provides nourishment, shelter, and protect the cynipids [3]. Besides, the gall has been used in folk medicine for having various therapeutic properties since ancient times [7]. In the ancient Sumerian (5000 BC) and Babylonian periods, the galls of *Andricus infectorius* (Hartig) (known as *Turkish Gall*) were presented in the prescriptions for typhoid cure. Furthermore, it was used in the cure of bleeding and respiratory diseases in ancient Syria [8, 9]. In Traditional Uyghur Medicine, it is seen that the gall (*A. infectorius*) was widely used in the cure of intestinal dysmotility, dysentery, functional enteritis, hemorrhagic sores, alopecia areata, dental caries, periodontitis, halitosis, pharyngolaryngitis and tympanitis [10, 11]. According to last two decades pharmacological researches, the various biological activities of the oak galls such as antibacterial, antiviral, antifungal, antioxidant, astringent, antidiabetic, antiparkinsonian, antitumor, antidiabetic, local anaesthetic, antipyretic, anti-inflammatory and many more [10, 12-16] have been revealed. In addition, there are many types of research on antioxidant activities, total phenolic and flavonoid compounds of *A. infectorius* galls. The gall extracts possess a strong antioxidant activity against free radicals because of the greater amount of phenolic and flavonoid compounds [17-23].

Previous phytochemical investigations have shown that the oak galls contain high amounts of tannin (50-70%) [24-28]. Gallic acid, ellagic acid and some sugars are the main constituents found in the cynipid galls in addition to tannin [29-31]. The amount of tannin is more concentrated in gall tissues than leaf tissues. The tissues with lower tannins are used as nutrients by cynipid larvae [32-34]. There is a highly significant positive correlation between the tannin level in oak and gall wasp diversity or abundance. The cynipids, along with their defence development against oak tannins, used them for their benefit. Tannins protect the cynipid larvae to fungal pathogens, hyper-parasites, leaf herbivores and some pathogenic factors [35].

The study aimed to investigate the amounts of the total secondary metabolites of the gall and host plant extracts. Besides, it reveals some biological characteristics (antioxidant activities) and phenolic components of all extracts for future investigations. In this context, some biological characteristics of the oak gall and the host plant are considered to be an accessible and important data source for antioxidant activity. In short, to determine in which areas (like the food industry, medicinal and pharmaceutical) the benefits of the cynipid gall used in traditional medicine, can be used.

2. MATERIAL and METHODS

2.1. Collection and Preparation of Gall and Host Plant Extracts

Andricus quercustozae (Bosc, 1792) asexual gall and the leaves of the host oak (non-galled leaves), *Q. infectoria*, were collected from Denizli, Turkey in May 2015. The species identified by the Entomology Laboratory in Pamukkale University. The galls and leaves of the host oak were dried in the shadow, broken into small pieces with an electric blender. And then gall and leaves were extracted with acetone and ethanol using the previous method [36] and stored in Secondary Metabolite Laboratory, Pamukkale University, Turkey.

2.2. Determination of Total Bioactive Components

2.2.1. Quantification of Total Phenolic Content

The Folin–Ciocalteu method [37] with slight modification was used to determine the total phenolic contents of each extract. The sample solution (1 mg/mL) was mixed with diluted Folin–Ciocalteu reagent (1 mL) and dH_2O (46 mL). After 3 min, sodium carbonate solution (3

mL, 2%, Na_2CO_3) was added. The absorbance of the mixture was measured at 760 nm after the incubation (in the dark, 2 hours, room temperature). The total phenolic content was expressed as equivalents of gallic acid (mgGAEs/g).

2.2.2. Quantification of Total Flavonoid Content

The total flavonoid contents of each extract were analysed according to the method [38]. Briefly, aluminium trichloride (1 mL, 2% $AlCl_3$) was mixed with the same volume of extract solution (2 mg/mL). The absorbance was measured at 415 nm after the incubation (10 min, room temperature). The total flavonoid content was expressed as equivalents of quercetin (mgQEs/g).

2.2.3. Quantification of Total Tannin Content

The vanillin method [39] with slight modification was used for analysing the total tannin content. The solution (0.5 mL) was mixed with vanillin reagent (1.5 mL, 1% in 7 M H_2SO_4) in an ice bath. The solution absorbance was measured at 500 nm after the incubation (15 min, room temperature). The total tannin content was expressed as equivalents of (+)-catechin (mgCEs/g).

2.3. Radical Scavenging Activity

2.3.1. Determination of DPPH Radical-Scavenging Activity

The radical scavenging activity of the extracts was determined using the method [40]. Different concentration (5 to 25 µg/mL) of the extracts (1 mL) was mixed with 4 mL of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical methanolic solution. The absorbance was measured at 517 nm after 30 min. Synthetic antioxidant BHT (butylated hydroxytoluene) was used as a positive control. The results were expressed as IC_{50} values.

2.3.2. Determination of ABTS Radical-Scavenging Activity

The method [41] with slight modification was used to determine the radical scavenging activity of the extracts. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) cation was produced by reacting ABTS solution (7 mM) with potassium persulfate (2.45 mM) and then the mixture to stand in dark (12-16 hours, at the room temperature). The mixture was diluted with ethanol to give an absorbance of 0.700 ± 0.02 units at 734 nm for the study. The extract solution (1 mL) and ABTS solution (2 mL) were mixed. The mixture absorbance was read at 734 nm after the incubation period (15 min, at room temperature). The results were expressed as IC_{50} values.

2.4. Total Antioxidant Activity

2.4.1. β–Carotene–Linoleic Acid Method

The total antioxidant activity of the extracts was analyzed using the β–carotene–linoleic acid method [42] with slight modifications. β-Carotene (0.2 mg) was dissolved in chloroform (1 mL) and added linoleic acid (20 µL) and Tween–20 (200 mg). The chloroform was evaporated using a rotary evaporator. The mixture was diluted with dH_2O (100 mL). As soon as the emulsion (4.8 mL) and 1 mL extracts (1 mg/mL) were placed into test tubes, initial absorbance was measured at 470 nm. The measurement was carried out at 30 min intervals for 2 hr. BHA and BHT were used as standards. The antioxidant activity was calculated using the equation below:

$$AA = \left[1 - \left(\frac{A_0 - A_t}{A^\circ_0 - A^\circ_t}\right)\right] x\ 100$$

Where A_0 and A^o_0 are the absorbance values measured at the initial incubation time for samples and control, respectively. While A_t and A^o_t are the absorbance values measured in the samples or standards and control at 2 hr.

2.4.2. Phosphomolybdenum Method

The phosphomolybdenum method [43] with slight modification was used to determine the antioxidant activity of the extracts. Extract solution (0.3 mL) was mixed with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The mixture absorbance was read at 695 nm after the incubation period (90 min, at 95 °C). The total antioxidant capacity was expressed as equivalents of ascorbic acid (mgAEs/g).

2.5. Reducing Power

2.5.1. Activity Cupric Ion Reducing (CUPRAC) Method

The method [44] was used to determine the cupric ion reducing activity (CUPRAC). Extract solution (0.5 mL) was added to premixed reaction mixture containing $CuCl_2$ (1 mL, 10 mM), neocuproine (1 mL, 7.5 mM) and NH_4Ac buffer (1 mL, 1 M, pH 7.0). Similarly, a blank was prepared by adding sample solution (0.5 mL) to the premixed reaction mixture (3 mL) without $CuCl_2$. The mixture and blank absorbances were measured at 450 nm after the incubation period (30 min, at room temperature). The absorbance of the blank was subtracted from that of the sample. CUPRAC activity was expressed as equivalents of Trolox (mgTEs/g).

2.6. Quantification of the Phenolic Compounds by HPLC

Phenolic compounds were analyzed by high performance liquid chromatography (HPLC) according to the method [45] with some modification. Detection and quantification were performed with a diode array detector (SPD–M20A), a LC–20AT pump, a CTO–10ASVp column heater, SIL–20ACHT auto sampler, SCL–10Avp system controller and DGU–14A degasser. The mobile phases were A: 3.0% formic acid in distilled water and B: methanol. Methanol was used to dissolve samples, and then 20 µL of this solution was injected into the column. Gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, 2,5-dihydroxybenzoic acid, chlorogenic acid, vanillic acid, epicatechin, caffeic acid, *p*-coumaric acid, ferulic acid, rutin, ellagic acid, naringin, quercetin, and cinnamic acid were used as standards. The differentiation and quantitative analysis were made by comparing the standards. The quantity of each phenolic compound was expressed as µg per gram of the extract.

2.7. Statistical Analysis

The SPSS Statistical Package program were used to analyze the results. The results were presented as mean ± std. Differentiations between the extracted groups were tested using Analysis of Variance and Tukey method were performed ($p < 0.05$).

3. RESULTS and DISCUSSION

3.1. Determination of Total Bioactive Components

The calibration curve generated from the analysis of the standard (gallic acid) was linear with $y=0.0016x+0.0407$; $r^2=0.983$. The *A. quercustozae* gall extracts showed the highest phenolic content, acetone (479.56 ± 45.36 mgGAE/g) and ethanol (437.27 ± 3.14 mgGAE/g), while *Q. infectoria* extracts showed the lowest contents. Among the studied extracts, total phenolic content ranged from 79.35 to 479.56 mgGAE/g (Table 1). Phenolic contents of the gall extracts were found to be more than the extracts of host oak (*Q. infectoria*). In addition, there were no differences between the acetone and ethanol extracts for each species ($p>0.05$), but the gall extracts and host plant extracts were found to be statistically different from each other ($p<0.05$).

In the present study, total flavonoid contents were analysed with the spectrophotometric method [38], and the flavonoid contents ranged from 33.19 to 110.28 mgQE/g (Table 1). Statistical differences among each group were found ($p<0.05$).

Table 1. Total Phenolic, Flavonoid and Tannin Contents (mean±SD) of the Extracts.

Species	Solvent	Total Phenolic Content (mgGAEs/g)	Total Flavonoid Content (mgQEs/g)	Total Tannin Content (mgCEs/g)
A. quercustozae asexual gall	Acetone	479.56±45.36^{b}	47.84±3.06^{b}	23.10±1.22^{b}
	Ethanol	437.27±3.14^{b}	33.19±1.91^{a}	7.03±0.02^{a}
Q. infectoria leaf	Acetone	87.90±8.97^{a}	110.28±3.47^{d}	34.04±0.75^{c}
	Ethanol	79.35±6.13^{a}	75.89±0.71^{c}	32.81±1.62^{c}

GAE = Gallic Acid Equivalents; QE = Quercetin Equivalents; CE = Catechin Equivalents; Data were given as the mean of the measurements ± std. The letters after the mean values in each column refers to statistically different than the others ($p<0.05$).

Tannin content was analysed using the vanillin method and these results were evaluated as catechin equivalents. The total tannin content of the extracts varies from 7.03 to 34.04 mgCE/g (Table 1). There were no differences ($p>0.05$) between the acetone and ethanol extracts of the host plant, but the gall extracts and host plant extracts were found to be statistically different from each other ($p<0.05$). Unlike phenolic compounds, *Q. infectoria* extracts showed the highest flavonoid and tannin content while the gall extracts showed the lowest contents. These results suggested that the phenolic, flavonoid and tannin contents were best extracted with acetone for both the gall and the host plant (Table 1).

Secondary metabolite constituents are known as phenolic, flavonoid and tannin etc. that serve as powerful antioxidants [46]. Moreover, there are important biological properties as antiallergenic, antimicrobial, antiartherogenic, antithrombotic, antiinflammatory, vasodilatory and cardioprotective effects [47-50]. Similar to the previous studies [21, 51-53], this study also shows that the cynipid gall and the host plant contain high amounts of phenolic, flavonoid and tannin.

3.2. Radical Scavenging Activity (DPPH and ABTS Assays)

The free radical scavenging activities of the samples of *A. quercustozae* gall and *Q. infectoria* leaves were tested by DPPH and ABTS assays. The DPPH and ABTS are known as radicals, they can be readily undergone scavenging by an antioxidant [54, 55]. The lower IC_{50} reflected a higher antioxidant activity in both assays (Table 2). In DPPH assay, the gall extracts more than the leaf extracts, have stronger scavenging activity. Among all the extracts, only ethanolic gall extract exhibited the highest radical scavenging capacity with IC_{50}: 8.67 µg/mL, followed by acetonic gall extract. The lowest scavenging activity was observed in ethanolic leaf extract with a very high IC_{50} value of 54.37 µg/mL. The ABTS scavenging capacity of the extracts was determined and IC_{50} values are given in Table 2.

The extracts showed scavenging activities in the range of 19.75 to 52.27 µg/mL. The acetonic leaf extract showed significantly stronger ABTS scavenging capacity (IC_{50}: 19.75 ± 0.92 µg/mL) than that of all other extracts ($p<0.05$). In compared extracts, *A. quercustozae* gall extracts had higher antioxidant capacity than *Q. infectoria* leaf extracts. The results of the strong radical scavenging capacity of the gall extracts are related to the high concentration of phenolic compounds in the gall extracts (Tables 1, 2).

3.3. Total Antioxidant Activity (β–carotene–linoleic Acid and Phosphomolybdenum Methods)

The total antioxidant capacities of the extracts were evaluated by using β–carotene–linoleic acid assay. The extracts of both samples exhibited generally high antioxidant activities (Table 2). However, the leaf extracts had strong antioxidant activity (91.05 ± 1.29% and 89.45 ± 1.72%) more than the gall extracts (80.74 ± 7.39% and 87.49 ± 1.27%). When compared with the inhibition values of all extracts, the acetone extract of the leaf (91.05 ± 1.29%) had a higher

value than others, but lower than synthetic antioxidants (BHA and BHT). In phosphomolybdenum assay, the extracts obtained from the gall had a higher antioxidant capacity than leaf extracts (p<0.05). Furthermore, the acetone extracts showed the highest antioxidant activity while the ethanol extracts showed the lowest activity (Table 2). Therefore, both the gall and the leaf extracts can be considered as natural inhibitors in the food industry. It must be the first study to compare the antioxidant capacities of the gall and the host plant.

Table 2. Antioxidant Properties of the Extracts.

Species	Solvent	ABTS (IC_{50}) (µg/mL)	DPPH (IC_{50}) (µg/mL)	β–carotene /linoleic acid method (%)	Phosphomolybdenum method (mgAEs/g)	CUPRAC assay (mgTEs/g)
A. quercustozae asexual gall	Acetone	52.27±4.49^{c}	11.00±0.39^{a}	80.74±7.39^{a}	78.20±1.63^{c}	237.74±5.55^{c}
	Ethanol	44.97±2.56^{bc}	8.67±0.58^{a}	87.49±1.27^{ab}	75.68±0.44^{c}	245.82±1.06^{c}
Q. infectoria leaf	Acetone	19.75±0.92^{a}	46.16±4.60^{b}	91.05±1.29^{b}	57.83±3.55^{b}	146.73±1.77^{a}
	Ethanol	40.71±2.19^{b}	54.37±3.61^{c}	89.45±1.72^{ab}	37.76±2.52^{a}	155.62±2.29^{b}
BHA	-	nt	nt	100.00±0.00	nt	nt
BHT	-	12.05±0.44	18.00±0.30	92.89±0.52	nt	nt

AE: Ascorbic Acid Equivalents; TE: Trolox Equivalents; nt: not tested; Data were given as the mean of the measurements ± std. The letters after the mean values in each column refers to statistically different than the others (p<0.05).

Table 3. Phenolic Compounds in the Ethanolic Extracts (µg/g extract).

No.	Phenolic Component	RT (min)	*A. quercustozae* asexual gall	*Q. infectoria* leaf
1	Gallic Acid	6.8	113.384	46.337
2	3,4-dihydroxybenzoic acid	10.7	210.835	84.87
3	4-hydroxybenzoic acid	15.7	383.299	100.285
4	2,5-dihydroxybenzoic acid	17.2	6002.886	2181.12
5	Chlorogenic acid	18.2	132.297	40.314
6	Vanillic acid	19.2	1002.53	360.975
7	Epicatechin	21.3	27286.511	3679.685
8	Caffeic acid	22.7	31979.335	8982.126
9	*p*-Coumaric acid	26.1	2.203	89.31
10	Ferulic acid	30.1	1.311	90.41
11	Rutin	45.6	< LOD	< LOD
12	Ellagic acid	47.7	2854.833	2635.375
13	Naringin	49.7	29.915	911.861
14	Quercetin	70.4	< LOD	285.237
15	Cinnamic acid	71.1	6.316	97.219

LOD = Limit of Detection; RT: Retention Time.

3.4. Reducing Power (CUPRAC Method)

Cu^{2+} reduction is used to determine electron donation activity which is known an important mechanism of antioxidant. Therefore, in order to analyse extracts' electron-donating power, their ability to reduce Cu (II) was tested. The high values of TEs reflected a high reducing activity. The reducing power activities of the extracts are presented in Table 2. When compared to the cupric reducing ability of all extracts, the ethanol extracts had a high cupric reduction potential for both samples. Moreover, the gall extracts exhibited higher values than the leaf extracts (p<0.05). The high reducing power of the gall extracts might relate to the high phenolic compounds that act as electron donors.

There are investigations on the antioxidant activities of both the host plant [51] and the cynipid gall [17-20, 22, 56, 57]. For the first time, a different species (*A. quercustozae*) and its host plant (*Q. infectoria*) were compared with this study. This and similar studies should reveal the important biological characteristics of many cynipid galls and host plant, which has been used since ancient times against many diseases, in terms of human health.

3.5. Phenolic Composition (HPLC)

In the study, the phenolic components of the ethanolic extracts of *A. quercustozae* asexual gall and *Q. infectoria* leaf were determined using the HPLC method (Table 3). For both species' extracts, caffeic acid has the highest concentration followed by epicatechin. Caffeic acid and epicatechin are abundant in medicinal plants and possess many biological effects such as antioxidant, anti-aging [58, 59]. Moreover, other phenolic compounds found in the extracts such as gallic acid also possess beneficial effects on human health. Radical scavenging activity can thus be explained by the presence of epicatechin and caffeic acid.

4. CONCLUSION

The results reported in this study revealed that tested total phenolic compounds were significantly found more in the gall extracts. However, the gall extracts were strong radical scavenging because of the highest level of phenolics. The non-galled leaves extracts have the highest flavonoid and tannin contents while the gall extracts showed the lowest contents. These results suggested that the phenolic, flavonoid and tannin contents were best extracted with acetone for both the gall and the host plant. Due to the high antioxidant activities were observed, it is suggested that they can be used as a natural agent in food, medicinal and pharmaceutical applications. This study clearly indicates that the gall derived remedies may have distinct therapeutic effect as compared with analogues produced from other parts of the host plant. The galls can also be used for the prevention and treatment of various diseases. Although cynipid diversity is rich in worldwide, only gall extracts of a few species have been studied so far. Further studies are necessary to determine some biological characteristics of other cynipid galls extract for the food industry and medicine.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

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