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Research Article

Determination of secondary metabolite in galls of some cynipid wasps (Hymenoptera: Cynipidae) and characterization of the phenolic compound

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Abstract: The galls of cynipid species (Hymenoptera: Cynipidae) have been used since ancient times as an important source of bioactive compounds. Many researchers have evaluated the medicinal potential of some cynipid galls and found that these galls have numerous ethnomedical uses. The aim of this study was to determine the total bioactive (phenolic, flavonoid and tannin) compound amounts of gall extracts, to reveal the phenolic compound contents by HPLC method and to set ground for future pharmaceutical studies. The galls of cynipid wasps (totally 24 taxa) on host plants were collected from the Eastern Black Sea Region of Türkiye. Acetone, ethanol, methanol, and water extracts of these galls were prepared for quantity analysis and HPLC. The phenolic compound amounts (phenolic, flavonoid and tannin) of the cynipid gall extracts were determined, and their phenolic compound contents were also revealed. Some phenolic compounds in ethanol gall extracts were analyzed using HPLC, and some of these compounds were detected for the first time in the cynipid galls. 2,5-dihydroxybenzoic acid, caffeic acid, epicatechin, and ellagic acid are the most abundant in the ethanolic gall extracts. Total phenolic, flavonoid and tannin amounts of the cynipid gall extracts showed high variation. All these studies on quantification and characterization of phenolic compound are the first detailed studies on these taxa of cynipid galls and show that these cynipid galls might pharmaceutically be an important source for human and animal health.

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1. INTRODUCTION

Phenolic compounds, known more than 8.000 structures, are the most widely distributed secondary metabolites in plants (Del Rio *et al*., 2013; Mammadov, 2014; Vuolo *et al*., 2019). The accumulation of phenolic compounds, which play very important roles in metabolism, in the plant cell is important for the life of the plant (Mammadov, 2014). The production of the phenolic compound by plants enables them to cope with changing environmental challenges (intense light, low temperature, nutrient deficiency, etc.) throughout the course of evolution (Lattanzio, 2013). Phenolic compounds act as protective agents, inhibitors, natural toxic substances, and pesticides to defend natural plants against herbivores, nematodes, phytophagic

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insects and pathogens (fungi and bacteria) (Cornell & Hawkins, 2003; Bhattacharya *et al*., 2010; Lattanzio, 2013). In addition, they contribute to the nutritional, colour, and sensory properties of vegetables and fruits (Chikezie *et al*., 2015). Currently, numerous scientific literature reports considered as important compounds for human health owing to their antioxidant activity, antidiabetic, antimutagenic, anti-inflammatory, cardioprotective, neuroprotective, antitumor, and antiaging properties, etc. (Zhang *et al*., 2018; Vuolo *et al*., 2019).

Host plants defend their own tissues through secondary metabolite production and accumulation instead of staying silent against herbivorous insects (Fürstenberg-Hägg *et al*., 2013). Gall-inducing groups in insects affect plant tissues for their own benefit (Mani, 1964; Stone & Schönrogge, 2003; Giron *et al*., 2016; Oliveira *et al*., 2016). It is thought that the secondary metabolite accumulation induces gall development on host plant (Oliveira *et al*., 2014). The host plants form a new functional structure (gall) to protect their vascular bundles (Isaias *et al*., 2013). The gall provides a microenvironment in which insects can feed and develop (Price *et al*., 1986, 1987; Stone & Schönrogge, 2003). As a result, plants have found the best way in the evolutionary process by offering them food sources instead of chemical counterattack against gall-inducing insects (Stone & Schönrogge, 2003).

In accordance with the histochemical research, it was revealed that the most common secondary metabolite groups in galls are: (1) phenolic compounds, (2) terpenes and (3) alkaloids (Kuster *et al*., 2020). In the inner and outer tissues of galls, phenolic compounds are found in different proportions. Phenolic compounds, which are rare in inner tissues, allow insect larvae to feed on these tissues (Abrahamson *et al*., 1991; Bronner 1992; Isaias *et al*., 2000; Cuevas-Reyes *et al*., 2004; Detoni *et al*., 2010; Ferreira *et al*., 2017). While the metabolites accumulated in the outer tissues of the galls protect the gall-inducing insects, the inner tissues serve as a food source (Bragança *et al*., 2017). The species-specific morphological structure of galls depends on the storage of phenolic compounds in different gall tissues. The conservative feature of gall tissues due to their phytochemical content is seen in all galls, regardless of the herbivore taxa (Kuster *et al*., 2020).

Gall wasps or cynipids belonging to Cynipidae (Insecta: Hymenoptera) are one of the important insect groups that induce gall on host plants. The most important host plants of gall-inducer cynipids, which has roughly 1.400 species in the world (Buffington *et al*., 2020) and 165 species in Türkiye (Azmaz & Katılmış, 2017, 2020a, 2020b, 2021a, 2021b; Azmaz, 2021; Bayrak & Avcı, 2019; Mutun *et al*., 2020; Demirel *et al*., 2022, 2023; Fatih & Gençer, 2022; Tataroğlu & Katılmış, 2022), are oaks (*Quercus* L.), other Fagaceae genera closely related to oaks (*Castanea* Miller, *Castanopsis* (D.Don) Spach, *Chrysolepis* Hjelmq., *Lithocarpus* Blume, *Notholithocarpus* Manos, Cannon & S.H.Oh), and roses (*Rosa* L.). Besides, other plant families (such as Asteraceae, Lamiaceae, Rosaceae and Papaveraceae) are host plant groups for gall wasps (Ronquist *et al*., 2015; Buffington *et al*., 2020).

The cynipid galls have been used in folk medicine owing to their therapeutic properties since ancient times (Oefele, 1933; Schimitschek, 1953; Imtiyaz *et al*., 2013; Iminjan *et al*., 2014; Elham *et al*., 2021). Much research on cynipid gall extracts in the last two decades has revealed various biological activities of the cynipid galls (Gao *et al*., 2018; Iylia Arina & Harisun, 2019; Azmaz *et al*., 2020; Hu *et al*., 2020; Kılınçarslan Aksoy *et al*., 2020; Yusof & Abdullah, 2020). It is very important to determine the phytochemical (phenolic compound, flavonoid etc.) contents of the cynipid galls, which have high tannin content and different phenolic compounds (Taper & Case, 1987). Therefore, the study aimed to investigate the amounts of the total phenolic compounds (phenolic, flavonoid, and tannin) of the gall extracts belonging to different cynipid species. Besides, it was aimed to compare their amounts of phenolic compounds of all extracts, and to reveal characterization of the phenolic compounds for future studies as well.

2. MATERIAL and METHODS

2.1. Collection of Cynipid Galls and Preparation of Gall Extracts

Galls of cynipid species (Cynipidae) were collected from host plants (mostly oak species) distributed in the Eastern Black Sea Region, Türkiye between 2017 and 2019. In total, the galls of 20 different cynipid species were collected from their host plant to obtain the extract (Table 1). In addition, the galls of the four cynipid species (*Andricus assarehi*, *A. kollari*, *A. quercustozae*, and *Cynips quercusfolii*) were also collected from different host plants for comparison (Table 1). The cynipid species were identified by the Entomology Research Laboratory at Pamukkale University. Extracts and secondary metabolite studies were carried out in the Secondary Metabolite Laboratory, Pamukkale University.

Table 1. Cynipid galls collected and their host plants from the study area.

Cynipid Galls	Host Plant	Abbreviations of Gall Extracts in Tables 2-6
Asexual galls of *Andricus assarehi* Melika & Sadeghi, 2008	*Q. macranthera* subsp. *syspirensis*	AAQM
Asexual galls of *A. assarehi*	*Q. petraea* subsp. *iberica*	AAQP
Asexual galls of *A. caputmedusae* (Hartig, 1843)	*Q. macranthera* subsp. *syspirensis*	ACQM
Asexual galls of *A. fecundatrix* (Hartig, 1840)	*Q. petraea* subsp. *iberica*	AFQP
Asexual galls of *A. infectorius* (Hartig, 1843)	*Q. petraea* subsp. *iberica*	AIQP
Asexual galls of *A. kollari* (Hartig, 1843)	*Q. macranthera* subsp. *syspirensis*	AKQM
Asexual galls of *A. kollari*	*Q. petraea* subsp. *iberica*	AKQP
Asexual galls of *A. lignicolus* (Hartig, 1840)	*Q. petraea* subsp. *iberica*	ALQP
Asexual galls of *A. mitratus* (Mayr, 1870)	*Q. petraea* subsp. *iberica*	AMQP
Asexual galls of *A. polycerus* (Giraud, 1859)	*Q. macranthera* subsp. *syspirensis*	APQM
Asexual galls of *A. quercustozae* (Bosc, 1792)	*Q. infectoria*	AQQI
Asexual galls of *A. quercustozae*	*Q. macranthera* subsp. *syspirensis*	AQQM
Asexual galls of *Aphelonyx cerricola* (Giraud, 1859)	*Q. cerris*	ACQC
Asexual galls of *Ap. persica* Melika, Stone, Sadeghi & Pujade-Villar, 2004	*Q. cerris*	APQC
Sexual galls of *Biorhiza pallida* (Olivier, 1791)	*Q. petraea* subsp. *iberica*	BPQP
Asexual galls of *Cynips baskalei* Azmaz & Katılmış, 2020	*Q. petraea* subsp. *iberica*	CBQP
Asexual galls of *C. korsakovi* Belizin, 1961	*Q. macranthera* subsp. *syspirensis*	CKQM
Asexual galls of *C. quercus* (Fourcroy, 1785)	*Q. petraea* subsp. *iberica*	CQQP
Asexual galls of *C. quercusfolii* (Linnaeus, 1758)	*Q. macranthera* subsp. *syspirensis*	CQFQM
Asexual galls of *C. quercusfolii*	*Q. petraea* subsp. *iberica*	CQFQP
Sexual galls of *Diplolepis fructuum* (Rübsaamen, 1895)	*Rosa canina*	DFRC
Sexual galls of *D. mayri* (Schlechtendal, 1876)	*R. canina*	DMRC
Sexual galls of *D. rosae* (Linnaeus, 1758)	*R. canina*	DRRC
Sexual galls of *Synophrus politus* Hartig, 1843	*Q. cerris*	SPQC

Thirty-sixty galls without adults/larvae (depending on the gall size) belonging to each cynipid species were dried in the shadow and broken into small pieces with an electric blender. Samples of small gall part were transferred into beakers. Acetone, ethanol, methanol and water (dH_2O) were separately added in the ratio of 1:10 and were put in water bath at 55ºC for 6 h. The extraction mixture was separated from the residue using filter paper. This process was

repeated twice. Then, the solvents (acetone, methanol, and ethanol) of extract samples were removed using a rotary evaporator (BUCHI, rotavapor R-210/R-215, Germany). Also, the water extract was lyophilized using a freeze dryer (Thermosavant Modulyo D, USA). After removing the solvents, the gall extracts were obtained (Mammadov *et al*., 2011) and stored in the Entomology Research Laboratory, Pamukkale University, Türkiye.

2.2. Determination of Total Phenolic Components

2.2.1. *Quantification of total phenolic compound*

The Folin–Ciocalteu method (Slinkard & Singleton, 1977) with slight modification was used to determine the total phenolic amounts of each gall extract prepared with different solvents (acetone, ethanol, methanol, and water). 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 h, room temperature). The total phenolic amount was expressed as equivalents of gallic acid (mgGAEs/g).

2.2.2. *Quantification of total flavonoid compound*

The total flavonoid amounts of each gall extract prepared with different solvents (acetone, ethanol, methanol and water) were analyzed according to the method by Arvouet-Grand *et al*. (1994). 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 amount was expressed as equivalents of quercetin (mgQEs/g).

2.2.3. *Quantification of total tannin compound*

The vanillin method (Bekir *et al*., 2013) with slight modification was used for analyzing the total tannin amount of gall extracts prepared with different solvents (acetone, ethanol, methanol and water). 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 amount was expressed as equivalents of (+)-catechin (mgCEs/g).

2.3. Characterization of Phenolic Compounds

The phenolic compound amounts of the ethanolic gall extracts were analyzed by high-performance liquid chromatography (HPLC) according to the method described by Caponio *et al*. (1999) with some modifications. Ethanol gall extracts were preferred for the HPLC method because ethanol dissolves only polar substances (like phenolic compounds) owing to it being a polar solvent. Detection and quantification were performed with a diode array detector (SPD–M20A), a pump (LC–20AT), a column heater (CTO–10ASVp), autosampler (SIL–20ACHT), the system controller (SCL–10Avp) and degasser (DGU–14A). 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 extract.

2.4. Statistical Analysis

The SPSS Statistical Package program (SPSS Statistics Version 25) was used to analyze the results. The results were presented as mean ±SD. Since the data did not show normal distribution (Kolmogorov-Smirnov Test, $p>0.05$), differentiation among the extracted groups

was tested using the Kruskal-Wallis H Test, which is a non-parametric test used for many groups, was performed. Besides, it was statistically performed pairwise comparisons among groups. As a result of pairwise comparisons, the same letters indicate two groups with statistically significant differences (p<0.05). It is stated that there is no significant difference in the groups without any letter (p>0.05).

3. RESULTS

The amounts of total phenolic, total flavonoid, and total tannin compounds of cynipid gall extracts were determined. When acetone extracts of the cynipid galls were compared, the extract of the *A. assarehi* galls (collected from *Q. macranthera*) had the highest amount of total phenolic (378.73±13.6 mgGAEs/g), while the extract of the *A. polycerus* galls had the highest amount of total flavonoid (108.85±3.37 mgQEs/g) and the extract of *B. pallida* galls had the highest amount of total tannin (205.05±5.55 mgCEs/g). It was observed that there were significant differences among the groups in terms of the amounts of phenolic, flavonoid and tannin of acetone gall extracts (p<0.05). On the other hand, there were no statistically significant differences among the acetonic extracts of the galls belonging to the same cynipid species collected from two different host plants (p>0.05) (Table 2).

Table 2. Total amounts of phenolic, flavonoid and tannin compounds of acetone gall extracts.

Extracts of Cynipid Galls	Total Amount of Phenolic (mgGAEs/g)	Total Amount of Flavonoid (mgQEs/g)	Total Amount of Tannin (mgCEs/g)
AAQM	378.73±13.6 ab	35.9±2.33	21.04±1.13 c
AAQP	148.10±9.81	29.90±0.50	90.68±14.84
ACQM	273.93±13.70	49.44±0.63	169.22±9.94
AFQP	184.77±4.01	59.41±1.27	14.61±0.03 ab
AIQP	281.64±7.63	59.19±0.25	25.73±1.18
AKQM	249.35±18.58	55.83±2.92	30.69±0.17
AKQP	284.56±10.29	64.29±0.84	192.83±2.88 a
ALQP	295.39±0.35	101.66±0.38 a	149.50±5.77
AMQP	87.89±9.38	9.90±0.27 ab	113.12±0.13
APQM	225.60±17.63	108.85±3.37 bc	113.20±0.00
AQQI	189.77±8.32	35.81±1.25	180.33±3.62
AQQM	103.31±10.84	29.10±0.41	30.68±4.17
ACQC	217.27±16.03	50.24±1.45	121.99±7.63
APQC	294.98±13.75	59.30±0.44	37.55±0.19
BPQP	239.98±8.48	50.44±0.67	205.05±5.55 bc
CBQP	216.43±14.07	43.73±0.02	97.55±6.47
CKQM	113.31±4.96	12.08±0.22	67.74±5.84
CQQP	73.93±5.55 b	24.01±1.43	82.83±5.83
CQFQM	82.06±4.88	11.77±0.17	121.16±3.81
CQFQP	167.68±11.97	11.48±0.13 c	84.11±2.82
DFRC	279.98±22.72	31.01±1.16	39.40±3.61
DMRC	271.44±17.50	25.16±0.25	35.81±2.00
DRRC	227.27±5.01	31.35±0.01	25.50±0.71
SPQC	1.43±0.62 a	33.34±1.12	83.24±0.95

GAE: Gallic Acid Equivalents, QE: Quercetin Equivalents, CE: Catechin Equivalents; values (the mean of the measurements ±SD). As a result of pairwise comparisons, the same letters indicate two groups with statistically significant differences (p<0.05). It is stated that there is no significant difference in the groups without any letter (p>0.05).

When ethanol extracts of the cynipid galls were compared, the extract of the *A. assarehi* galls (collected from *Q. macranthera*) had the highest total phenolic compound (349.35±15.94 mgGAEs/g), while the extract of the *A. lignicolus* galls had the highest total flavonoid compound (102.01±0.32 mgQEs/g) and the extract of *A. quercustozae* galls (collected from *Q. infectoria*) had the highest total tannin compound (112.55±8.38 mgCEs/g). It was revealed that the amounts of phenolic, flavonoid and tannin compound of ethanol gall extracts were significantly different from each other (p<0.05). However, it was not found statistically significant differences among the ethanol extracts of the galls belonging to the same cynipid species collected from two different host plants (p>0.05) (Table 3).

Table 3. Total amounts of phenolic, flavonoid and tannin compounds of ethanol gall extracts.

Extracts of Cynipid Galls	Total Amount of Phenolic (mgGAEs/g)	Total Amount of Flavonoid (mgQEs/g)	Total Amount of Tannin (mgCEs/g)
AAQM	349.35±15.94 cd	42.57±0.27	5.98±0.30
AAQP	165.60±8.48	40.60±0.19	21.07±0.84
ACQM	244.56±8.67	76.41±2.06	34.77±1.73
AFQP	192.27±9.39	63.84±0.11	8.52±0.15
AIQP	217.27±18.46	81.35±0.64 b	10.87±0.72
AKQM	237.47±11.61	54.31±0.49	1.82±0.47 b
AKQP	314.14±4.60 a	72.37±0.66	70.61±7.69
ALQP	288.93±10.32	102.01±0.32 cd	38.94±5.02
AMQP	128.73±5.08	19.39±0.11	36.28±0.80
APQM	207.69±9.01	83.06±4.81 a	12.60±0.27
AQQI	145.18±7.20	41.41±0.02	112.55±8.38 abc
AQQM	185.18±12.07	32.03±0.27	10.25±0.21
ACQC	155.81±13.91	61.88±0.41	76.16±2.20
APQC	314.56±12.07 b	67.91±0.82	3.18±0.08
BPQP	240.81±4.10	63.83±0.03	71.16±3.62
CBQP	148.72±8.86	60.56±0.53	74.22±5.54
CKQM	146.64±3.65	14.46±0.29	1.80±0.19 c
CQQP	52.48±3.44	10.76±0.27 d	66.16±5.83
CQFQM	30.81±2.50 d	11.09±0.08	15.33±1.44
CQFQP	73.94±2.50	9.08±0.24 abc	1.26±0.11 a
DFRC	286.22±11.27	23.87±0.28	20.22±0.42
DMRC	271.85±8.39	32.78±0.04	11.58±0.67
DRRC	261.22±0.72	44.47±0.23	9.66±0.23
SPQC	7.06±1.25 abc	16.58±0.13	14.90±0.81

GAE: Gallic Acid Equivalents, QE: Quercetin Equivalents, CE: Catechin Equivalents; values (the mean of the measurements ±SD). As a result of pairwise comparisons, the same letters indicate two groups with statistically significant differences (p<0.05). It is stated that there is no significant difference in the groups without any letter (p>0.05).

When methanol extracts of the cynipid galls were compared, the extract of the *A. assarehi* galls (collected from *Q. macranthera*) had the highest total phenolic amount (403.73±1.57 mgGAEs/g), while the extract of the *A. lignicolus* galls had the highest total flavonoid amount (102.17±0.19 mgQEs/g) and the extract of *A. caputmedusae* galls had the highest total tannin amount (109.22±5.09 mgCEs/g). It was found that phenolic, flavonoid and tannin amounts of methanol gall extracts were significantly different from each other (p<0.05), while there were

no statistically significant differences among the methanol extracts of the galls belonging to the same cynipid species collected from two different host plants (p>0.05) (Table 4).

Table 4. Total amounts of phenolic, flavonoid and tannin compounds of methanol gall extracts.

Extracts of Cynipid Galls	Total Amount of Phenolic (mgGAEs/g)	Total Amount of Flavonoid (mgQEs/g)	Total Amount of Tannin (mgCEs/g)
AAQM	403.73±1.57 bc	51.36±0.43	7.91±1.35
AAQP	255.81±11.57	52.84±1.73	15.31±0.87
ACQM	233.94±8.75	62.03±0.47	109.22±5.09 bc
AFQP	205.39±3.65	76.52±1.96	7.54±0.53
AIQP	227.27±17.69	97.27±0.90 a	9.73±0.29
AKQM	234.14±12.63	71.07±0.97	2.62±0.19
AKQP	264.98±7.91	73.82±0.74	63.94±4.11
ALQP	388.52±6.56 a	102.17±0.19 bc	43.66±2.88
AMQP	106.23±4.15	20.34±0.33	43.55±2.41
APQM	283.31±10.95	58.02±0.21	12.05±0.30
AQQI	200.81±7.80	42.87±0.67	78.94±6.36
AQQM	188.31±6.25	42.21±0.17	12.11±0.13
ACQC	113.94±1.25	88.46±0.41	78.66±8.03
APQC	343.93±9.20	78.99±1.22	2.77±0.20
BPQP	222.89±3.14	65.36±0.65	101.16±1.66 a
CBQP	187.48±19.59	71.83±1.68	67.83±4.63
CKQM	185.81±12.17	11.67±0.16 c	1.90±0.17 c
CQQP	75.39±3.76	10.58±0.39 ab	59.22±1.73
CQFQM	42.68±4.09 c	16.43±0.27	6.44±1.27
CQFQP	99.14±4.51	12.56±0.40	1.65±0.09 ab
DFRC	243.93±21.00	35.59±2.06	20.16±1.07
DMRC	277.89±3.14	50.80±1.14	14.87±0.31
DRRC	270.18±2.72	55.68±0.37	10.91±0.10
SPQC	22.89±1.90 ab	17.64±0.16	25.82±0.82

GAE: Gallic Acid Equivalents, QE: Quercetin Equivalents, CE: Catechin Equivalents; values (the mean of the measurements ±SD). As a result of pairwise comparisons, the same letters indicate two groups with statistically significant differences (p<0.05). It is stated that there is no significant difference in the groups without any letter (p>0.05).

When water extracts of the cynipid galls were compared, the extract of the *A. kollari* galls (collected from *Q. petraea*) had the highest total phenolic content (342.06±6.58 mgGAEs/g), while the extracts of the *A. assarehi*, *Ap. cerricola*, *B. pallida* and *C. baskalei* galls had the highest total flavonoid content and the extract of *Ap. cerricola* gall had the highest total tannin content (84.22±8.39 mgCEs/g). It was determined that phenolic, flavonoid and tannin contents of water gall extracts were significantly different among groups (p<0.05). On the other hand, there was not a statistically significant difference among the water extracts of the galls belonging to the same cynipid species collected from two different host plants (p>0.05) (Table 5).

Table 5. Total amounts of phenolic, flavonoid and tannin compounds of water gall extracts.

Extracts of Cynipid Galls	Total Amount of Phenolic (mgGAEs/g)	Total Amount of Flavonoid (mgQEs/g)	Total Amount of Tannin (mgCEs/g)
AAQM	241.23±3.20	53.67±0.21	3.27±0.16
AAQP	262.89±5.63	118.42±0.00 a	5.71±0.68
ACQM	176.22±6.88	74.25±0.47	82.55±3.36 b
AFQP	205.60±0.95	97.45±0.96	5.32±0.10
AIQP	176.43±17.12	109.65±0.45	8.42±0.68
AKQM	221.23±7.55	73.85±1.07	2.12±0.19
AKQP	342.06±6.58 bc	100.39±2.65	80.05±4.28 a
ALQP	260.81±4.88	103.44±0.37	37.55±3.15
AMQP	186.85±5.24	117.35±1.85	8.95±0.64
APQM	187.27±7.50	96.47±2.96	7.46±0.28
AQQI	242.89±8.88	61.19±1.44	48.66±2.88
AQQM	178.10±3.14	58.75±0.87	7.13±0.39
ACQC	199.67±5.94	118.40±0.02	84.22±8.39 c
APQC	327.68±1.65 a	112.93±0.43	4.03±0.31
BPQP	162.89±3.20	118.40±0.02	71.16±3.81
CBQP	136.85±10.48	118.40±0.02	68.67±5.00
CKQM	242.27±3.76	20.00±0.56	2.65±0.19
CQQP	44.35±5.39	17.09±0.20	47.83±5.83
CQFQM	40.60±2.52 c	12.07±0.27 a	22.83±1.66
CQFQP	64.98±1.30	13.05±0.14	0.41±0.00 abc
DFRC	219.98±13.92	41.95±1.19	19.51±0.65
DMRC	231.22±13.82	52.47±1.33	15.01±1.30
DRRC	259.77±7.71	84.11±1.86	6.70±0.19
SPQC	34.56±0.62 ab	14.03±0.24	16.55±0.20

GAE: Gallic Acid Equivalents, QE: Quercetin Equivalents, CE: Catechin Equivalents; values (the mean of the measurements ±SD). As a result of pairwise comparisons, the same letters indicate two groups with statistically significant differences ($p<0.05$). It is stated that there is no significant difference in the groups without any letter ($p>0.05$).

The characterization of the phenolic compounds of ethanolic gall extracts were determined by HPLC method using 15 standard phenolic compounds. 2,5-dihydroxybenzoic acid, caffeic acid, epicatechin and ellagic acid were the most abundant compounds in the gall samples. Caffeic acid was detected in 15 of the samples, followed by epicatechin in eight samples and 2,5-dihydroxybenzoic acid in only one sample (Table 6). The amount of standard phenolic compound of gall samples is given in Table 6.

Table 6. Phenolic compound characterization of ethanol gall extracts.

Extracts of Cynipid Galls	Gallic Acid (µg/g)	3,4-dihydroxybenzoic Acid (µg/g)	4-hydroxybenzoic Acid (µg/g)	2,5-dihydroxybenzoic Acid (µg/g)	Chlorogenic Acid (µg/g)	Vanillic Acid (µg/g)	Epicatechin (µg/g)	Caffeic Acid (µg/g)	*p*-coumaric Acid (µg/g)	Ferulic Acid (µg/g)	Rutin (µg/g)	Ellagic Acid (µg/g)	Naringin (µg/g)	Quercetin (µg/g)	Cinnamic Acid (µg/g)
AAQM	146.1	321.6	43.7	2958.1	68.0	515.7	5951.9*	5514.6	0.03	7.4	< LOD	2154.8	34.5	42.6	21.9
AAQP	1021.1	270.3	634.0	3909.8	103.0	626.9	26612.4	39786.0*	< LOD	< LOD	< LOD	5026.2	245.8	< LOD	286.7
ACQM	193.2	309.1	680.2	4664.9	39.8	452.5	133082.8*	37228.8	28.8	145.0	10.6	1525.1	2182.9	409.0	0.1
AFQP	781.0	534.2	604.5	7329.1	489.8	524.0	13833.8	203835.8*	0.03	13.2	365.0	1281.1	0.8	58.0	138.0
AIQP	1371.7	1333.3	1495.8	6692.1	1678.5	412.0	3185.8	192029.6*	145.0	239.6	789.8	5092.1	1619.1	2967.0	213.6
AKQM	196.3	298.8	161.2	2773.3	196.2	501.2	4425.9	8026.5*	8.0	< LOD	< LOD	479.2	89.4	19.7	17.4
AKQP	656.9	756.8	757.3	11696.4	531.4	1150.9	123037.3*	12812.5	20.2	87.3	89.5	2010.3	53.1	7.4	22.8
ALQP	557.5	784.3	999.7	23380.3	764.4	1064.2	129063.5*	48674.2	77.2	280.5	4.6	4885.5	6573.5	295.4	804.5
AMQP	0.1	2.2	1.4	36.1	0.1	7.3	62.8*	55.3	0.05	< LOD	< LOD	36.4	4.8	< LOD	0.7
APQM	1539.0	261.9	990.2	14137.6	855.7	2639.3	18985.5	74183.1*	< LOD	< LOD	< LOD	11704.1	154.9	1041.7	644.9
AQQI	277.3	291.7	540.9	2839.0	63.4	238.7	36106.6*	33252.7	31.5	132.3	874.9	1807.9	814.7	27.6	11.3
AQQM	958.0	106.9	10.4	3091.9	100.1	479.2	5284.1	5290.0*	9.5	42.1	< LOD	1130.2	249.5	110.1	5.0
ACQC	1645.5	222.8	498.7	5356.2	1006.5	457.9	740.7	7354.5*	12.0	13.3	190.9	713.5	0.7	324.8	86.7
APQC	1458.0	2235.0	699.3	31286.9*	1592.5	597.6	3382.8	29320.6	29.1	60.8	770.5	4410.8	20.9	214.5	262.5
BPQP	531.3	678.1	587.5	2838.9	33.8	256.5	27879.8*	12955.1	66.4	529.2	273.6	1536.2	1646.9	378.8	224.1
CBQP	1610.5	228.7	1006.5	5334.8	786.3	992.1	3501.1	115369.9*	3.3	< LOD	105.4	2161.8	0.7	554.9	15.4
CKQM	1455.8	426.8	263.5	8058.9	3859.4	513.7	19662.6	90831.9*	< LOD	< LOD	< LOD	1439.3	9069.7	< LOD	2.0
CQQP	585.6	189.5	195.6	5447.3	267.2	1023.9	20441.0	70714.4*	43.9	149.3	716.5	444.3	5023.6	3.2	13.0
CQFQM	446.6	192.9	206.6	3257.7	77.4	598.9	29164.7*	7757.2	22.2	4.2	234.5	288.2	2736.6	0.2	5.6
CQFQP	28.7	97.0	629.1	8614.3	386.2	690.5	26219.8	83604.7*	< LOD	< LOD	< LOD	2217.1	150.1	17.4	62.3
DFRC	32.6	298.0	454.5	7821.2	258.8	2979.0	2408.5	10828.2*	21.8	17.5	< LOD	2994.3	6.0	12.0	13.2
DMRC	289.6	2949.8	6732.5	82015.2	2636.4	13172.9	402573.4	701264.4*	961.1	383.3	43.1	53626.2	724.1	2.1	748.6
DRRC	1004.3	1611.1	1519.1	44437.5	1261.0	1057.8	866.4	68135.7*	0.3	70.8	427.9	8926.7	3270.9	412.5	278.9
SPQC	128.3	41.6	37.6	988.6	31.8	60.2	2039.9	3383.8*	< LOD	< LOD	< LOD	369.2	0.9	3.8	26.2
Retention Time (min)	6.8	10.7	15.7	17.2	18.2	19.2	21.3	22.7	26.1	30.1	45.6	47.7	49.7	70.4	71.1

LOD: Limit of Detection; * Maximum Value

4. DISCUSSION and CONCLUSION

In the past, the cynipid galls have been used in both Western and Eastern folks in traditional medicine against various diseases and have taken their place in codex books (The British Pharmaceutical Codex, etc.) (Galla, 1911; Larew, 1987; Yılmaz Sarıözlü & Kıvanç, 2011). In ancient Chinese medical sources, the cynipid galls have been used against many diseases such as cancer (Gao *et al*., 2018). Many studies have been carried out about the bioactive compounds and biological properties of the galls in especially oriental countries (Asif *et al*., 2012; Noori *et al*., 2015; Iylia Arina & Harisun, 2019; Kot *et al*., 2019; Azmaz *et al*., 2020; Sukor *et al*., 2020). The cynipid galls are known to have both a greater variety and greater amounts of bioactive compounds compared to their host plant (Hartley, 1998; Gao *et al*., 2018). However, although most of the phytochemical studies on galls today are about the identification or isolation of tannin compounds, the subject of future studies should be on other groups of secondary metabolites (flavonoids, terpenoids, etc.) contained in the galls.

Experiments carried out to reveal the amounts of phenolic compounds (phenolic, flavonoid and tannin) of different cynipid gall samples showed that gall samples prepared using different solvents contained large amounts of phenolic compounds and were different from each other in terms of their amounts of compound.

The gall samples with the high amount of phenolic, flavonoid, and tannin mostly belong to the same genus (*Andricus*). The amounts of phenolic compounds of galls developed both in oak buds or acorns (mostly *Andricus* spp. galls) and galls formed in *Rosa* fruits were found to be higher than galls formed in oak leaves. It has been determined that *S. politus* gall contains very low levels of phenolic compounds. The amounts of phenolic compounds (phenolic, flavonoid, tannin) contained in galls are related to where the galls develop on the host plant. In young plant tissues, secondary metabolites are synthesized in higher amounts than older plant parts due to their diverse metabolic activities (Achakzai *et al*., 2009; Barton & Koricheva, 2010; Chomel *et al*., 2016; Hussein & El-Anssary, 2019; Kariñho-Betancourt *et al*., 2019). Numerous studies have shown the effect of galling organisms on the host plant metabolism, either by inhibiting, maintaining or inducing the synthesis of new compounds (Rokas *et al*., 2003; Stone & Schönrogge, 2003).

The amount of phenolic compound in cynipid gall extracts (*A. assarehi, A. lignicolus, Ap. persica*) was found to be high. It is well established that secondary metabolites amount and types are affected by soil type (Eyüpoğlu, 1999; Özyazıcı *et al*., 2013; Mammadov, 2014). We consider that the differences in the amounts of phenolic compounds of gall extracts may be due to the soil type where the plant of gall is grown. In addition, the amount of phenolic compounds in galls is related to the part of the plant where the galls develop and also depends on host plant species. As a result, due to some factors such as these, differences in phenolic amounts of gall extracts were detected.

Historically, the cynipid galls have been used as a natural therapeutic resource in traditional medicine with more ethnopharmacological applications than modern medicine. In this study, cynipid galls belonging to different cynipid species were different and had the amounts of high phenolic compounds (phenolic, flavonoid, tannin) and because of this reason, they may be useful in pharmaceutical applications against various diseases, however further studies are required to test this hypothesis.

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Declaration of Conflicting Interests and Ethics

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

Authorship Contribution Statement

Musa Tataroglu: Investigation, Resources, Visualization, Methodology, Software, Formal Analysis, and Writing - original draft. **Ozge Kilincarslan Aksoy:** Investigation, Resources, Visualization, Methodology, Software, Formal Analysis, and Writing - original draft. **Yusuf Katilmis:** Resources, Methodology, Supervision, Writing-Reviewing, and Validation. **Ramazan Mammadov:** Resources, Methodology, Supervision, Writing-Reviewing, and Validation.

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