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PHYTOCHEMICAL PROFILE OF *AGRIMONIA EUPATORIA* L. FROM BULGARIA AND EFFECTS OF ITS EXTRACTS ON *GALLERIA MELLONELLA* (L.) (LEPIDOPTERA: PYRALIDAE) LARVAE

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ABSTRACT

Plants contain polyphenolic compounds such as phenolic acids, flavonoids, stilbenes, lignans, essential oils, *etc*., which are endowed with antioxidant properties. This study aimed to correlate the total phenolic compound and flavonoid content of *Agrimonia eupatoria* L. with its antioxidant properties and to determine some mineral elements (Na, K, Ca, Mg, P). The antioxidant capacity of the extract was tested by three methods (DPPH, FRAP, and TEAC). According to the results, the antioxidant content of *A. eupatoria* was found to be high (IC_{50}=38.03±0.01µg/mL). Total phenolic and flavonoid contents were found as 13.66±0.38 mg GAE/g and 4.65±0.01mg QE/g, respectively. Besides, the major components found in *A. eupatoria* were α-pinene (62.72%), n-hexadecanoic acid (11.41%), (5E,9E)-farnesyl acetone (6.64%), and (5E,9Z)-farnesyl acetone (3.65%). Heavy metal content in *A. eupatoria* was found within WHO limits. It was also investigated whether *A. eupatoria* has toxic effects. Because a medicinal plant is not supposed to harm metabolism, for this investigation, larvae stage of *Galleria mellonella* had selected as a test organism. In order to investigate the toxic effects, oxidative stress parameters (SOD, CAT, GST, GPx, MDA) and AChE activity were measured. And no harmful effects were observed at the doses administered at 24, 48, and 72 h.

1.Introduction

The vast variety of medicinal plants in Bulgaria, used in different forms, is increasing. Over 80% of the world's population uses various herbal supplements and infusions for treatments. The genus *Agrimonia* are perennial herbaceous flowering plants classified in Division Magnoliophyta, Magnoliopsida class, Rosaceae family, and the *Agrimonia* genus. Two plant species, *Agrimonia eupatoria* L., and *Agrimonia procera* Wallr., are naturally spread in Bulgaria (Petrova *et al*., 1999).

Polysaccharides, tannins, flavonoids, catechins, procyanidins, phytosterols, B vitamins, and vitamin K have been previously identified in *A. eupatoria* (Garcia-Oliveira *et al*., 2020). Polyphenols constituents in the plant species reduce the risk of several diseases or cause a curative effect (Pamukov, 1989). The content of flavonoids and other biologically active components correlates with other secondary metabolites and increases plant species' antioxidant potential and extracts.

Therefore, they form specific structural parts that inactivate free radicals in the body (Pamukov, 1989; Muruzovic *et al*., 2016).

Agrimony is well known for its beneficial effects on various diseases such as liver complaints, gall-bladder stones, diarrhea, edemas, and kidney diseases. Thanks to its diuretic properties, the herb is widely used against atony of the bladder and dysuria. Many other uses of Agrimony in Bulgarian folk medicine are known, such as rheumatism, hemorrhoids, bleeding gums, varicose ulcers; laryngitis; pulmonary and cutaneous tuberculosis. The extracts could be used externally as compressor gargle and internally as an infusion (Pamukov, 1989).

It was reported that more than 50 components have been identified in the essential oil composition of the plant's leaves, flowers, and roots. The major constituents in the wild growing leaf oil were β-caryophyllene, caryophyllene oxide, α-humulene, and E-β-farnesene, and in the cultivated leaf oil were α-pinene, β-caryophyllene, E-α-farnesene, and cumin aldehyde. The main constituents of the essential oil obtained by the wild-growing flowers were β-caryophyllene, E-β-farnesene, and a-humulene, while β-caryophyllene, caryophyllene oxide, and α-copaene were among the main constituents in the cultivated flowers (Muruzovic *et al.,* 2016; Navaei and Mirza, 2009).

The essential oil constituents from the aerial part of *A. asiatica* Juz. were obtained using a steam-distillation method in wild-growing conditions in Kazakhstan. The essential oil extracted from the aerial part of the plant was analyzed by gas chromatography-mass spectroscopy, and its major components amounting to 100% were found to be β-selinene (36.370%), α-panasinsene (21.720%), hexadecanoic acid (7.839%), and 1,2-nonadiene (6.199%) (Kozykeyeva *et al*., 2020).

Galleria mellonella (L.) (Lepidoptera: Pyralidae) is a greater wax moth and found throughout the world. *G. mellonella* has four life stages and eggs are laid in the spring. Investigating the toxicity of medicinal plants is a necessity prior their usage. The vertebrate model organisms used for this objective are often subject to ethical considerations. This has led researchers to prefer invertebrate models. Invertebrate models, including *Galleria mellonella*, are used to assess the toxicity of various products. The larvae of *G. mellonella* are also often used as a model organism in researchs (Arsene *et al.*, 2021).

A detailed phytochemical profile of medicinal plants was necessary due to the local differences in their composition. The studies contributed to the popularization of medicinal plants in Bulgaria and provided an opportunity for their application in phytotherapy and pharmacy. The discovery of new biologically active components in the composition of plant species gives a strong impetus to creating herbal pharmaceutical prototypes in modern medicine.

Therefore, the present study aimed to analyze the phytochemical profile of a traditional Bulgarian phytotherapy species - *Agrimonia eupatoria* L. In addition, since the toxicity tests of medicinal plants are essential for safe use, it was also investigated whether *A. eupatoria* has harmful effects on *Galleria mellonella* larvae.

2. Materials and methods

2.1. Materials

2.1. Plant material

The plants were collected in October 2016 from South Bulgaria (at 553 m elev., 42.85 ° N 26.15 ° E) - these are the coordinates of the village of Bozhevtsi. The samples were collected by hand and dried in ventilated rooms without direct sunlight, and the room air temperature was regulated (18±2°C). After drying, the samples were placed in paper bags for storage. The aerial plant parts were separated into leaves before analysis.

2.2. Methods

2.2.1. Chemical composition of the plants

The moisture of the plants was determined by drying up to the constant weight at 105°C and the results from the chemical analyses were given on a dry weight (DW) basis. The ash

content was determined according to AOAC (2005), by mineralization of the samples at 550°C for 5 h.

2.2.2. Isolation of essential oil

The air-plants (50 g) were cut to a size of 0.5 cm. The essential oil was isolated by hydrodistillation (ratio plant:water = 1:10) for 3 h in a Clevenger-type laboratory glass apparatus (Balinova and Diakov, 1974). The oil obtained was dried over anhydrous sodium sulfate and stored in tightly closed dark vials at 4°C until analysis.

2.2.3. Chromatography–Mass Spectrometry (GC-MS) analysis

The compounds of the essential oil were detected with gas chromatography (GC) (Agilent 7890A), HP-5 column MS (30 m × 250 mm × 0.25 μm), temperature: 35°C/3 min, 5°C/min to 250°C for 3 min, 49 min in total, helium as carrier gas, 1 mL/min constant speed, 30:1 split ratio. A gas chromatography–mass spectrometric (GC/MS) analysis was carried out on an Agilent 5975C mass spectrometer, helium as a carrier gas, column and temperature the same as in the GC analysis. The identification of the chemical compounds was made by comparison to their relative retention time and library data (NIST 08 database; own libraries) (Adams and Robert, 2007). Components were listed according to their retention (Kovat's) indices, calculated using a standard calibration mixture of C_8 - C_{40} n-alkanes in n-hexane. Compound concentration was computed as percentage of the total ion current (TIC).

2.2.4. Protein content

The total protein content was analyzed according to the method of AOAC (2016) with a UDK 152 Kjeldahl System (Velp Scientiffica, Italy). The samples 1.0 g each, were mineralized in 15 mL concentrated H_2SO_4 and catalysts: anhydrous K_2SO_4 and CuSO4. The process was run at 420°C for 60 min. With this method, 40% NaOH was used to produce an alkaline distillation medium and 4% H_3BO_3 in order to collect the distilled ammonia. The titrations were carried out with a standard HCl (0.2 N) solution.

2.2.5. Cellulose content

The content of cellulose (crude fiber) in leaves was determined by a modification of the method by Brendel *et al.* (2000). Hydrolysis of cellulose and hemicellulose was carried out by boiling 1 g of leaves with 16.5 mL of 80% CH_3COOH and 1.5 mL concentrated HNO_3 for 1.5 h. After filtration of the suspension, the solid residue was dried at 105°C for 24 h and weighed.

2.2.6. Total chlorophylls and carotenoid content

For evaluation of chlorophyll a, chlorophyll b and the total carotenoids content, 0.5 g of fresh leaf sample was homogenized with 10 ml extract (80% alkaline acetone) and stored in the dark at 25°C for 24 h. After that, the homogenate was centrifuged at 1500 g for 10 min. Absorbance was measured at 470 nm, 645 nm and 663 nm; then, the results were calculated by the corresponding formulas (Côrte-*Real et al.*, 2017):

Chlorophyll a (mg/L) = 9.784*A663-0.990*A645 (1)

Chlorophyll b (mg/L) = 21.426*A645-4.650*A663 (2)

Total carotenoids content (mg/L) = 4.695*A470-0.268*(chl a + chl b) (3)

Finally, mg/L unit was converted into μg/g.

2.2.7. Nutrient contents

Plant samples (areal part) were dried and 0.5 g was weighed. Then, each sample put into a porcelain crucible. All samples were burned until gray ash (550°C). After burning the 0.5 g weighed samples, the ashes were dissolved in 4 mL 0.1 N HCl and filtered (Whatman No 1), and completed with distilled water (10 mL) (Kaçar and İnal, 2010). Mineral and heavy metal contents was determined by Yozgat Bozok University, Science and Technology Application and Research Center using iCAP-Qc ICP-MS spectrometer (Thermo Scientific).

2.2.8. Total Phenolic Contents

Folin-Ciocalteu Reagent (FCR) method was used to determine the total phenolic content of the extracts (Singleton *et al.,* 1999). The prepared samples were incubated at room temperature (20±1°C) for 2 h and absorbance

measurement was performed at 765 nm. Gallic acid was used for standard phenolic substance control. The values obtained are expressed as gallic acid conjugate. Spectrophotometric measurements to determine the total phenolic content PerkinElmer Lambda 25 UV / VIS made in spectrophotometer device.

2.2.9. Determinations of Total Flavonoid Assay

The total flavonoid compound amounts of the 40 mL methanol extracts were determined by optimizing the aluminum chloride colorimetric method of Biju *et al*., 2014. Then absorbance was measured at 510 nm. As result of quercetin equivalents (QE) g-1 of extract was calculated.

2.2.10. DPPH Free Radical-Scavenging Activity

The plant (leaf) sample (4 g) was mixed by methanol (40 mL) (1/10 w/v). The prepared samples were incubated for 24 h at 40°C in an oven (Electo-mag M 5040 P). Then, it was filtered into balloon flasks (Whatman No 1 filter paper). The methanol in the samples was removed with the help of a rotary evaporator (Heating Bath B-491, BUCHI). The balloon bottles, which were blown up, were kept in the oven for 24 h and completely dried. The extracts were taken into falcon tubes and closed with parafilm and stored at +4°C to be used in the analysis.

The free radical activities of the extracts were determined using DPPH (1,1-diphenyl-2-picrilhydrazyl) free radical, a known and commonly used radical (Gezer *et al*., 2006). Firstly, the amount of extract that defines a certain amount of DPPH radical has been determined, and a comparison has been made between these samples. 16 mg DPPH radical solution was prepared in 100 mL CH_3OH. The DPPH solution to be used in the analysis was prepared as 0.1 µM. By setting 517 nm in the spectrophotometer, DPPH reading was done and dilution was made with methanol until the absorbance value was 1.000. One mg/mL extract solution was prepared as main stock and 6 different concentrations were obtained by dilution. 3 mL samples were taken from each concentration (50, 75, 100, 150, 200, 300) and 1 mL 0.1 µM DPPH was added on top. The reaction mixture was incubated for 30 min in the dark. BHT (butyl hydroxytoluene) and BHA (butyl hydroxyanisol) were used as reference. Radical scavenging activity DPPH was determined as the inhibition percentage and the following formula is used:

Radical scavenging activity DPPH %= [A blank −A sample)/A blank] x100

Spectrophotometric measurements for DPPH radical scavenging activity determination were performed with the aid of PerkinElmer Lambda 25 UV / VIS spectrophotometer device.

2.2.11. Ferric reducing antioxidant power assay (FRAP)

The antioxidant capacity of samples was detected by Benzie and Strain's FRAP assay. Dried samples (4 g) were extracted with distilled water (40 mL) at a temperature from 80 to 105°C for 20 min for fraction I. The residues were extracted with distilled water (60 mL) at a temperature from 100 to 130°C for 30 min for fraction II. After cooling to 25°C, both fractions were filtered. They were combined and dried at 40°C and weighed to detect the yield (Benzie and Strain, 1999). This method measures the ability of antioxidants to reduce ferric iron. This assay evaluates the alteration in absorbance at 620 nm because of the generation of FeII-tripyridyltriazine from oxidised FeIII. The reagent was made ready via mixing acetate buffer (300 mmol/L) with 2,4,6-tripyridyl-s-triazine (10 mmol/L) (TPTZ) in HCl (40 mmol/L) and with ferric chloride (20 mmol/L) at low pH. As the standard, Trolox® was used. Samples were quantified by a spectrophotometer (PerkinElmer Lambda 25 UV / VIS).

2.2.12. Trolox equivalent antioxidant capacity (TEAC) assay

TEAC assay is consisted in the reducing of the absorbance of the ABTS+ (Re *et al*., 1999) at 734 nm. Dried samples (4 g) were extracted with distilled water (40 mL) for 20 min at a temperature of 80 to 105°C to give Fraction I. The residues were extracted with distilled water (60 mL) at a temperature of 100 to 130°C for 30 min to give Fraction II. After cooling to 25°C, both fractions were filtered. These were

combined and dried at 40°C and weighed to determine yield (Benzie and Strain, 1999).

ABTS+ was prepared by reacting ABTS solution with potassium persulfate (2.45 mM). ABTS+ solution was diluted with phosphate buffer for obtaining an absorbance of 0.7±0.02 at 734 nm. Diluted ABTS+ was added to Trolox® standard or biological sample, then this admixture was incubated for 15 min. After this step, at 734 nm, the inhibition in absorbance was evaluated. All evaluations were performed in 6 repetitions. Samples were quantified by a spectrophotometer (PerkinElmer Lambda 25 UV / VIS).

2.2.13. Determination of hydroxyl radical scavenging capacity

Hydroxyl radical scavenging capacity was assessed by detected the ability of leaf sample extracts to reduce the generation of 2-hydroxyterephthalate which is a strongly fluorescent in a reaction between terephthalic acid and hydroxyl radical (Gutteridge and Halliwell, 2010).

2.2.14. Determination of superoxide scavenging capacity

Superoxide scavenging capacity was determined as the superoxide radical inhibition caused decreasing of nitro blue tetrazolium to formazan (McCord and Fridovich, 1999).

Cultivation of Galleria mellonella and application of Agrimonia eupatoria extract to Galleria mellonella larvae

Galleria mellonella were taken from the stock culture of Kırşehir Ahi Evran University, Faculty of Agriculture, Department of Plant Protection. Corn flour, water, bran, milk powder, honey, glycerol, yeast, honey nutrients were used in the cultivation of *G. mellonella*. The last stage larvae were taken from the prepared cultures and used in the experiments. Cultures were placed in an incubator adjusted to 28±2°C, 65±5% relative humidity.

Four groups are formed for this study (control, 5% extract concentration group, 10% extract concentration group and 20% extract concentration group). Different concentrations of *A. eupatoria* extract (5%, 10%, 20%) were injected into the application groups with a microinjector in the amount of 5 µl to the left last leg of each larva. The same amount of distilled water was given to the control group. The duration of the experiment is 24 h, 48 h and 72 h. 20 insects were used for each group. 3 repetitions were made.

Measurement of malondialdehyde (MDA) levels, acetylcholinesterase (AChE), superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and glutathione peroxidase (GPx) activities

Oxidative stress parameters (MDA levels, SOD, CAT, GST, GPx) and AChE activities were measured according to following procedures.

MDA: Ohkawa *et al.* (1979)
GST: Habig *et al.* (1974)
SOD: Marklund and Marklund (1974)
CAT: Aebi (1984)
GPx: Paglia and Valentine (1961)
AChE: Ellman *et al.* (1961)

2.2.15. Statistics

All measurements (antioxidants and mineral matters) were carried out in triplicates. The results were expressed as mean ± SD and analyzed using MS-Excel software. The data obtained in the study (Galleria mellonella analyses) were evaluated using the One-Way Analysis of Variance (ANOVA) and Tukey test in the Windows SPSS 26.0 computer program. A P value of <0.05 was considered statistically significant.

3. Results and discussions

3.1. Chemical composition

A. eupatoria has been used to treat various diseases for centuries in many parts of the world, especially in Europe. It is also widely used as an herbal tea due to the plant's flavor (Tomlinson *et al*., 2003). The chemical composition of *A. eupatoria* is presented in Table 1.

According to Table 1, the essential oil (EO) rate was found to be 0.02%. According to the studies, the oil yield based on the dry weight of samples was 0.2% per leaf (Muruzović *et al*., 2016; Kozykeyeva *et al*., 2020). *A. eupatoria* also contains 1.2-1.6% of flavonoids, which belong to different subgroups. According to the

literature, the amount of polyphenol in the. *A. eupatoria* plant was obtained as 124.5±0.032 GAE/g (Lee *et al*., 2010; Ciobanu *et al.*, 2018).

The antioxidant activity of *A. eupatoria* is shown in Table 2. DPPH radical scavenging activity was determined as 38.03 µg/mL and also in comparison with BHA and BHT samples. BHA and BHT are synthetic antioxidants used as food additive to prevent deterioration (Norhasidah *et al*., 2014). Some extracts contain less essential oil flavonoid antioxidants and their observed antioxidant activity is comparable to BHT such as ginger, rosemary, and sage (Sekretar *et al*., 2004). A study of the antioxidant activity of *A. eupatoria* (Agrimony) extracts was measured and evaluated in the DPPH radical scavenging and ABTS radical decolorization reaction systems. The radical scavenging capacity of *A. eupatoria* extracts varied in a wide range (9.1-97.5% in DPPH reaction and 6.7-79.5% in ABTS reaction) depending on the polarity of the solvent used to obtain the extract (Venskutonis *et al.*, 2007). A study found the most significant antioxidant capacity for *Agrimonia* herba (IC_{50} 45.55 µg/mL) (Ciobanu *et al*., 2018). Our results are lower than that reported by Muruzović *et al.* (2016) who studied the concentration of total phenols, flavonoids, the antioxidant activity of the water, diethyl ether, acetone, and ethanol extracts of *A. eupatoria*. The concentration of total phenols was obtained ranged from 19.61 mg GA/g to 220.31 mg GA/g. The concentration of flavonoids was obtained ranged from 20.58 mg RU/g to 97.06 mg RU/g. Differences obtained for the antioxidant activity of the extracts and their phenolic and flavonoid content could be due to the geographical characteristics of the plants and differences in the methods used for examining the activities.

The *A. eupatoria* essential oil chemical composition is shown in Table 3.

Table 1. The chemical composition of *A. eupatoria*

Parameters	Leaves
Moisture, %	6.69
Yield of essential oil, % (v/w)	0.02
Protein, %	14.13
Cellulose, %	21.01
Ash, %	7.48
Chlorophyll a, µg/g dw	72.22
Chlorophyll b, µg/g dw	1456.00
Total carotenoids, µg/g dw	82.51
Total phenol contents, mg GAE /g	13.66 ± 0.38
Total flavonoid assay, mg QE/g	4.65 ± 0.01

Table 2. Antioxidants activity of *A. eupatoria*

Methods	Leaves
FRAP assay, µmol/L	864.00± 0.01
TEAC assay, µmol/L	57.97± 0.02
IC50 value, µg/mL	38.03± 0.01
BHA	19.66± 0.0
BHT	13.81± 0.0
Hydroxyl radical scavenging capacity, mM EtOH/mL	16.8 ± 5.8
Superoxide scavenging capacity, unit SOD/mL	**14.6 ± 3.7**

Table 3. Chemical composition of *A. eupatoria* essential oil

№	RT, min	RI a	Compounds	Content (% of TIC b)
1	10.05	930	α-Pinene	62.72 ± 0.61
2	11.50	976	β-Pinene	1.23 ± 0.01
3	12.31	999	n-Octanal	0.33 ± 0.0
4	13.22	1024	D-Limonene	0.71 ± 0.0
5	15.57	1099	n-Nonanal	0.85 ± 0.0
6	16.28	1110	6-Campholenol	0.52 ± 0.0
7	25.24	1450	Geranyl acetone	0.98 ± 0.0
8	26.00	1480	methyl-γ-Ionone	1.37 ± 0.01
9	26.78	1512	Tridecanal	0.57 ±0.0
10	27.88	1562	(E)-Nerolidol	0.51 ±0.0
11	28.37	1577	Spathulenol	0.75 ±0.0
12	28.58	1581	Caryophyllene oxide	1.44 ± 0.01
13	29.29	1630	γ-Eudesmol	1.38 ± 0.01
14	34.17	1861	(Z,Z)-Farnesyl acetone	1.72 ± 0.01
15	34.49	1883	(5E,9Z)-Farnesyl acetone	3.65 ± 0.03
16	36.30	1922	(5E,9E)-Farnesyl acetone	6.64 ± 0.016
17	36.69	1957	n-Hexadecanoic acid	11.41 ± 0.11
18	39.73	2130	Linoleic acid	0.46 ± 0.0
19	39.95	2141	Oleic acid	1.05 ± 0.0
20	40.03	2152	Linolenic acid	0.12 ± 0.0
Oxygenated aliphatics,%				15.02
Monoterpene hydrocarbons,%				65.70
Oxygenated monoterpenes,%				2.93
Oxygenated sesquiterpenes,%				16.35

a RI – retention (Kovat's) index; b TIC – total ion curren

A. eupatoria EO obtained from flowers was characterized by 20 constituents representing 98.41% of the total oil content. Ten of the EO constituents were with concentrations above 1%. The main EO constituents (over 3%) were: α-pinene (62.72%), n-hexadecanoic acid (11.41%), (5E,9E)-farnesyl acetone (6.64%), and (5E,9Z)-farnesyl acetone (3.65%). The distribution of significant groups of aroma substances in oils is shown in Table 3. Monoterpene hydrocarbons (65.70%) are the dominant group in the oil, followed by oxygenated sesquiterpenes (16.35%), oxygenated aliphatics (15.02%), and oxygenated monoterpenes (2.93%).

Due to its wide application opportunities, the *A. eupatoria* essential oil was studied by several researchers. Navaei and Mirza (2009) analyzed the essential oils of leaves and flowers in wild and cultivated forms of *A. eupatoria* distributed in Iran. As a result of the study, they reported the main components of wild leaves as β-caryophyllene (59.6%), caryophyllene oxide (10.4%), and the main components of culture leave as α-pinene (28.2%), β-caryophyllene (20%).

Jin *et al.* (2010) obtained the chemical composition of essential oils of *A. pilosa* collected from 3 different regions of China by micro distillation and traditional distillation methods and compared them by making their

analyzes. In total, 49 compounds were identified. They determined hexadecanoic acid with 11.83-41.18% as the main component. Other components were linolenic acid methyl ester (1.93-13.45%), α-curcumene (trace-7.88%), p-propenylanisol (trace-6.55%), and α-bisabolol (0.94% 6.27%) (Wang *et al*., 2012). The essential oil obtained by water distillation of the leaves and roots of *A. eupatoria* grown in the Zhejiang region of China was analyzed by GK-KS, and 68 compounds from the root and 65 compounds from the leaves were identified. Common main components in both leaves and roots were determined as cedrol (14.37%), α-pinene (8.31%), and linalool (5.72%) (Feng *et al.*, 2013). The essential oil obtained by steam distillation of the aerial part of *A. aitchisonii* (Rosaceae) collected from India was examined by GK/KS and NMR spectroscopy, and it was reported that the oil was rich in methyl mirtenate (62.4%). Other major constituents were defined as limonene (7.2%), linalyl acetate (5.9%), linalool (4.8%), mirtenyl acetate (4.6%), and zingiberene (2.4%) (Melkani *et al.*, 2007).

3.2. Nutrient contents

Today, the analytical determination of heavy metals in medicinal and aromatic plants is among the most important quality parameters in determining these plants' purity, safety, and efficacy.

In our study, macro-and microelements of *A. eupatoria* are presented in Figure 2 and Figure 3. With regard to total dry matter, the order of limiting nutrients was K > Ca >Mg> P >Na for macro-elements and Mn > Fe > Cu > B for micro-elements. Also, the order of heavy metals contents were Sr > Co >Rb > Zn> Ba >Ni>Cr (Fig 4).

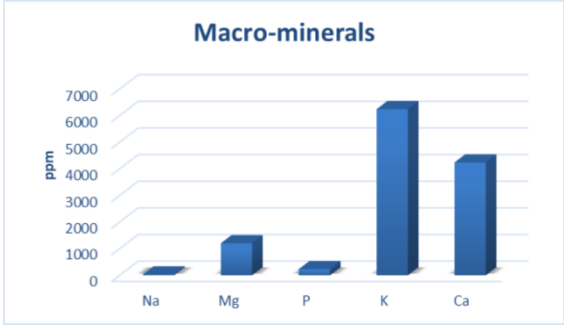

Figure 2. Macro- minerals in *Agrimonia eupatoria* L. (ppm)

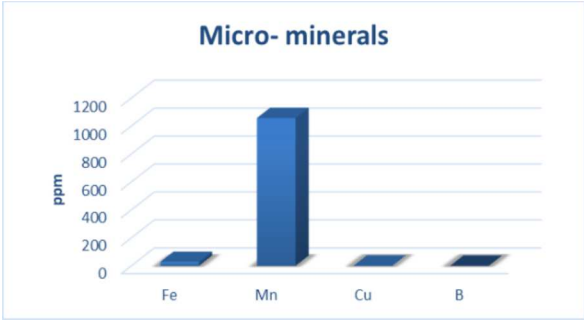

Figure 3. Micro- minerals in *Agrimonia eupatoria* L. (ppm)

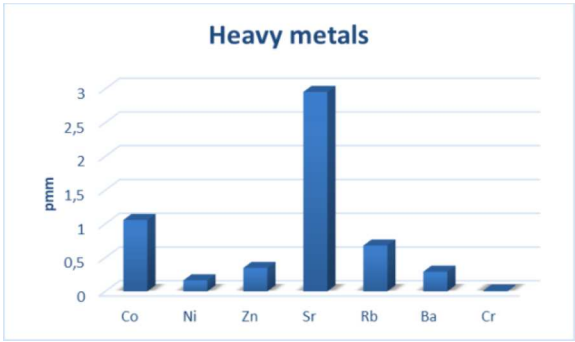

Figure 4. Heavy metals in *Agrimonia eupatoria* L. (ppm)

The limit values for Cr, Ni, and Zn determined by the WHO/FDA are 1.30, 0.02, and 50 ppm. Zinc (Zn) is an essential trace element that plays a vital role in many organisms' physiological and metabolic processes. Nevertheless, higher zinc

concentrations can be toxic to the organism (Doğan, 2020; Şenkal *et al.*, 2019).

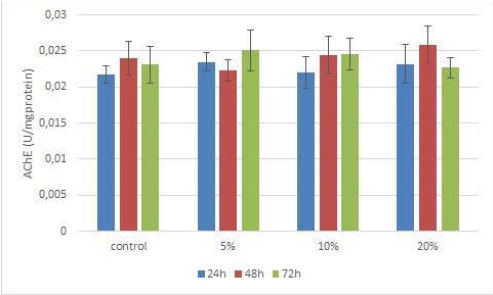

Figure 5. Effects of *A. eupatoria* extract on AChE activities of larvae of *Galleria mellonell*a. (There are no statistical differences between groups.)

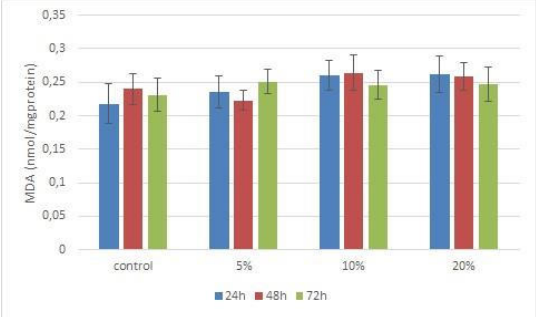

Figure 6. Effects of *A. eupatoria* extract on MDA levels of larvae of *Galleria mellonella*. (There are no statistical differences between groups.)

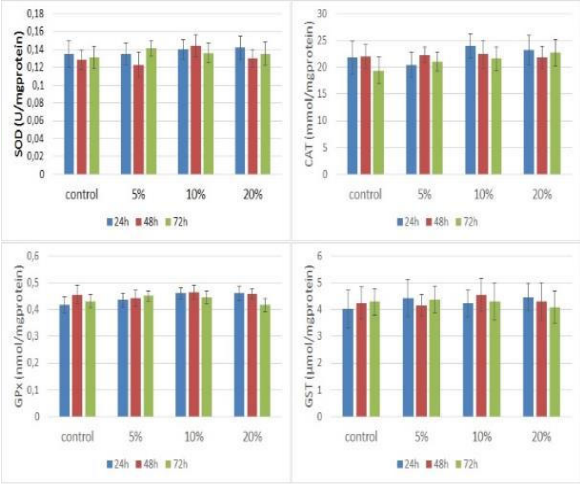

Figure 7. Effects of *A. eupatoria* extract on SOD, CAT, GST and GPx activities of larvae of *G. mellonella*. (There are no statistical differences between groups.)

LPO is the main event that plays an important role in xenobiotic toxicity (Apaydin *et al*., 2014). Since MDA is the end product of LPO, increased MDA is an important indicator of LPO (Kara *et al*., 2016). Cells have various defense mechanisms against oxidative damage, and these enzymatic antioxidants in tissues neutralize the oxidative stress that occurs due to the formation of free radicals (Bas and Pandır, 2016). Therefore, if the antioxidant enzyme activity is insufficient in the cell, the free radical level increases; for this reason, the activity determination of these enzymes is important in the determination of oxidative stress and so toxicity of a substance (Bas *et al.*, 2014). No change in MDA level and antioxidant enzyme activities depending on the dose applied in this study is proof that extract of *A. eupatoria* doesn't cause oxidative stress.

Acetylcholine esterase (AChE), found in tissues, is an enzyme that can hydrolyze acetylcholine. Xenobiotics can also exert their toxic effect by inhibiting AChE. As a result of inhibited AChE, acetylcholine accumulation occurs in the synapses, and therefore continuous stimulation occurs in the cholinergic system (Hazarika *et al.*, 2003). If this enzyme is inhibited, acetylcholine molecules can send the muscles to contract continuously, causing partial or general paralysis. Xenobiotics often work on this principle. No change in AChE activity due to increasing application doses in this study suggests that extract of *A. eupatoria* can be used as a medicinal plant.

4. Conclusions

Based on the study of *A. eupatoria* essential oil for their chemical composition, phenolic content, flavonoid content, and antioxidant activity, it could be concluded that all the studied samples are important sources of biologically active compounds. We can reveal that *A. eupatoria*, which has a high antioxidant effect, will also be a pioneer for toxicological studies. Toxicological tests on medicinal, aromatic plants are limited, and for their safe use, toxicological tests are required. Therefore, we

conducted this research on *G. mellonella* larvae, as a model organism. As a result, it was determined that the applied doses did not affect the parameters observed. These results will also be a beginning study for future toxicological research.

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