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A study on the bıoactıvıty of plant extracts obtaıned from *Arum maculatum* leaves by dıfferent extractıon techniques

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Abstract

Arum maculatum is a highly known plant worldwide for traditional use. The aim of this study is to evaluate the bioactivity of the plant extract obtained using different techniques and solutions. Total fenolic, flavonoid components and antioxidant, antimicrobial and enzyme inhibition activity of the plant extracts (Boiling in water, fermenting in water and USB in methanol) were investigated. Additionally, oil components of the extracts was analysed in GC-MS. As a result of the GC-MS analysis, 18 different fatty acid were determined. Major fatty acid components of extracts were palmitic acid (19.57%), oleic acid (15.25%), linoleic acid (21.84%) and alpha-linolenic acid (15.95%). The plant extracts were also found to be consisting of omega 3-6-9 fatty acid. The results showed that methanolic extracts in USB is produced better and more effective findings than the other extracts. According to the antimicrobial activity experiments, E. coli and S. aureus were the only strains inhibited by the all extracts obtained three different methods. The highest inhibition was recorded against Bacillus subtilis with USB methanol extracts. The only antifungal activity was observed against C. albicans with extracts obtained by boiling in water. Enzyme inhibition activity was very limited with all extracts. Amylase activity was slightly inhibited (14.1%) up to 30 min.

Keywords: *Arum maculatum, antioxidant activity, antimicrobial activity, enzyme inhibition, omega fatty acids*

Introductıon

People around the world still resort to traditional medicine to prevent and treat many diseases. In traditional practices, botanical supplements are preferred because of few or no side effects (Al-Shmgani et al., 2019). Plants with antioxidant properties are widely used today as a preventive treatment, functional and therapeutic foods, nutraceuticals, in the food industry and in human nutrition (Wasowicz et al., 2004). Because the intake of such food or food products is generally considered beneficial for the prevention and treatment of cancer and cardiovascular, inflammatory, microbial and age-related diseases. In fact, with an ordinary diet, we consume most of these beneficial compounds with various physical and chemical properties (Kurt et al., 2018). In addition, while synthetic antioxidants are inexpensive, they are not found safe. Hence, the popularity of natural antioxidants is increasing in recent years. Therefore, herbs, spices, fruits, vegetables, seeds and teas, and various plant extracts rich in polyphenols, which are potential sources of antioxidants, are widely studied (Wasowicz et al., 2004).

The genus Arum (Araceae) consists of 29 species of tuberous plants native to Asia, Europe and North Africa. Arum species are the plants that have a therapeutic effect on various ailments and used in folk medicine and therefore worth to investigate further (Farahmandfar et al., 2019). Archaeological evidence shows that Arum has been used by humans since ancient times (Azab, 2017). *Arum maculatum* L., one of the species belonging to the Arum genus, has been known as a medicinal plant for centuries. Throughout the history it has been used to heal snake bites and wounds, malaria, rheumatism, abdominal pain, antihypertensive, diabetes, gout, sore throat, kidney stones, colitis, liver disease, hemorrhoids, and colds (Kozuharova et al., 2020). This plant is called also as yılan yastığı, nivik otu, livik otu, ayı kulağı, kabargan, tirşik, Andırın doktoru, yılan dili, yılan pıçağı in Turkey locally (Erbil et al., 2018; Atalay and Yıldız, 2020). All parts of this wild plant have been reported to cause irritation to the skin, mouth, tongue and throat, causing swelling of the throat, difficulty breathing, burning pain and stomach ache. However, these effects disappear if the plant is boiled or dried (Al-Shmgani et al., 2019; Atalay and Yıldız, 2020). However, its therapeutic significance has been reported for kidney and liver injuries, hemorrhoids, and as a pain reliever for many diseases. *A. maculatum* extract has shown antimicrobial and antifungal activities against a wide variety of Gram-positive, Gram-negative bacteria and fungi (Al-Shmgani et al., 2019). It was observed that the extracts prepared from the leaves of the plant had higher antimicrobial effects than the extracts prepared from the fruit, but the antifungal effect could not be determined (Erbil et al., 2018).

The objectives of this study are (a) extracting total phenolic and flavonoid compounds from *A. maculatum* species by three different methods, namely fermentation, boiling and ultrasound, (b) comparing the extraction efficiency with various solvents and different methods, (c) investigation of antioxidative effects of *A. maculatum* with DPPH radical scavenging activity and FRAP, (d) to evaluate the antimicrobial properties and enzyme activity of *A. maculatum* extracts, and (e) to determine the fatty acid profile of *A. maculatum* leaf extracts.

Materıal and methods

Sample Preparation

The samples of *A. maculatum* used in this study were collected in February 2019 from the natural areas by the local people in Iskenderun region of Hatay province. The collected above-ground parts were brought to the laboratory without waiting and used freshly in the experiments. After washing with tap water they were chopped finely for extraction.

Extraction method

Three different methods were used for the extraction. Two of them were traditional (boiling and fermentation) and the other was modern

(ultrasonic bath). In traditional methods, the usage of this plant by the public has been taken into consideration. Therefore water was used for extraction solvent. In the fermentation method (**FW**: Fermented in Water), water at a temperature of 100 °C was added to the fresh plant (leaf water ratio: 1:2). It was hermetically sealed and stored at room temperature for 24 hours. Filtration was carried out after 24 hours. The second extraction method (**BW**: Boiled in waterwas carried out by boiling the plant with water for 60 minutes (leaf water ratio: 1:2). Methanol was used as solvent in the third extraction method (**USB**: Ultrsonic Water Bath Extract) (Comlekcioglu and Kutlu, 2021). Methanol (500 mL) was added to the plant sample (50 g) and sonicated for 1 hour in the Ultrasonic Water Bath at room temperature. After centrifugation (3500 rpm, 15 min), the separated liquid part was collected in another tube. The extraction procedure was repeated twice. After combining the extracts the solvent was removed in a vacuum rotary evaporator tilla dry extract was obtained. The dried plant extract was stored at -20 ° C until analysis.

Determination of oil content and fatty acid composition of leaf extracts

Analysis of the fixed oils was made with GC-MS according to Comlekcioglu and Kutlu (2021). Oil extraction was carried out in FOSS Soxtec 2055 device using 100 mL of hexane to 3 g of plant sample. GC-MS analyzes were performed with the Schimadzu GC 2025 system ®. A TRCN-100 (60m x 0.25 mm x 0.20 µm film thickness) SE-54 fused silica capillary column was used. Electron energy is 70 eV. The injection amount is 1 µl. Analysis of the samples was increased by 5°C per min after being kept at 80°C for 2 min. After reaching a temperature of 140°C, it was kept for 2 minutes. Following this process, it was kept for 5 more minutes at 240°C with an increase of 3°C per minute. The total analysis time was set as 61 minutes. The injections were carried out in split mode (1:50) at 240°C and the detector temperature was 250°C. Helium is used as carrier gas and its flow rate is adjusted to 30mL/min. The gas flows used were determined as H2 = 40mL min^{-1} and dry air = 400mL min^{-1}.

Determination of total phenolic content

The total phenolic content of the samples was made by using the Folin-Ciocalteu's Reagent method the procedure of Obanda and Owuor (1997). Gallic acid (Sigma) was used as standard. The prepared solutions were read at 750 nm in a spectrophotometer (Perkin-Elmer Lambda EZ 150, USA). The absorbance values obtained are given in terms of mg gallic acid equivalent (GAE) / g dry sample weight with the help of the calibration curve created with gallic acid solutions. All experiments were done in triplicate.

Determination of total flavonoid content

Total flavonoid content in plant extracts was determined spectrophotometrically according to Chang et al. (2002). The standard solution was calculated with different concentrations (25-200 µg mL^{-1}) of quercetin (Sigma) prepared according to the above procedure. Absorbance was read in a spectrophotometer at 415 nm. The absorbance values obtained were converted into µg quercetin equivalent/g dry sample weight. All experiments were done in triplicate.

Determination of antioxidant capacity

Antioxidant capacity (reduction capacity of free radicals) was determined the DPPH method defined by Brand-Williams et al. (1995). Five different concentrations of solutions were prepared by diluting each plant extract. The results were shown as IC50, in which -the concentration was required to reduce 50% of DPPH free radicals. All experiments were done in triplicate and ascorbic acid was used as positive control.

Antioxidant capacity: %AA=[(Acontrol-Asample) /Acontrol] x 100FRAP method

FRAP analysis was carried out according to Benzie and Strain (1996). Total 50 µL of plant extracts were transferred to 2 mL eppendorf tubes and 600 µL of FRAP agent was added on it. Absorbance was measured at 593 nm. Results were calculated as µmol ascorbic acid equivalent / g dry plant weight using ascorbic acid (100–1000 µmol/ L^{-1}) calibration graph. Results are given as µmol/g dry plant weight. All experiments were done in triplicate.

Antimicrobial activity assay

Antimicrobial activities of the plant extract were determined with well diffusion method. The test organisms used were *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6633), *Sarcina lutea* (ATCC 9341NA), *Escherichia coli* (ATCC 309628), and the clinical isolates *Enterococcus faecalis, Enterococcus faecium, Candida parapsilosis and Candida albicans.* Bacterial cultures maintenance were carried out in Nutrient broth (Oxoid) and the yeasts were in Sabouraud dextrose broth (Oxoid). Mueller Hinton agar dishes were cultured with a standardized inoculums mcFarland 0.5 turbidity giving $1.5x10^8$ cfu/mL of bacterial suspension and also Sabouraud dextrose agar were cultured with each of yeast strains (Collins et al., 1989). The plant extracts (50 µL) were loaded into the wells prepared with a 6 mm cork borer aseptically after inoculation of test microorganisms with a sterile cotton swab. Then the plates were incubated at 37°C for 24 h and 48 h. At the end of the incubation period, the diameter of inhibition zones were measured. DMSO (%10) was also used as solvent control. The extracts showing inhibition zone were then tested for determination of MIC values in micro-well plates (Collins et al., 1989).

Enzyme inhibiton assay

Enzyme inhibition activity of three plant extracts of *A. maculatum* were tested against alpha-amylase enzyme showing the optimum temperature and pH as 40°C and 7.6, respectively. Dried plant extracts were dissolved (16 mg/mL) in 100 mM phosphate buffer at pH 7.6. The inhibition activity of extracts on amylase was studied by mixing and preincubating the extract (20µL) with enzyme (50µL) in different periods. Then, the remaining amylolytic activity was assayed by adding 400 µL of soluble starch (1%) (Merck) and incubating the final mixture for 15 min at 40°C. Finally reaction was stopped by addition of 500 µL 3,5-dinitrosalicylic acid reagent (Bernfeld, 1955) and absorbance (550 nm) was measured in a spectrophotometer (Perkin Elmer Lambda EZ 150). The activity detected without preincubating the enzyme mixture in the absence of extracts was taken to be 100%. The results were recorded as the mean values of triplicates.

Results and dıscussıon

Results on fatty acid composition

The data of the fatty acid composition of *A. maculatum* extracts are given in Table 1. While the amount of oil in the plant extract was found to be 2.08%, a total of 18 fatty acids were determined, 8 of which were saturated and 10 were unsaturated. Considering the average values, it was seen that the main components of the fixed oil of plant extracts were palmitic acid (19.57%), oleic acid (15.25%), linoleic acid (21.84%) and alpha-linolenic acid (15.95%). According to the results, stearic (5.20%), gamma-Linolenic (7.64%) and cis-4,7,10,13,16,19-Docosahexaenoic (5.07%) acids were found above 5%. Other fatty acids remained below 5%.

According to the analysis, polyunsaturated fatty acids (PUFA) were found to be the highest, and saturated fatty acids (SFA) were almost half of the polyunsaturated fatty acids while monounsaturated fatty acids (MUFA) were the least. The main fatty acids in the sample, oleic (C18:1), linoleic (C18:2) and alpha linolenic (C18:3) acids have 53% of the oil and thus contribute to higher levels of unsaturated fatty acids.

Table 1. *Fatty acid compositions of Arum maculatum (%)*

Carbon Numbers	Fatty Acids	Amount (%)
C4:0	Butyric acid	1.05 ± 0.01
C14:0	Myristic Acid	1.37 ± 0.00
C16:0	**Palmitic Acid**	**19.57 ± 0.02**
C18:0	Stearic Acid	5.20 ± **0.01**
C20:0	Arachidic Acid	0.23 ± 0.00
C21:0	Heneicosanoic Acid	0.37 ± 0.00
C22:0	Behenic Acid	0.72 ± 0.00
C24:0	Lignoceric Acid	0.41 ± 0.00
C14:1	Myristoleic Acid	0.28 ± 0.00
C16:1	Palmiteloic Acid	0.82 ± 0.00
C17:1	Cis-10-Heptadecanoic Acid	0.90 ± 0.00
C18:1	**Oleic Acid**	**15.25 ± 0.02**
C18:2	**Linoleic Acid**	**21.84 ± 0.02**
C18:3	gama-Linolenic Acid	7.64 ± 0.01
C18:3	**alfa-Linolenic Acid**	**15.95 ± 0.02**
C20:3	Cis-8,11,14-Eicosatrienoic Acid	0.73 ± 0.01
C20:5	cis-5.8.11.14.17-Eicosapentaenoic Acid	2.61 ± 0.01
C22:6	cis-4,7,10,13,16,19-Docosahexaenoic	5.07 ± 0.01
Ratio of saturated fatty acid		28.93
Ratio of monounsaturated fatty acid		17.24
Ratio of polyunsaturated fatty acid		53.83

Table 2. *Total phenolic and flavonoid content and antioxidant activity values*

	BW*	FW*	USB*
Phenol (mg GAE g^{-1})	4.83 ± 0.48	4.79 ± 0.70	12.44 ± 0.02
Flavonoid (µg QE g^{-1})	2.06 ± 0.07	1.96 ± 0.09	3.03 ± 0.08
FRAP (mmol AAE g^{-1})	3.82 ± 0.12	4.11 ± 0.005	5.89 ± 0.05
IC50 Value (%DPPH) (mg mL^{-1})	3.20 ± 0.12	12.91 ± 0.15	1.97 ± 0.05

*: (**BW**: Boiled in water Exctract; **FW**: Fermented in Water Extract; **USB**: Ultrsonic Water Bath Extract)

PUFAs have been shown to activate genes that reduce adipose tissue mass and suppress the development of obesity more strongly than MUFA or SFA (Moussavi et al., 2008). In this context, considering the effects of PUFAs in the prevention of cardiovascular diseases and cancer, obesity and diabetes, the importance of vegetable oils rich in these fatty acids increases (Pelliccia et al., 2013; Jabeur et al., 2017). The rich MUFA and PUFA (71.7% in total) content of the studied *A. maculatum* makes the plant stand out in terms of these features.

While there are few studies in the literature on the seed fatty acid profile of *A. maculatum* and some species belonging to the genus Arum, no study was found on the above ground part used as food. Christie (2003) studied *A. maculatum* and Saglık et al. (2003) studied the fatty acid profile of *A. italicum* seed oils. The seed fatty acid profiles of *A. italicum* and *A. maculatum* plants were similar. Compared to the results obtained from this study, palmitic, oleic and linoleic acids were found to be similarly high, while palmitoleic and tridecanoic acids were not found in the plant extract studied. As a result, a similar fatty acid profile was observed, although the proportions varied. However, it is surprising that the seed oil of another species, *A. cyrenaicum*, contains very high amounts of myrestic acid (62.9%) (Zargoun et al., 2020). As a result, it is possible to say that the origin of the plant samples, the region where it grows and the plant organ analyzed have a great effect on the fatty acid composition of the Arum.

Results of antioxidant activity

The antioxidant activity of three different extracts of *A. maculatum* was evaluated using four different in vitro experiments (Table 2). Phenolic compounds are powerful antioxidants due to their ability to scavenge free radicals. These abilities of phenolic compounds are due to substituted hydroxyl groups in their aromatic rings (Farahmandfar et al., 2019). According to the results, the phenol amounts of the plant were found to be 12.44 mg/g in USB extracts. Although asimilar amount of phenolic was detected with the extracts prepared with water, it was much more less than the USB extract. The results of the other three analyzes were also similar to the phenol results. The values obtained from the methanolic extract obtained by USB gave the highest yield in terms of quantity and antioxidant power. The solvent type plays an important role in the structural diversity of antioxidant compounds found in raw extracts. Therefore, the choice of solvent and extraction method are crucial for determining antioxidant activity.

Farahmandfar et al., (2019) found the total phenolic content value between 39.85-55.25 mg g^{-1} and the total flavonoid content value between 1.67-4.42 mg g^{-1} in the leaf extracts of *A. maculatum*. Erbil et al., (2018) found that the phenolic content of *A. maculatum* leaf extracts was 1.35 mg g^{-1}. As a comparison, the values obtained in this study were very low than that of Farahmandfar et al., (2019), however, the

Table 3. Antimicrobial activity of A. maculatum extracts against some microorganisms

	Extraction Methods			Control	
Microorganisms	**BW⁺**	**FW⁺**	**USB⁺**	**CXM**	**Nys**
Bacillus subtilis ATCC 6633	-	-	21±1.33	-	NT
S.aureus ATCC 29213	20±0.92	12±0.12	14±0.14	23±0.22	NT
*Enterococcus faecium**	-	-	-	-	NT
S. Lutea ATCC 9341NA	-	-	-	50±0.51	NT
E.coli ATCC 309628	15±1.13	11±0.21	15±0.14	-	NT
*Enterococcus faecalis**	-	-	6±0.32	30±0.15	NT
*Candida parapsilosis**	-	-	-	NT	-
*Candida albicans**	9±0.11	-	-	NT	32±0.13

⁺**: BW**: Boiled in water Exctract; **FW**: Fermented in Water Extract; **USB**: Ultrsonic Water Bath Extract; Cxm: Cefuroxime sodium (30µg)-Oxoid; ; Nys: Nystatine 100U; NT: Not Tested; *Clinic Isolate

Table 4. MIC values of A. maculatum extracts

	Extracts (mg mL^{-1})		
Microorganisms	BW⁺	FW⁺	USB⁺
B. subtilis ATCC 6633	-	-	2
S. aureus ATCC 29213	4	4	16
E. coli ATCC 309628	16	4	8
Candida albicans	16	-	-

⁺: **BW**: Boiled in water Exctract; **FW**: Fermented in Water Extract; **USB**: Ultrsonic Water Bath Extract

values were found to be higher than Erbil et al., (2018). Since different researchers used different methods in analysis and calculation, especially in antioxidant activity studies, the difference between the values are inevitable, as in this study. Therefore, it would be convenient to evaluate the study in itself by comparing extractions and focusing on differences in the methods. In this study, water and methanol, two of the most preferred and recommended solvents for the extraction of antioxidant compounds, were preferred. The results obtained from this study confirmed the researchers who have reported the efficacy of methanol extracts of plant species (Arab et al., 2011; Comlekcioglu and Kutlu, 2021). Water extracts of both applications (BW and FW) exhibited the lowest antioxidant activity. There are studies describing in vitro and in vivo antioxidant properties in extracts of some species of Arum using different extraction methods, solvents and different tests. Uguzlar et al., (2012) and Karahan et al., (2015) prepared extracts from *A. dioscorides* using different solvents such as water, ethanol, methanol and acetone. Among these extracts, they stated that methanolic extract was more active and had the highest antioxidant content. In another study, methanolic and aqueous extracts were prepared from *A. elongatum* and *A. hygrophilum* and tested for phenolic content and antioxidant activity. It was reported that the methanolic extract was more active too (Jaradat and Abualhasan, 2016).

Antimicrobial activity of Arum maculatum

The antimicrobial activity and MIC values of the *A. maculatum* extracts obtained in different ways presented in Table 3 and 4. The extracts produced different antimicrobial activities in varying degrees against different microoganisms tested. Three of the organisms, *E. faecium, S. lutea, C. parapsilosis,* were not affected from all of the extracts. *S.aureus* and *E. coli* were the only strains inhibited with all extracts. While FW extracts produced an inbitory action only against *B. subtilis* and *E. faecalis*, BW extract produced inhibitory action against *C. albicans* in addition to *B. subtilis* and *E. faecalis*. But USB extracts of *A. maculatum* was found to be the most effective against majority of the bacteria except the yeast tested. The components of Arum extracts were identified and revealed containing tannins, phenolics, saponnins and flavonoidal glycosides by researchers. Their antimicrobial activities were also investigated (Colak et al., 2009; Farahmandfar et al., 2018; Zargoun et al., 2020). The antimicrobial activity of Arum could be due to higher amount of phenolic components (Table 2) since natural phenolic compounds from different plants shows potential antimicrobial activity (Nitiema et al., 2012). Although the different solvent and techniques used for extraction, Farahmandfar et al. (2018) demonstrated that the highest extraction yield was obtained with ultrasonic method. Here in this study the phenolic content extraction was improved by USB method and consequently, antimicrobial activity were more evident as in Farahmandfar et al. (2018) findings. Antimicrobial activity of phenolic contents could be due to their ability to change the membrane permeability (Shahidi and Naczk, 1995). Therefore, USB method could be the choice of extraction for a broad spectrum of antibacterial activity for *A. maculatum*.

If the subject is antimicrobial efficacy, BW extracts has a broad range activity by producing antifungal action. Among the microorganisms group, Gram positive strains were the most sensitive one affected from the extracts, in terms of both inhibiton zone values and strain number. Our results are compatible with the findings of Safari et al., (2014), Mansour et al. (2015), Farahmandfar et al. (2019) as they have reported that the inhibiton of *Staphylococcus spp.*, *E. coli* and *Candida albicans*. Among the solvent of extracts, methanol was the best one producing antimicrobial activity which is similar to Mansour et al. (2015)'s finding. As *A. maculatum* extracts could produce inhibition on microorganism in variying degrees, Naseef et al. (2017) and Jaber et al. (2020) reported no inhibition of *A. euxinum, A. palaestinum* and *A. hygrophilum*, respectively. Therefore, *A. maculatum* is the most promising species for further bioactive investigations.

Enzyme inhibiton assay

Enzyme Inhibition activity determination of *A. maculatum* extracts, the enzyme, alpha amylase, was preincubated at 40 °C for 0, 15 and 30 min with each extracts prior to enzyme acticity determination. The activity was measured were the remaning activity (Figure 1). According to the results, the Arum extracts slightly inhibited amylase in the range of 4.8% and 14.1%. Among the extracts, the highest enzyme inhibiton was occured with USB method obtained. The enzyme has lost its original activity by 9.1%, 10.2% and 14.1% within 0, 15 and 30 min, respectively. On the other hand, the least inhibition, average 7.4%, was detected with FW extracts. As a results, amylase enzyme was not significatly reduced enzimatic reaction of amylase in the presence of the *A. maculatum*

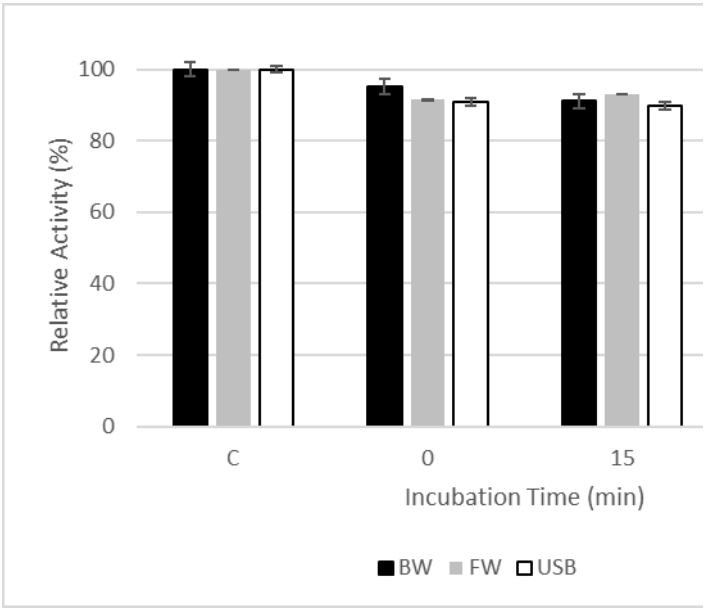

*Figure 1. Inhibitory Activity of Different Extracts of Arum maculatum on Alpha Amylase (**BW**: Boiled in water Exctract; **FW**: Fermented in Water Extract; **USB**: Ultrasonic Water Bath Extract)*

extracts up to 30 min.

Plantal drogs containing bioactive metabolites are commonly used worldwide for many purposes. Most of these are easy accessible natural products and still remain unexplored for their biological activity in living organisms. Enzymes are the molecular target for most drugs due to their protein nature (Copeland, 2013). These potential natural-therapeutic products should be screened for enzyme inhibiton for determination of their biological role.

Conclusıons

Interest in plant-based chemicals is increasing day by day due to the increasing resistance of microorganisms against the antibiotics used, as well as the limited development of new antibiotics. In this study, phytochemical content analysis was carried out by considering the consumption of *A. maculatum* collected from Hatay / Iskenderun region by the people who believe that it has the potential to be beneficial for health. The results demonstrated the presence of bioactive ingredients responsible for the plant's antioxidant and antimicrobial capacity. It was determined that especially the methanolic extracts obtained by USB have strong antioxidant activity due to their low IC50 value. Since extracts obtained by using methanol compared to water have higher antioxidant power, it can be suitable for the research of natural, environmental and healthy antioxidant for the pharmaceutical and food industries. It has an important profile with omega 3 (α-Linolenic acid, eicosapentaenoic acid and docosahexaenoic acid, omega 6 (linoleic and gamma-linolenic acid) and omega 9 (oleic acid) fatty acids. The results also showed that the relation between the phenolic contents, antioxidant activity and antimicrobial activity properties. On the other hand, the inhibition ratio of the enzyme showed that the bioactive metabolites of *A. maculatum* have the potential to be used as drugs and food additives etc. However, further chromatographic analysis is required in order to obtain more precise data to clarify the bioactivity of this medicinal plant. Various factors affect the bioactivity of plant extracts, especially the solvent and extraction method. In this study, it was seen that modern techniques give better results than the techniques commonly used by the public on the bioactivity of Arum. This study highlights the importance of modern techniques in the industrial use of common and valuable plant material.

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