

# LC-MS/MS analysis and biological activities of endemic *Achillea sieheana* Stapf from Türkiye

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**ABSTRACT:** In this study, *in vitro* ABTS, DPPH, 5-lipoxygenase, and  $\alpha$ -glucosidase inhibitory activities of *n*-hexane (ASH), dichloromethane (ASD), ethyl acetate (ASE), and methanol (ASM) extracts from the aerial parts of endemic *A. sieheana* were assayed for the first time. The phytochemical content of ASE, the most active extract, has been first determined by LC-MS/MS. Also, the total amount of phenolic compounds of extracts were calculated.

ASE demonstrated significant antioxidant activity with IC<sub>50</sub> values of 0.096 and 0.156 mg/mL for ABTS and DPPH, respectively. ASD and ASE with IC<sub>50</sub> values of 0.045 and 0.089 mg/mL exhibited significant anti-inflammatory activity. ASE showed moderate  $\alpha$ -glucosidase inhibitory activity with an IC<sub>50</sub> value of 0.774 mg/mL. Also, the total phenolic content was found to be highest in the ASE. Feruloylquinic acid, luteolin, luteolin glucoside, isorhamnetin, isovitexin, methoxyflavonoid like chrysoeriol and its glycoside derivative in the ASE were detected by LC-MS/MS. The results demonstrated that ASE had significant anti-inflammatory, antioxidant and moderate  $\alpha$ -glucosidase inhibitory potential.

**KEYWORDS:** *A. sieheana*; LC-MS/MS; antioxidant; anti-inflammatory;  $\alpha$ -glucosidase.

## 1. INTRODUCTION

The genus *Achillea* L., which is one of the most important members in the Compositae (Asteraceae) family and consists more than 100 species in different parts of the world. The main habitats of this genus are placed in different parts of Iran, Turkey, Serbia, and Eastern regions of Europe [1]. *Achillea* L. has 6 sections and 49 species (58 taxa), and 24 species of them are endemic with an endemism ratio 49% in the Flora of Turkey [2].

Many *Achillea* species are rich sources of flavones, other flavonoids, phenolic glycosides, non-saturated carboxylic acids, sesquiterpenes, sesquiterpene lactones, lignans and triterpene alkaloids [1]. It has also been reported that coumarin and sesquiterpene coumarin ether derivatives have been isolated from some *Achillea* species [3,4,5]. In traditional medicine, *Achillea* species have been utilized for treating various diseases such as rheumatic complaints, cancer, respiratory diseases, blood sugar and cholesterol problems, and hemorrhoids [6].

Numerous inflammatory illnesses, including arthritis, allergy, asthma, psoriasis, inflammatory bowel disease metabolic syndrome, skin and cardiovascular renal neurological disorders including Alzheimer's disease are linked to LOX-s [7]. The capacity of a bioactive substance to efficiently remove free radicals, suppress lipid peroxidation events, and stop other oxidative damage is referred to as antioxidant activity. In addition, it serves as the basis for several other biological processes including anti-aging, anti-cancer, and anti-inflammation. More significantly, antioxidant activity has been linked to the protection of several chronic illnesses, including cancer, diabetes, and cardiovascular disease [8].

Diabetes is a diverse collection of illnesses that affects proteins, carbohydrates, and fats are metabolized. It causes insufficient or nonexistent insulin production as well as diminished tissue sensitivity to insulin. By 2025, 300 million individuals are expected to have the condition, and by 2030, it may affect 366 million people. Plants have a vital part in managing diabetes mellitus and are proven to be safe and less expensive than synthetic medications. The World Health Organization (WHO) has advised evaluating

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traditional plant remedies for diabetes since they are efficient, non-toxic, have few to no side effects, and are thought to be an important source for research on hypoglycaemic drugs [9].

In this study, it is aimed to conduct antioxidant, anti-lipoxygenase,  $\alpha$ -glucosidase inhibitory activity studies on different extracts obtained from aerial parts of endemic *Achillea sieheana* plant and to determine the chemical content of most active extract between all extracts by LC-MS/MS for the first time. To the best of our knowledge, there is no publication has been found in the literature the chemical content and different biological activity studies on the endemic *A. sieheana*.

## 2. RESULTS

### 2.1. Antioxidant activity

An antioxidant is a chemical that, when present in comparably small amounts to an oxidizable substrate, considerably retards or prevents the oxidation of that substrate based on [10]. Antioxidants slow down lipid peroxidation and the progression of many chronic illnesses. The common spectrophotometric methods for determining the antioxidant capacity of extracts include assays based on the usage of DPPH +, ABTS +, DMPD +, and  $O_2^-$  radicals. Because of their ease of use, speed, sensitivity, and reproducibility, the DPPH radical and ABTS + scavenging algorithms have been extensively adopted for assessing compounds' antioxidant activity [11].

In the study, *in vitro* antioxidant activities of all extracts were evaluated according to the DPPH and ABTS methods. As can be seen from **Table 1**, ASE demonstrated significant antioxidant activity with half maximal inhibition concentration ( $IC_{50}$ ) values of 0.096 and 0.156 mg/mL for ABTS and DPPH, respectively and also the highest content of phenolic content (59.736 GAE) was found in ASE extract.

**Table 1.** The antioxidant activities and total phenolic contents of extracts from aerial parts of *A. sieheana*

Extracts&Standart	DPPH radical scavenging activity $IC_{50}$ (mg/mL)	ABTS radical scavenging activity $IC_{50}$ (mg/mL)	Total phenolic content (mg GAE/ g extract)
ASH	1.778 $\pm$ 0.010	0.311 $\pm$ 0.001	19.502
ASD	0.516 $\pm$ 0.002	0.113 $\pm$ 0.001	48.505
ASE	0.156 $\pm$ 0.003	0.096 $\pm$ 0.001	59.737
ASM	0.191 $\pm$ 0.001	0.117 $\pm$ 0.001	46.265
Ascorbic acid	0.018 $\pm$ 0.001	0.015 $\pm$ 0.001	

((Each value in the table is represented as mean  $\pm$  SD (n=3). Total phenolic content was expressed as gallic acid equivalent (GAE)).

### 3.2. Anti-inflammatory activity

Dioxygenases that do not contain sulfur, do not produce heme, and contain iron are known as lipoxygenases (LOXs). LOXs are widely present in fungi, animals, and plants. There are five different types of LOXs: 5-, 8-, 11-, 12-, and 15-. Additionally, linoleic and -linolenic acids serve as the substrate for 5- and 15-LOXs, which are crucial for plants [7]. According to the 5-LOX evaluation of all extracts, ASD and ASE with  $IC_{50}$  values of 0.045 and 0.089 mg/mL exhibited significant anti-inflammatory activity (**Table 2**). In addition, indomethacin was used as positive control and it had  $IC_{50}$  value of 0,022  $\pm$  0.001 mg/mL.

### 3.3. $\alpha$ -Glucosidase inhibition assay

Because the final step in the digestion of carbohydrates is catalyzed by the enzyme alpha-glucosidase,  $\alpha$ -glucosidase inhibitors may delay the use of dietary carbohydrates to reduce postprandial hyperglycemia (PPHG). Acarbose, miglitol, and voglibose are  $\alpha$ -glucosidase inhibitors that are known to lower PPHG primarily by interfering with the enzymes that break down carbohydrates and delaying glucose absorption [12]. In this study, acarbose was used as positive control with  $IC_{50}$  value of 0.040  $\pm$  0.002. ASE showed moderate  $\alpha$ -glucosidase inhibitory activity with an  $IC_{50}$  value of 0.774  $\pm$  0.003 mg/mL. A lower effect was observed in other extracts than ASE.

The anti-inflammatory and  $\alpha$ -glucosidase inhibitory activity results of *A. sieheana* extracts were expressed in **Table 2**.

**Table 2.** The anti-inflammatory and  $\alpha$ -glucosidase inhibitory activity results of extracts from aerial parts of *A. sieheana*

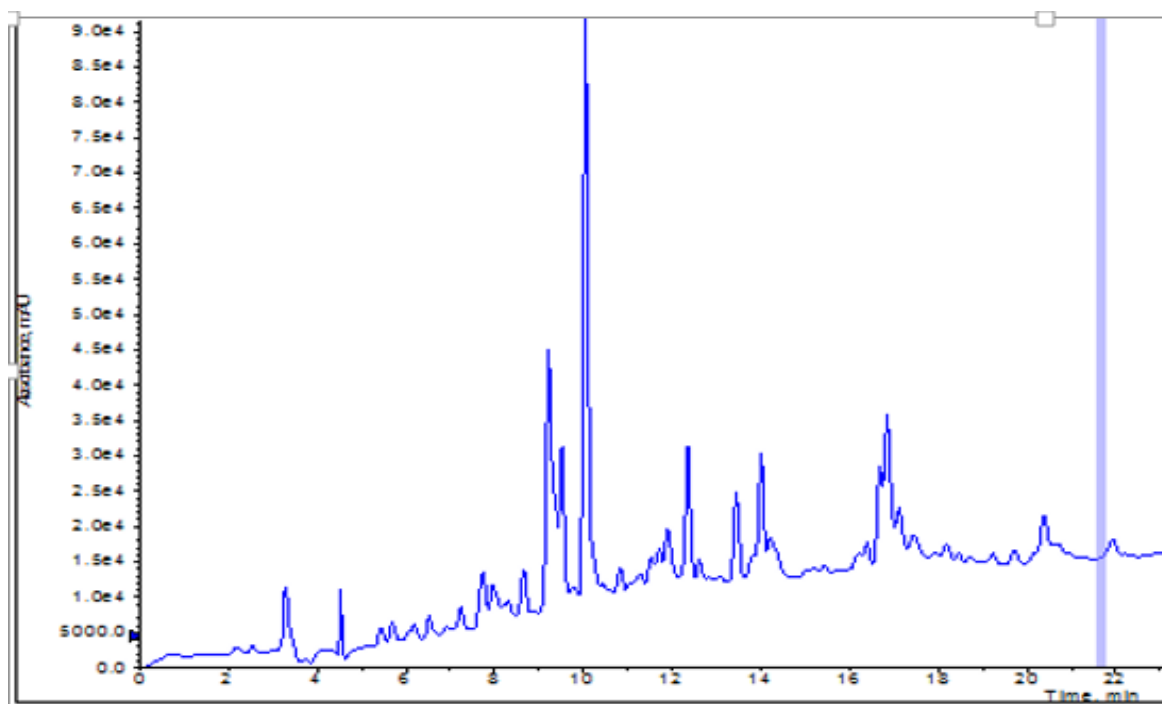
Extracts&Standart	The anti-inflammatory	$\alpha$ -glucosidase inhibitory
	IC <sub>50</sub> (mg/mL)	IC <sub>50</sub> (mg/mL)
ASH	0.168 ± 0.008	1.538 ± 0.033
ASD	0.045 ± 0.005	1.253 ± 0.017
ASE	0.089 ± 0.001	0.774 ± 0.003
ASM	0.257 ± 0.004	1.396 ± 0.006
Indomethacin	0.022± 0.001	
Acarbose		0.040 ± 0.002

(Each value in the table is represented as mean ± SD (n=3))

### 3.4. LC-MS/MS analysis

The biological activity studies revealed that the most active extract among all extracts was ethyl acetate (ASE) when the examined activity results were evaluated. Therefore, it was decided to analyze the chemical content of the ASE extract by LC-MS/MS analysis (**Figure 1**). Also, the results of detected phenolic compounds were showed in **Table 3**. Feruloylquinic acid, hydroxycinnamic acid derivative; luteolin, isorhamnetin, isovitexin, chrysoeriol flavone derivatives were determined in ASE extract.

Feruloylquinic acid showed a signal at  $m/z$  367 and MS ions at  $m/z$  191 and 173. Ions at  $m/z$  191 and 173 are deprotonated quinic acid and dehydrated quinic acid, respectively, while ion at  $m/z$  193 corresponds to ferulic acid [13]. Luteolin was identified with a molecular ion peak at  $m/z$  285 which fragmented to ion at  $m/z$  133. Luteolin glucoside having 162 amu higher molecular weight than luteolin demonstrated [M-H]<sup>-</sup> value at  $m/z$  447 and the product ion at  $m/z$  285 representing luteolin aglycone through the absence of a glucoside moiety [14,15]. Isorhamnetin gave [M-H]<sup>-</sup> value at  $m/z$  315 and the product ion at  $m/z$  300.



**Figure 1.** LC-MS/MS chromatogram of ethyl acetate (ASE) extract of *A. sieheana*

**Table 3.** The characterization of phenolic compounds in the ASE extract of *A. sieheana* by LC-MS/MS analysis

Retention Time (min.)	[M-H] m/z	Fragments	Identification	Reference
9.2	367	191, 173	Feruloylquinic acid	[13,16,17]
9.6	431	341, 311	Apigenin-6-C-Glucoside (isovitexin)	[18]
10.0	447	285	Luteolin glucoside	[2,14,15,16]
10.0	461	298, 284	300MW methoxyflavonoid hexoside like Chrysoeriol O-hexoside	[18]
12.3	461	299, 284	Kaempferol-methyl ester-O-hexoside or Luteolin-methyl ester-O-hexoside	[18]
13.4	475	299, 175	300MW methoxyflavonoid glucuronide like Chrysoeriol glucuronide	[18]
17.0	285	133	Luteolin	[14,15,16]
17.0	315	300	Isorhamnetin	[15,18]

### 3. DISCUSSION

In the literature, there is only the chemical content and biological activities of the essential oil of *A. sieheana* [19,20]. The antioxidant activity of the methanol extract was investigated by phosphomolybdenum,  $\beta$ -carotene bleaching and DPPH methods and it was found to have a good DPPH radical scavenging effect with an  $IC_{50}$  (value of 87.04  $\mu\text{g/mL}$  and high reducing effect on the oxidation of  $\beta$ -carotene with 71.08% value [20]. Unlike in this study, the biological activities (antioxidant, anti-lipoxygenase, antidiabetic) of hexane, dichloromethane, ethyl acetate and methanol extracts obtained from aerial parts of *A. sieheana* by fractional percolation method and the phytochemical content of ethyl acetate extract were evaluated by LC-MS/MS. Ethyl acetate extract (ASE) is a good antioxidant with  $IC_{50}$  values of  $0.156 \pm 0.003$  mg/mL for DPPH and  $0.096 \pm 0.001$  mg/mL for ABTS. It showed a moderate antidiabetic effect with an  $IC_{50}$ ,  $0.774 \pm 0.003$  mg/mL and a total phenolic content of 59.737 mg GAE/g. According to the anti-inflammatory activity results, ASD and ASE showed significant activity with  $IC_{50}$  values of  $0.045 \pm 0.005$  mg/mL and  $0.089 \pm 0.001$  mg/mL, respectively.

In studies with other *Achillea* species, the ethanol extract of *A. cucullata* showed DPPH radical scavenging activity with an  $IC_{50}$  value of  $132 \pm 0.026$   $\mu\text{g/mL}$ . The total phenolic and flavonoid content was found as  $53.807 \pm 0.059$  (mg GAE/g),  $21.372 \pm 0.026$  (mg QE/g). Also, the extract strongly inhibited  $\alpha$ -glucosidase enzymes with  $IC_{50}$  value of 24.75  $\mu\text{g/mL}$  [21]. Venditti et al., studied antioxidant and  $\alpha$ -glucosidase inhibitory activities of *A. tenorii* ethanol extract.  $IC_{50}$  values of the extract was found as  $31.41 \pm 3.13$   $\mu\text{g/mL}$ ,  $22.49 \pm 2.01$   $\mu\text{g/mL}$ , 32  $\mu\text{g/mL}$  for DPPH, ABTS and  $\alpha$ -glucosidase inhibitory assays, respectively. In addition, phenolic acid and flavonoid derivatives were isolated from the extract and pure luteolin was determined which is a well known  $\alpha$ -glucosidase inhibitor with  $IC_{50}$  value of  $47 \pm 1.5$   $\mu\text{M}$  (13.23  $\mu\text{g/mL}$ ) [22]. The ethanolic extract of *A. fragrantissima* showed higher  $\alpha$ -glucosidase inhibitory activity ( $IC_{50}=26.66 \pm 1.02$   $\mu\text{g/mL}$ ) than the aqueous extract. Chloroform and n-butanol fractions obtained from ethanolic extract exhibited significant activity ( $IC_{50}$  129.7  $\pm$  2.01 and 102.6  $\pm$  1.12  $\mu\text{g/mL}$ , respectively). The flavonoid derivatives; quercetin-3,6,7-trimethyl ether ( $IC_{50}$ , 14.59  $\pm$  0.89  $\mu\text{g/mL}$ ), isovitexin-4'-methyl ether ( $IC_{50}$ , 83.57  $\pm$  0.59  $\mu\text{g/mL}$ ), isovitexin ( $IC_{50}$ , 34.37  $\pm$  1.09  $\mu\text{g/mL}$ ), acacetin-6-C-(6''-acetyl- $\beta$ -D-glucopyranoside) ( $IC_{50}$ , 1.5  $\pm$  0.09  $\mu\text{g/mL}$ ) were purified from these fractions and they showed more significant activity than fractions [23]. The *A. schurii* extract demonstrated a good antioxidant capacity ( $IC_{50}$ , 58.87  $\pm$  2.12  $\mu\text{g/mL}$ ) and high phenolic contents (76.93 mg/g polyphenols, 18.61 mg/g flavonoids and 41.48 mg/g caffeic acid derivatives, respectively). Four phenolic acids (gentisic, caffeic, chlorogenic, p-coumaric acids) and five flavonoids (isoquercitrin, rutin, quercetin, apigenin, and luteolin) compounds were identified in this extract [24].

In this study, 5-LOX evaluation of all extracts was performed *in vitro*. Mostly *in vivo* antiinflammatory studies were recorded in the literature studies. In research on *A. santolina*, the plant's methanol extract shown substantial antihyperalgesic and anti-inflammatory effects during pretreatment and short-term therapy in another investigation. A comparison of the effects of 200 and 400 mg/kg of this extract showed no significant difference. *A. santolina* defatted extract did not significantly affect CFA-induced inflammation at any point in the treatment process ( $P>0.05$ ). Indomethacin was shown to be less effective than methanolic extract when given short-term for edema, hyperalgesia, and lowering serum IL-6 levels at a dosage of 200 mg/kg [25]. Aqueous and methanol extracts from *A. ageratum* have inflammatory inhibitory effect because

they inhibited carrageenan oedema in both the earlier phase of inflammation (0-3 h after injection) and the later phase of inflammation. This activity was higher at 5 h than at 3 h with ED<sub>50</sub> values of 215.68 ± 41.59 for the aqueous extract and 292.57 ± 40.42 for the methanol extract [26].

Apart from these reports, a large number of the previously reports have been published reports *in vitro* and *in vivo* on antioxidant, antiinflammatory and antidiabetic activities of *Achillea* spp. and about investigating the phytochemical characterization of different *Achillea* species by LC-MS/MS [14,15,27,28,29].

*Achillea* spp. have a broad spectrum of biological activity [30]. The various biological activities of *Achillea* extracts are probably associated with its phenolic contents. Most of the species belonging to the *Achillea* genus contain flavonoids: flavonols and flavones and their derivatives. In different species of *Achillea*, aglycons (apigenin, luteolin, quercetin), monoglycosides (mainly O-glucosides, C-glucosides, and O-glucuronides), diglycosides (O-diglucosides, C-diglucosides, O-rutinosides, 6-C-glucosyl-8-C-arabinosyl, 6-C-arabinosyl-8-C-glucosyl, luteolin-6-C-apiofuranosyl-(1→2)-glucoside, 3-O-arabinosyl-(1→6)-glucoside), and methyl derivatives were found [27]. Many investigations have reported on flavones and 3-hydroxyflavones; among them, luteolin, apigenin, and quercetin with their corresponding glycosides appear to be major chemicals in a large number of *Achillea* species. Numerous C-glycosylflavones with luteolin and apigenin skeletons accumulate in the species [30].

In this study, feruloylquinic acid, luteolin and its glucoside form, isorhamnetin, isovitexin, chrysoeriol flavone derivatives in the ASE were detected by LC-MS/MS. These detected molecules are known that have antioxidant, anti-inflammatory, antidiabetic, and other biological properties [12,31,32,33].

Flavonoids are a class of secondary plant phenolics and have various bioactivities, including antioxidant, anti-inflammatory properties, anti-diabetic effects and many other effects. For radical scavenging activity, substituents on the B ring are more important than those on rings A and C. The C2=C3 double bond conjugated with the 4-oxo group on ring C is responsible for electron delocalization from the B-ring and also, glycosylation attenuates the scavenging activity. For anti-inflammatory activity, a hydroxyl group at C-3 is and C2=C3 double bond has been found to be a very important moiety for the inhibition of LOX activity [34]. The total of hydroxyl groups, hydroxyl configuration, C2=C3 double bond, and C-4 ketonic functional group are the essential features in the manifestation of bioactivity of flavonoids especially for antidiabetic effect [35].

A flavone molecule called luteolin is found in many therapeutic plants. The flavones are a subclass of flavonoids and are among the most prevalent secondary metabolites in plants. It is a potent inhibitor of the 5-LOX enzyme, which is a crucial step in the formation of leukotriene B<sub>4</sub>, a mediator of a number of inflammatory disorders. Since both lipoxygenases and cyclooxygenases are ROS-producing enzymes, inhibition of both enzymes can be linked to suppression of ROS. Luteolin is an anti-inflammatory substance that works in a variety of ways, including antioxidant and ROS scavenging processes [36]. Luteolin controls many cells signaling pathways and reduces inflammation in the brain tissues. Additionally, because oxidative stress and inflammation play a significant role in the onset, development, and mortality of neurodegenerative disorders, antioxidants and anti-inflammatory drugs like luteolin can be employed as innovative therapeutic agents for the treatment of neurodegenerative diseases [32].

Luteolin and luteolin 7-O-glucoside shown substantial inhibition against yeast  $\alpha$ -glucosidase in research, with luteolin having the greatest inhibitory action among the substances examined. Yeast  $\alpha$ -glucosidase inhibitory activity of luteolin was stronger than acarbose with IC<sub>50</sub> of about 5 mg/mL, a drug used clinically, and its IC<sub>50</sub> was 0.5~1 mg/mL. Luteolin and foods containing the compound might improve the symptoms caused by hyperglycemia in type II diabetic patients. However, there is a possibility that its inhibitory activity against human  $\alpha$ -glucosidase may be different from that against the yeast enzyme. It also inhibited porcine pancreatic  $\alpha$ -amylase activity strongly and its IC<sub>50</sub> was in the range of 50 to 500  $\mu$ g/mL while IC<sub>50</sub> of acarbose against alpha-amylase ranged 5 to 50  $\mu$ g/mL [12].

Many disorders, including osteoarthritis and periodontitis, which can limit inflammatory responses, are affected by isorhamnetin's anti-inflammatory properties. Isorhamnetin's anti-inflammatory properties also aid in the prevention of acute lung damage, TB, and kidney protection. The mechanism is connected to controlling the production of cytokines, ROS, and inflammatory mediators. Isorhamnetin has anti-oxidant action by scavenging DPPH and ABTS radicals and inhibiting lipid peroxide of liver mitochondria *in vitro*. Due to its antioxidant properties, isorhamnetin can also suppress HIF-1 $\alpha$ , which prevents cancer cells from migrating and invading *in vitro*. Because of its many beneficial effects on PI3K/AKT, NF- $\kappa$ B, and other signaling pathways as well as cytokines, isorhamnetin is used to treat a variety of disorders [37].

Vitexin and isovitexin, which are bioactive components, have been taken out of several therapeutic plants. As a result of their distinct chemical make-up, they share the bioactivities of the popular flavone

apigenin. For example, the presence of C-8 glucoside boosts vitexin's anti-oxidant efficacy by decreasing the negative charge on the oxygen atom at C-3, and their shared o-di-hydroxyl structure in the A ring has been shown to contribute to its effectiveness as a radical scavenger. The inflammation effects of vitexin and isovitexin have been drawing more attention, which may be due to their inhibition in the pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , etc. and increase the anti-inflammatory cytokine such as IL-10. As is widely recognized, inflammation contributes to a number of diseases, including cancer and problems of the neurological and cardiovascular systems [38].

#### 4. CONCLUSION

To sum up, ethyl acetate (ASE) extract demonstrated significant antioxidant, anti-inflammatory and moderate  $\alpha$ -glucosidase inhibitory activity than other extracts due to having its chemical constituents. Also, it was found that the total amount of phenolic compounds of the ASE extract was higher than the other extracts. Phytochemical characterization of ASE, the most active extract, has been determined by LC-MS/MS for the first time. The phenolic aglycones and glycoside components of ASE extract have a major role to play in the biological activities. The results of this study could serve as basic scientific research for necessary development of drugs and nutraceuticals from natural resources as a source of natural antioxidant, antidiabetic, anti-inflammatory agents in the future.

#### 5. MATERIALS AND METHODS

##### 5.1. Plant material

*A. sieheana* Stapf aerial parts (**Figure 2**) were collected in July 2018 from Develi-Kayseri, Turkey. It was identified by Prof. Dr. Şükran Kültür (Istanbul University Herbarium, ISTE No: 116351).



**Figure 2.** *A. sieheana* (photo by T. Dikpınar)

##### 5.2. Extraction

The aerial part of *A. sieheana* were air dried at room temperature and powdered. The amount of 960 g plant material was fractionally extracted with 8 L of n-hexane, dichloromethane, ethyl acetate and methanol solvents until colorless by percolation method to be used in both pharmacological activities and isolation analysis in further studies. All extracts were obtained under vacuum by rotary evaporator and were kept in the freezer at +4 °C.

### 5.3. Antioxidant activities

#### 5.3.1 DPPH radical scavenging activity

The DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical scavenging activities of all extracts were evaluated by a method described by Zou et al [39]. One of the substances that has a proton free radical with a distinctive absorption is DPPH. When exposed to proton radical scavengers, this absorption significantly decreases. In their reaction with DPPH, antioxidants reduce the same number of DPPH molecules as there are available hydroxyl groups [40].

#### 5.3.2 ABTS radical scavenging activity

The ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] assay of all extracts were evaluated by Zou et al [39]. The ability of test compounds to reduce the color of reactions with the ABTS radical, which is an intensely colored radical cation of ABTS, is used to determine an antioxidant's capacity. In comparison to DPPH radicals, ABTS + radicals are more reactive. Ionic strength has no effect on the solubility of ABTS +, which is soluble in both aqueous and organic solvents [11].

#### 5.3.3. Determination of total phenolic contents

The extracts were investigated regarding their composition by a colorimetric technique, Folin-Ciocalteu assay according to Gao et al [41].

### 5.4. Anti-inflammatory activity

The *in vitro* 5-lipoxygenase inhibitory (5-LOX) activities of extracts were evaluated to the method described by Phosrithong et al [42]. Indomethacin was used as a reference standard. Percent inhibition was calculated according to the following equation:

$$\% \text{ inhibition} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

A dose-response curve was plotted to determine IC<sub>50</sub> values. The IC<sub>50</sub> is defined as the concentration of the extract sufficient to inhibit 50% of the enzyme's activity.

### 5.5. $\alpha$ -Glucosidase inhibition assay

*In vitro*  $\alpha$ -glucosidase inhibitory activity of extracts was evaluated to the method described by Ramakrishna et al [43].

### 5.6. LC-MS/MS analysis

LC-MS/MS (Liquid chromatography linked to tandem mass spectrometry) analysis was carried out using an Absciex 3200 Q trap MS/MS detector. Experiments were performed with a Shimadzu 20A HPLC system coupled to an Applied Biosystems 3200 Q-Trap LC- MS/MS instrument equipped with an ESI source operating in negative ion mode. For the chromatographic separation, a GL Science Inertsil ODS - 3 250  $\times$  4.6 mm, 5  $\mu$ m particle size, analytical column operating at 40 $^{\circ}$  C has been used. The solvent flow rate was maintained at 0.5 mL/min. Detection was carried out with PDA detector. The elution gradient consisted of mobile phases (A) acetonitrile:water:formic acid (10:89:1, v/v/v) and (B) acetonitrile:water:formic acid (89:10:1, v/v/v) was used. The composition of B was increased from 10% to 100% in 40 min. LC-ESI-MS/MS data were collected and processed by Analyst 1.6 software.

### 5.7. Statistical analysis

The results were analysed by the GraphPad Prism 5 program and expressed as mean  $\pm$  standard deviation.

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Materials – T.D., L.B.; Data Collection and/or Processing – T.D., L.B., F.G.; Analysis and/or Interpretation – T.D., L.B., F.G.; Literature Search – T.D., L.B., F.G.; Writing – T.D., L.B.; Critical Reviews – T.D., L.B., F.G.

**Conflict of interest statement:** The authors have no conflict of interest to declare within this article.

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