International Journal of Secondary Metabolite
2021, Vol. 8, No. 4, 312–320
https://doi.org/10.21448/ijsm.950087
Published at https://dergipark.org.tr/en/pub/ijsm
Research Article

Investigation of *in vitro* Enzyme Inhibitory Properties and Antioxidant Activity of *Moltkia coerulea* (Willd.) Lehm. (Boraginaceae) Growing in Raman Mountain - Batman

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Abstract: The province of Batman is located in the Southeastern Anatolia Region of Turkey, and it is significant in terms of its ecosystem and plant biological diversity. Recently, researching economically important plant species has become a necessity in the province. In this context, while the members of the Boraginaceae genus have found a wide application area in traditional medicine in many countries from ancient times until today, they have been used for many purposes in Turkey. Most of the members of this family are medically important plants containing secondary metabolites such as flavonoids, terpenoids, alkaloids, fatty acids, glycosides, phytosterols, and various proteins. *Moltkia coerulea* (Willd.) Lehm. is found Anatolia, Lebanon and Crimea. This study aimed to determine the enzyme inhibition and antioxidant activity of *M. coerulea* (Willd.) Lehm, which has not been studied before, and grows in the untouched Raman Mountain in Batman. α-amylase and α-glucosidase inhibition results of methanolic (MeOH) and aqueous (Aq) extracts of *M. coerulea* were calculated as acarbose equivalents (ACAEs/g extract). Tyrosinase inhibition results of MeOH and Aq extracts of *M. coerulea* were calculated as kojic acid equivalent (mmol KAEs/g extract). Additionally, the extracts were tested against the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) free-radical to analyze their antioxidant activity. The highest antioxidant activity was found in the leaf extract (MeOH) as 61.2 % with for the DPPH• method. These results showed that *M. coerulea* could be used as a potential source of natural antioxidant.

ARTICLE HISTORY
Received: June 09, 2021
Revised: Sept. 06, 2021
Accepted: Sept. 26, 2021

KEYWORDS
Antioxidant activity,
Enzyme inhibition,
Moltkia Lehm.

1. INTRODUCTION

The Boraginaceae family is represented in the world with 130 genera and 2300 species, mainly annual, biennial or perennial herbs and shrubs, some trees and a few lianas, and it is distributed in temperate and subtropical areas of the Northern and Southern Hemispheres (Akçin & Binzet 2009; Tufa *et al*., 2019). In the last arrangement made by APG IV (2016), in light of molecular phylogeny, the Boraginaceae family was placed in the order Boraginales, which was formed within the clades of Eudicots – Superasterids - Asterids and Lamiids (Chacon *et al*., 2016).

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e-ISSN: 2148-6905 / **© IJSM** 2021

In Turkey, it includes 357 taxa, including 34 genera, 325 species, 16 subspecies, and 16 varieties. Turkey ranks in top 10 with the broadest distribution of taxa in the Boraginaceae family. Of these, 31 genera and 320 species naturally grow; 3 genera and 5 species are cultivated, 1 species is naturalized, and there are 2 types of hybrids. Among the natural species, the endemism rate was reported as 42.2 % (Davis 1978; Yıldırım 2000). Most plants belonging to the family are used as ornamental plants and obtain spices and dyestuffs (Pehlivan *et al*., 2001). The family includes important plants that have a wide range of use in medicine, pharmacology and cosmetics. The therapeutic effect of these herbs is related to their contents of many biologically active compounds, including naphthoquinones, flavonoids, terpenoids, and phenols. Components isolated from these plants exhibit antimicrobial, antitumor, antiviral, anti-inflammatory, cardiotonic, contraceptive and antiplatelet activities (Sharma *et al*., 2009; Papp *et al*., 2011; Taravati *et al*., 2014; Dresler *et al*., 2017). *M. coerulea*, which constitutes the biological material of this study, belongs to the Boraginaceae family. The vernacular name of this species is "Mavi kesen". In the Anti-Taurus Mountains in the province of Niğde in Turkey, the flowers of the plant are consumed by children because of their taste, while its flowers and roots are also used to treat diarrhea and abdominal pain (Özdemir & Alpınar 2010-2011; Özdemir & Alpınar 2015). In Sivas, the leaves of *M. coerulea* are consumed as food (Orhan *et al*., 2021).

The α-amylase and α-glucosidase enzymes are key enzymes involved in carbohydrate digestion. Oligosaccharides such as starch, α-dextrin, maltose are hydrolyzed by amylase. α-glucosidase hydrolyzes disaccharides and oligosaccharides into glucose units in the small intestines (Sing *et al*.,2010). Therefore, the inhibition of α-amylase and α-glucosidase enzymes is an important strategy in reducing increased blood glucose levels (Laube 2002). Drugs that lower blood glucose levels have side effects such as severe hypoglycemia, lactic acidosis, neurological disorders, upset stomach, headache, and even death. Considering the side effects of the long-term use of insulin and other hypoglycemic drugs, it is needed to develop safe and effective drugs, especially plant-based ones (Grover *et al*., 2002; Watkins 2003). Different traditional medical systems use raw plant extracts or active compounds derived from the plant to treat diabetes. Additionally, after the recommendation made by the World Health Organization (WHO), research on the hypoglycemic activities of medicinal plants has become more important (WHO 1980). The literature review in this study revealed that there are few studies (such as Zengin *et al*., 2017; Orhan *et al*., 2021) on *Moltkia* species that grow or are grown in Turkey. The fact that the area where the study material was collected had not been investigated reveals the necessity of shedding light on the properties of this plant in the area. Moreover, this study was needed for reasons such as differences like the altitudes of the habitats of plants and the soil structure affecting the secondary metabolites produced by plants. Thus, in this study, I aimed to investigate the *in vitro* enzyme inhibitory properties and antioxidant activity of the MeOH and aq extracts of the dried and ground parts (above-ground parts: leaves and flowers) of *Moltkia coerulea* (Willd.).

2. MATERIAL and METHODS

2.1. Plant Material

Moltkia coerulea which constitutes the study material, was collected from the campus of Batman University during the vegetation period in May 2020 (Figure 1). The botanical identity of the plant was confirmed by Dr. Alevcan Kaplan. Voucher specimens have been deposited at Batman University (voucher no.2020/12). The plants collected have been identified using the 6th volume of the Flora of Turkey (Davis 1978). The collected plant was washed to remove impurities and dried in the shade at room temperature. The samples obtained were ground in the blender.

2.2. Plant Extraction

The Aq and MeOH extracts of dried and ground plant parts (above-ground parts: leaves and flowers) were prepared. First of all, the aq extract was prepared. The dried and ground plant parts were extracted with 100 mL hot water (2 % w/v) for 6 hours on a heated magnetic stirrer and filtered. The residues were then recovered with 100 mL of water, again using the same procedure. The filtered aq extracts were combined, lyophilized and stored at +4 ºC pending experiments. After this, the MeOH extract was prepared. The dried and ground plant parts were treated with 200 mL of MeOH on a shaker for one day (24 hours) at room temperature and then filtered. This process was repeated with the same procedure. Finally, the extracts were pooled and concentrated using a rotary evaporator.

2.3. Enzyme Inhibitory Activity

2.3.1. α-amylase inhibitory activity

For determining α-amylase inhibition, a 25 µL sample of the extract (Aq and MeOH), and 50 µL of an α-amylase solution prepared in a sodium phosphate buffer (pH 6.9) were added to test tubes, and the samples were incubated at 37 ºC for 10 minutes. The reaction was initiated by adding 50 µL of a starch solution to the wells. A blank was prepared containing denatured enzyme samples. The samples and the blank were incubated at 37 ºC for 10 minutes. The reaction was stopped by adding 25 µL of 1M HCl, and 100 µL of a potassium iodide solution was added. Samples and blank were read at 630 nm. The α-amylase inhibition effect of the extracts were expressed in units of mmol Acarbose equivalent per g dry weight (mmol ACAEs/g extract) (Sarıkurkcu *et al*., 2018).

2.3.2. α-glucosidase inhibitory activity

For determining α-glucosidase inhibition activity, 50 µL of the extract (Aq and MeOH), 50 µL glutathione, 50 µL α-glucosidase solution prepared in a phosphate buffer (pH 6.8) and 50 µL 4-N-trophenyl-α-D-glucopyranoside. (PNPG) were added into test tubes, and the resulting samples were incubated at 37 ºC for 15 minutes. A blank containing denatured enzyme samples was also prepared. The reaction was stopped by adding 50 µL of 0.2 M sodium carbonate. The samples and the blank were read at 400 nm. Acarbose, an α-glucosidase inhibitor, was chosen as a reference. The α-glucosidase inhibition effect of the extracts were expressed in units of mmol Acarbose equivalent per g dry weight (mmol ACAEs/g extract) (Sarıkurkcu *et al*., 2018).

2.3.3. Tyrosinase inhibitory activity

To determine tyrosinase inhibition activity, 25 µL of the aq and MeOH extracts, 100 µL of a phosphate buffer (pH 6.8) and 40 µL of a tyrosinase solution were added, and the samples were incubated at 25 ºC for 15 minutes. The reaction was initiated by adding 40 µL of an L-DOPA solution to the tubes. A blank containing denatured enzyme samples was also prepared. The samples and the blank were read at 492 nm after waiting for 10 minutes and at 25 ºC. The tyrosinase inhibition activity of the extracts were expressed in mmol kojic acid equivalent per g dry weight (mmol KAEs/g extract) (Sarıkurkcu *et al*., 2018).

2.4. Antioxidant Activity

2.4.1. DPPH scavenging activity

Free radical activities of the extracts were determined using the DPPH free radical (Gezer *et al*., 2006). For the experiment, the concentrate was prepared by dissolving 4 mg DPPH in 100 mL MeOH. 3.2 mL of the DPPH radical and 200 µL (500 µg/mL) of the extract solutions were

added for each sample. After 30 minutes of incubation at room temperature in the dark, absorbance was measured at 517 nm. For control, 200 µL of the extract solution was added to the test tube. Each trial was made with triplicate. The following formula was used to determine the % DPPH radical scavenging activities of the samples.

$$\% \text{ DPPH scavenging activity} = [(A_{control} - E_{extract}) / A_{control}] \times 100$$

2.5. Statistical Analysis

All experiments in the study were carried out in triplicates. For the DPPH assay, the % scavenging activities of the samples were calculated using the Microsoft Excel program, and standard error bars were added to the plots. For the other analysis, the descriptive statistical data (Mean ± Standard Deviation) of the variables are shown in tables. The statistical analysis were performed using SPSS for Windows (version 21.0).

3. RESULTS and DISCUSSION

M. coerulea which constitutes the study material, was collected from the campus of Batman University during the vegetation period in May 2020 (Figure 1).

Figure 1. Natural photos of *Moltkia coerulea*: A- general view of plant, B- flowering part.

The yield amounts of the extracts are given in Table 1. Accordingly, when the effects of the solvents on the yield were examined based on due to the extraction process, the highest performance in terms of the two different solvents was obtained in the experiments using the aq as a solvent. A lower yield was obtained when MeOH was used as the solvent. The inhibitory effects of the Aq and MeOH extracts of *M. coerulea* against three different enzymes were determined (Table 2).

Table 1. Extract yields of *Moltkia coerulea* in the Aq and MeOH solvents.

Samples	Solvent system	%	mg /g dry matter
MCL	Aq	21.37	213.7
MCL	MeOH	11.12	111.2
MCF	Aq	21.65	216.5
MCF	MeOH	12.15	121.5

MCL: *Moltkia coerulea* leaf; *Moltkia coerulea* flower

± standard deviation was used. n: 3

Table 2. Enzyme inhibitory activities of the Aq and MeOH extract of *M. Coerulea*.

Sample	Part	Extract type	α-amylase inhibition activity (mmol ACAEs/g extract)	α-glucosidase inhibition activity (mmol ACAEs/g extract)	Tyrosinase inhibition activity (mmol KAEs/g extract)
M.coerulea	Leaf	Aq	0.296±0.12	0.481±0.06	0.860±0.00
		MeOH	0.325±0.01	0.610±0.01	0.761±0.00
	Flower	Aq	0.450±0.05	0.516±0.22	0.480±0.01
		MeOH	0.358±0.07	0.524±0.71	0.610±0.04

KAE: Kojic acid equivalent; ACAE: Acarbose equivalent
Values expressed are means ± S.D. of three parallel measurements

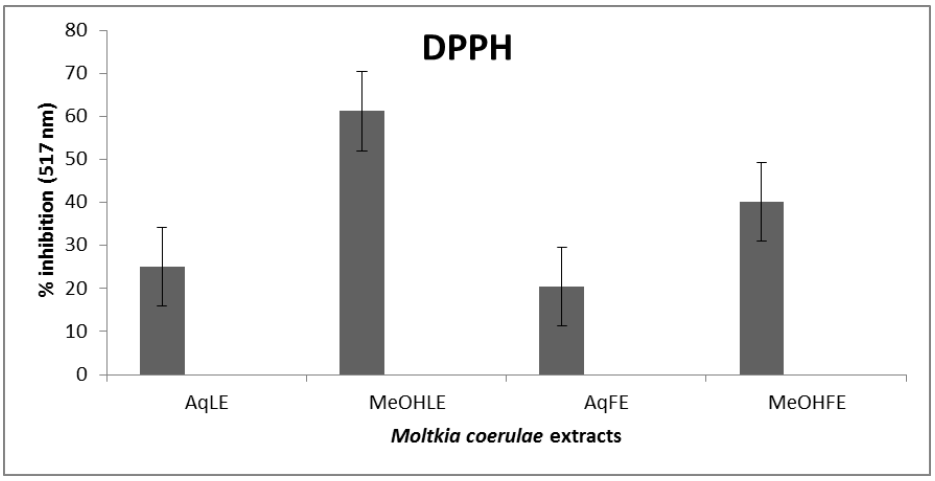
Diabetes is characterized by chronic hyperglycemia and has become a significant health problem all over the world. A consistently high glucose level in the blood leads to cardiovascular disease, neuropathy, retinopathy, nephropathy and other disorders. Hypoglycemic drugs used today manage to normalize serum glucose levels, but they cause gastrointestinal complaints. Thus, it is important to find effective therapeutic agents that inhibit α-amylase and α-glucosidase and have no side effects (Liu *et al*., 2017). The α-glucosidase and α-amylase inhibition effects of the Aq and MeOH extracts of the flowers and leaves of the *M. coerulea* plant are shown in Table 2. The α-amylase inhibition effect of the Aq and MeOH (0.450 and 0.358 mmol ACAEs/g extract, respectively) extracts of flowers was stronger than the Aq and MeOH (0.296 and 0.325 ACAEs/g extract, respectively) extracts of leaves. The α-glucosidase inhibition effect of the Aq and MeOH (0.481 and 0.610 mmol ACAEs/g extract, respectively) extracts of leaves was closest with the Aq and MeOH (0.516 and 0.524 mmol ACAEs/g extract, respectively) extracts of flowers. In general, the results obtained in this study on the α-amylase inhibition effects of the extracts were similar to those reported in the literature. The α-glucosidase inhibition effects of the extracts in this study were lower than those in some studies and higher than those in some others (Zengin *et al*., 2017;Orhan *et al*., 2020). The different substrates can explain these differences in the inhibition of two enzymes that affect sugar metabolism.

Likewise, in various studies investigating the enzyme inhibition effects of phenolic compounds, some phenolic compounds have been reported to have effective glycosidase inhibition activities (Kubola *et al*., 2008; Vadivel & Biesalksi, 2011; Wang *et al*., 2012). This also explains the inverse relationship between the consumption of foods rich in phenolic compounds and diabetes rates. Moreover, tyrosinase is a key enzyme that catalyzes the production of the pigment melanin that helps prevent damage induced by UV light exposure. Melanin synthesis occurs in melanocytes by the transformation of tyrosine into dihydroxy phenylalanine (DOPA), then DOPA-quinone, and then into black-brown eumelanin and yellow-red pheomelanin via the tyrosinase enzyme. With this function, tyrosinase is takes part in the formation of skin and hair color (Canovas *et al*., 1982; Rodriguez-Lopez *et al*., 1991; Cooksey *et al*., 1997). Its excess can lead to hyperpigmentation and neurodegenerative diseases such as Parkinson's disease. Although many synthetic inhibitors, especially kojic acid, have been developed to inhibit tyrosinase activity, the long-term toxic effects of these inhibitors have made their use questionable, and studies to determine natural inhibitors as alternatives to these have become the focus of attention (Tocco *et al*., 2009). Accordingly, in line with the data obtained it this study, the inhibitory effect of the Aq and MeOH (0.860 and 0.761 mmol KAEs/g extract, respectively) leaf extracts on the tyrosinase enzyme was stronger than that of the Aq and MeOH (0.480 and 0.610 mmol KAEs/g extract, respectively) flower extracts. Zengin *et al*. (2017), also determined that the aeiral parts of the plant samples' extract

of the same plant was more effective in inhibiting the tyrosinase enzyme (34.97 ± 0.50 mg KAEs/g extract). In addition, they stated that rutin, which is the major compound with phenolic groups, has been previously reported to inhibit tyrosinase through an inhibitory mechanism similar to that of copper chelators. That is, rutin competes competitively with the substrate L-DOPA in the active site pocket, inducing hydrophobic surface exposure (Si *et al*., 2012; Zengin *et al*., 2017).

Oxidative stress is recognized as the main pathological trigger for many diseases, including type II diabetes and Alzheimer's. Antioxidants are thought to be therapeutic tools to inhibit the activity levels and formation of oxidative stress (Liu *et al*., 2017). In this study, the antioxidant capacity of the MeOH and aq extracts of *M.coerulea* was determined using the DPPH test (Figure 2). The DPPH radical is widely used to evaluate free radical scavenging activity due to its ease of reaction. When the DPPH radical is scavenged with an antioxidant compound through hydrogen donation to form a stable DPPH-H molecule, the color of the solution turns from purple to yellow (Gangwar *et al*., 2014). If the results of this study are evaluated in general, it may be stated that the MeOH extracts of leaves and flowers (MeOHLE 61.2 % and MeOHFE 40.17 %, respectively) showed a better DPPH scavenging activity than the aq extracts (AqLE 25.1 %; AqFE 20.4 %). Koncˇic´ *et al*. (2010) collected the leaves of the *Moltkia petraea* plants from two regions named Sveti Jure and Snijezˇnica in Croatia, and they found that the leaves of the plants collected from the Snijezˇnica locality generally showed better antioxidant activity than the ones collected from the Sveti Jure locality. Orhan *et al*. (2021) investigated in the *in vitro* enzyme inhibition properties, antioxidant and phytochemical profiles antimicrobial and anti-tyrosinase activity of *M. aurea* and *M. coerulea*. As a result of their analysis of the total antioxidant capacity, they revealed that the ethyl acetate extracts exhibited a remarkable antioxidant potential compared to the extracts prepared using other solvents. While the superoxide scavenging activity of the water, MeOH and EA (ethyl acetate) extracts of the roots of both species was found promising, the MeOH extracts of all samples had significant DPPH free radical scavenging activities. The researchers stated that the antioxidant and antidiabetic effects of the extracts may have occurred due to their rutin and rosmarinic acid contents. It has been thought that the reason for the variations between *M. coerulea* species collected from different localities and between different species (such as *M. petraea*) in other studies was differences affecting these plants' biochemical and physiological structures such as species, organ, physiological age, harvest time, and locality. Consequently, in this study, it was determined that the plant extracts had a high rate of radical scavenging activity. Based on the results, it is thought that the extracts of *M. coerulea* can be used as natural source of antioxidants in areas such as food, cosmetics and pharmacology, in treatment, as a preservatives and as additives.

Figure 2. DPPH free radical scavenging activity of *Moltkia coerulea* extracts. AqLE; aqueous leaf extract, MeOHLE; methanol leaf extract, AqFE; aqueous flower extract, MeOHFE; methanol flower extract.



4. CONCLUSION

Due to the recently increasing concerns of the public regarding the effects of synthetic compounds on human health, natural compounds have gained a significant position. This field of research has revealed new, safe and natural sources for natural compounds, which are among the most popular topics in the scientific world. The results obtained in this study demonstrated that the extracts of *M. coerulea* can be considered as a source of natural biological agents. Therefore, these results show that the tested *M. coerulea* plant has a wide range of pharmaceutical uses.

Acknowledgments

The study was presented as an oral at International Eurasian Conference on Biotechnology and Biochemistry (BioTechBioChem 2020) on 16-18 June in Ankara by the online meeting.

Declaration of Conflicting Interests and Ethics

The author declares 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 author.

Authorship Contribution Statement

Alevcan Kaplan: Investigation, Resources, Visualization, Formal Analysis, and Writing - original draft.

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