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Doctoral Degree Thesis

Research on the anticancer potential of Turkish medicinal plants, and factors affecting the distribution along with phytochemical contents of *Glycyrrhiza glabra* L.

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Turkey has very rich plant resources of over 9000 species distributed throughout the country. In advancement of drug development, bioactive compounds isolated from the plants are still used as a lead for the discovery of a new drug. In addition, plant materials can be used as a supplement and/or prophylactic drug to avoid the emergent of serious health conditions which are difficult to detect such as cancer. Various researches were conducted on the therapeutic potential of Turkish plants against cancer. However, researches focusing on the advantages of these plants against gastric cancer are still limited. Furthermore, the phytochemical content of plants can be effected by the environmental factors which in turn make it difficult to obtain a plant with high quality. To challenge these issues, two main research objectives were proposed in this study. First objective is to focus on the therapeutic potential of selected Turkish plants against gastric cancer and the second is to investigate the effect of environmental, topographical, climatic, and geographical factors on the phytochemical content and habitat suitability of *Glycyrrhiza glabra*, commonly used as an herbal drug in Chinese medicine formulations, in the Hatay region of Turkey.

1. Therapeutic Potential of Turkish Medicinal Plants against Gastric Cancer

A. *Screening of the selected plants*

Gastric cancer is the fourth most common cancer in the number of cases and cause of deaths worldwide. In Turkey, the incidence and mortality of gastric cancer are 5.7% and 8.6 %, respectively. There are many risk factors that can be attributed to it such as poor diet (low fibers and high salt), chronic infection with *Helicobacter pylori*, alcoholism, and smoking. Early detection of gastric cancer is usually difficult to achieve, mainly because of its symptoms that are closely similar to stomach upset. Therefore, most of the patients do not start the treatment until the condition reaches an advance stage that is difficult to cure. One way to handle this is the usage of plant materials that has anticancer activity as a food and/or supplement on regular basis. In this study, 20 plant species were selected from Turkey and were extracted with water, 50% ethanol, and 95% ethanol to prepare 84 plant extract samples. Human gastric cancer cell line (AGS) was used to examine the ant-proliferative activity of the samples. Out of 84 samples, five plants (*Myrtus communis*, *Tanacetum macrophyllum*, *Trigonella foenum-graecum*, *Alchemilla mollis*, and *Quercus coccifera*) have shown high growth inhibition against gastric cancer. Normal gastric fibroblast cells were used to examine the toxicity

of *A. mollis*, *M. communis*, and *T. foenum-graecum* samples. The 95% ethanol extract of the aerial part of *A. mollis* (IC_{50} in AGS: 60.0 ± 6.9 µg/mL, IC_{50} in normal cells: 280.8 ± 1.2 µg/mL) and the 95% ethanol extract of the branches and stem parts of *M. communis* (IC_{50} in AGS: 78.0 ± 6.4 µg/mL, IC_{50} in normal cells: 110.7 ± 6.3 µg/mL) showed higher selectivity towards cancer cells than normal cell. In contrast, the 50% ethanol extract of the seed of *T. foenum-graecum* exhibit non selective inhibition to the growth of both normal and gastric cancer cells (IC_{50} in AGS: 28.6 ± 3.0 µg/mL, IC_{50} in normal cells: 2.4 ± 0.8 µg/mL). Further studies are needed to examine the mechanism of cell death and the responsible compounds for the activity. From this study, *M. communis* was considered for further research.

B. Extraction and Phytochemical Investigation on *Myrtus communis*

This plant belong to Myrtaceae family and has been used in Turkey to treat diarrhea, gastric ulcer, rheumatism, hemorrhoid, anxiety, skin disease, and antiseptics among other usages. For this study, the leaf and branches/stem parts were used. They were extracted with 99% ethanol and fractionated into four fractions (hexane, ethyl acetate, butanol, and water fractions). The fractions were examined for their ant-proliferative activity against gastric cancer. Butanol fraction showed the highest anticancer activity than other fractions. Isolation of the responsible bioactive compounds from the butanol fraction can provide a new lead compound for cancer treatment.

2. Effect of Various Factors on the Distribution and phytochemical contents of *Glycyrrhiza glabra*

Many environmental elements can affect the quality, growth, and distribution of licorice; hence, its cultivation is often unproductive. This study examined the important factors that influence the bioactive content of *Glycyrrhiza glabra* root and assessed appropriate growth zones from collection sites in Turkey. Preliminary investigations in the Hatay and the Nizip regions of Turkey endorsed the Hatay region as the main study area for this research due to its suitable soil condition and the existence of abundance amount of *G. glabra*. The contents of three bioactive compounds (glycyrrhizic acid, glabridin, and liquiritin), as well as the geographical data (aspect, curvature, elevation, hillshade, and slope), and soil features (pH, soil bearing capacity, and volumetric soil moisture content) were measured. Weather-related data (precipitation and temperature) were also acquired. An analysis of variance and multivariate analysis of variance were implemented. The results showed that aspect, curvature, and elevation, slope, soil bearing capacity, and volumetric soil moisture content have statistically significant impact on the glycyrrhizic acid and liquiritin contents. A GIS-based frequency ratio model with spatial correlations to the meteorological, soil, and topographical information was utilize to produce the habitat suitability zone map. This map classified the study area in the Hatay region into very high (15.1%), high (31.5%), moderate (40.3%), and low suitability (13.1%) zones. Further exploration and cultivation of *G. glabra* are recommended in areas within the high suitability zone.

Abbreviation

A	Aerial part
S	Seed
L	Leaf
Thr	Thorn
Br/st	Branches and stem
St	Stem
Fr	Fruit
R	Root
Fl	Flower
DMSO	Dimethyl sulfoxide
FBS	fetal bovine serum
PBS	Phosphate buffer
EtOH	Ethanol
Hex.fr.	Hexane fraction of the extract
EA.fr.	Ethyl acetate fraction of the extract
BuOH fr.	Butanol fraction of the extract
Water fr.	Water fraction of the extract
HPLC	High performance liquid chromatography
NMR	Nuclear magnetic resonance
PDA	Photo diode array
ANOVA	analysis of variance
MANOVA	multivariate analysis of variance
FR	Frequency ratio
RF	Relative frequency
DEM	digital elevation model
GPS	global positioning system
VSMC	Volumetric soil moisture content
HSZM	habitat suitability zone map
PR	Prediction ratio

ROC	Receiver operator characteristic
AUC	Area under the curve
GIS	Geographic information system
SRTM–DEM	Shuttle Radar Topography Mission–digital elevation model
IDW	inverse distance weighted

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Chapter 1. Introduction

1.1. Therapeutic Potential of Medicinal Plants against Gastric Cancer in Turkey

Gastric cancer is the fourth most common cancer in the number of cases and cause of deaths worldwide according to the 2018 database of World Health Organization (WHO). In Turkey, stomach cancer ranked sixth (5.7%) regarding the number of cases directly after the lung (16.5%), breast (10.6%), colorectal (9.5%), prostate (8.2%), and thyroid cancer (6.2%). It was also rated as the second most common cause of death from cancer together with the colorectal cancer having a mortality rate of 8.6%. In contrast to Turkey, Japan has higher incidence and mortality rate of stomach cancer, 13.1%, and 11.9%, respectively [1]. This can be the results of the aged population in Japan compared to Turkey as the incidence of gastric cancer increases with age [2].

There are multiple factors that play a major role in increasing the number of cases and death from stomach cancer. Among these, poor diet and bacterial infection by *Helicobacter pylori* are the most common risk factors for stomach cancer [3]. Inappropriate diet that has a lot of salt and low fibers can harm the body greatly by depleting it of the essential nutrient especially if incorporated with smoking and drinking alcohol excessively. It can also cause stomach upset and alter the gastric acidity which could lead to stomach ulcer and if continued for a long time, it can lead to gastric cancer. Infection with *H. pylori* can cause inflammation and irritation to the gastric mucosa, and gastric ulcer. If left untreated or relapsed, it can result in stomach cancer [2]. Turkey has shown an increase in the cases of gastric cancer compared to the previous years due to the increase exposure to the risk factors [4].

This cancer has several treatment methods such as; surgery, chemotherapy, and radiotherapy. However, most of these procedures will not be efficient without an early detection. This implies a serious problem since most of the signs and symptoms of this cancer are similar to the normal stomach upset. Therefore, many patients may not notice or cared to do diagnosis until it's too late [5]. One way to tackle this issues is to use plant materials that have ant-proliferative activity against cancer as food and/or supplements on daily basis. These plant materials will act as a prophylaxis against cancer.

The usage of plant materials as source to treat various aliments has been exercised in Turkey since the ancient times. It follows a system known as Unani system of medicine, which was practiced in Arabian countries as well as Iran, India, and Greek. This system depends on the diagnosis of the symptoms which proceeded by removal of the cause of illness, restoring the body balance of its internal fluids, and normalizing its temperature and/or organ. This is usually achieved by using medicine originated from herbal plants, animal tissues, and/or minerals. It also include treatment by non-herbal methods such as aromatherapy, cupping, massaging, etc. [6]. Using the plant materials as a medicine has helped in the development and discovery of drugs to treat numerous

illness by the detection of novel lead compounds from natural resources. Plentiful drugs have been synthesized from these compounds which proved to be safe and effective [7]. Furthermore, some of these compounds are considered as a great candidate for cancer research, drug discovery, and development.

Turkey has various geographical zones that have its own flora and eating habits [8]. One of these are the Mediterranean zone which mostly follows a Mediterranean diet that is rich in fiber and fresh vegetables and fruits. Remarkably, this region showed less incidence of gastric cancer compared to the other floral zone in the country [2]. Examples of plants in Hatay area that have ant-proliferative effect toward gastric cancer are *Achillea millefolium* L. [9,10,11], *Glycyrrhiza glabra* L. [12,13,14,15], *Eupatorium cannabinum* L. [16], *Olea europaea* L. [17,18,19], *Vitex agnus-castus* L. [20,21], *Viscum album* L. [22,23,24], etc.

1.2. Effect of Various Factors on the Distribution and phytochemical contents of Glycyrrhiza glabra

Turkey has a rich plant flora of more than 9000 plant species owing to its geographical location within three floristic regions (Europe–Siberian, Irano-–Turanian, and Mediterranean zones). This abundance and variation of plant materials have facilitated the advancement of the traditional medicine in the region. Hatay region of Turkey, which located within the Mediterranean zone, has more than 1300 plant species belonging to 110 plant families [25,8].

One of the plants that has wide distribution in Turkey is *Glycyrrhiza*, also known as licorice. It is a plant genus that belongs to the Fabaceae family and it is characterized as a perennial herbal plant. Around 20 known species of this genus have been distributed around the world. Due to its great economic value, it is cultivated in many countries [26]. Various bioactive compounds were isolated from *Glycyrrhiza*, such as Glycyrrhizic acid, glabridin, liquiritin, isoliquiritin, isoflavones, isoliquiritigenin, liquiritigenin and pinocembrin. These compounds are known to have various activities, for instance antioxidant, anti-inflammatory, and anti-proliferative effects towards cancer. Besides its pharmacological activities, it is commonly consumed due to its sweet tastes. Therefore, it is widely used in traditional medicines, cosmetics, and the food industry [27,28].

The percentage of bioactive compounds in *Glycyrrhiza* and theirs biological activities can widely varies in and between countries depending on geographical and environmental factors due to the extensive distribution of this plant [29,30]. Studies have shown that the bioactive contents of the plant depend on the environmental factors and soil conditions [31,32].

Researches have displayed that environmental factors and soil conditions are significant for plant growth and its contents of bioactive compounds [31,32]. For example, distress to the root growth can occur when moisture buildup in the soil. This will cause an increase of heavy metals and/or minerals to toxic levels that hinder the growth of the plant [33,34]. Furthermore, conditions, such as low oxygen content (low

aeration), low drainage, high redox potential, and high organic matter content are usually associated with a high level of moisture content. These conditions also cause the accumulation of magnesium and sulfides which negatively damages the plants [35,36].

To make these parameters more plausible and easier to understand, geographic information systems (GIS) have been utilized. Applying GIS-based methods can be helpful in determining the best growth setting of *G. glabra* by exploring the resources of this plant in countries where it grows naturally, and for classifying aspects that control its growth [37].

Hatay region in Turkey was selected as the main study area for this research. It is characterized by a Mediterranean weather and has sufficient amounts of micronutrients in the soil. Therefore, it is highly appropriate for the growth of *G. glabra* and other Mediterranean plants [29,38]. It also has more than 1300 plant species belonging to 110 plant families [25,8].

The current study investigated the soil, topographical, and environmental characteristics of the Hatay region. It also quantifies the glycyrrhizic acid, glabridin, and liquiritin in *G. glabra* with the intention of highlighting the factors that impact the bioactive contents and distribution of *G. glabra*. This survey also included Nizip region in Turkey and compared it to the Hatay region regarding the phytochemical content of *G. glabra* root and soil micronutrients.

1.3. Aim of the Study

The aims of the current study are:

a) to investigate the therapeutic potential of selected Turkish plants against gastric cancer
b) to investigate the effect of the environmental factors on the phytochemical content of *G. glabra*

Chapter 2. Materials and methods

2.1. Screening for Anti-proliferative Activity against gastric cancer

2.1.1. Plant Materials

In this study, twenty plant species were collected from Hatay and other regions in Turkey (**see Appendix Table A1. Plants collection sites in Turkey**). These plant materials are used as food, spice, and traditional medicine by people in Turkey.

Table. 1 shows the collected plant materials from Turkey. Twenty plant species belonging to 16 plant families were collected within the period of 2016-2019. The authentication of the plants was performed by Dr. Nazım Şekeroğlu (Department of Horticulture, Faculty of Agricultural Engineering, Kilis 7 Aralik University, Turkey), Dr. Faruk Karahan (Department of Biology, Faculty of Science and Literature, Hatay Mustafa Kemal University, Turkey), and Dr. Takashi Watanabe (Department of Medicinal plants, Kumamoto University, Japan).

Table. 1 Collected plant materials and their traditional usages

Scientific name	Family name	Collected part *	Traditional usage
Alchemilla mollis (Buser) Rothm	Rosaceae	A	A/ menopausal pain, diarrhea, stomach problems, skin complaint [39]
Ammi majus L.	Apiaceae	A	Fr / skin disorder [40]
Anastatica hierochuntica L.	Brassicaceae	S	Fr / infertility [41]
Capparis spinosa Linnaeus	Capparaceae	L, Thr, Br/st	R / kidney and liver diseases, mental disorder, spleen tumor A / diabetes, headache, fever, ulcer, etc. [42]
Clematis vitalba L.	Ranunculaceae	A	A / skin disorder, fever, eye infection, rheumatism, etc. [43]
Dioscorea communis (L.) Caddick & Wilkin	Dioscoreaceae	A	L / food [44]
Echium plantagineum L.	Boraginaceae	A	L / food [44]
Erica manipuliflora Salisb.	Ericaceae	A	A / urinary disorder, rheumatism, etc. [45]
Gentiana asclepiadea L.	Gentianaceae	A & R	R / liver disease, anorexia [39]

Scientific name	Family name	Collected part *	Traditional usage
Jacobaea aquatica (Hill) "G. Gaertn., B. Mey. & Scherb."	Compositae	A	A / contain toxic alkaloid [46]
Myrtus communis L.	Myrtaceae	L, Br/st	A / peptic ulcer, inflammation, diarrhea, skin disorder [47]
Nigella sativa L.	Ranunculaceae	S	S / flavoring agent, antibacterial, antihypertensive, skin disorder [48]
Pistacia terebinthus L.	Anacardiaceae	St, Fr, L	Fr & Br / ulcer [49]
Polygonatum multiflorum (L) All.	Asparagaceae	L, Fr, R, St	L & R / diarrhea, hemorrhage, skin inflammation [39]
Quercus coccifera L.	Fagaceae	L, Br/st	A / wound and burn healing [50]
Rhus coriaria L.	Anacardiaceae	Fr	Fr / antihypertensive, antiseptic [49]
Rubus sanctus Schreber	Rosaceae	A	L, Fl, & R / diabetes, pulmonary diseases, stomach pain, skin disorders [49]
Tanacetum macrophyllum (Waldst. & Kit.) Sch.Bip.	Compositae	L, St	Essential oil / antimicrobial [51]
Tilia platyphyllos Scop.	Malvaceae	A	Fl / common cold treatment [52]
Trigonella foenum-graecum L.	Leguminosae	S	S & L / diabetes, obesity, cancer, enhance breast feeding [53]

Note. * Indicates the plants parts, A, aerial parts; S, seed; Thr, thorn; St, stem; Br/st, branch and stem; Br, branches; Fr, fruit; Fl, flower; R, root; L, leaf.

2.1.2. Reagents

Accutase 0.5 mM EDTA (cell detachment solution) was bought from Innovative Cell Technologies, Inc. (California, USA). Cell line medium (RPMI–1640) with L–Glutamine, phenol red, and HEPES contain 10% fetal bovine serum (FBS), 0.25 w/v% trypsin–1 mmol/1 EDTA. 4Na solution with phenol red, and the remaining chemicals were purchased from Fujifilm Wako pure chemical corporation (Osaka, Japan).

2.1.3. Extraction Method

The plant material were dried for five days at 50°C using the dryer machine (ADVANTEC DRG400AA, Tokyo, Japan). Then it was converted to a fine powder by grinding it with a mill. Water, 50% Ethanol (EtOH), and 95% EtOH were used as the extraction solvents. For every 5 g of the plant powder, 100 mL of the extraction solvent was used. The plant mixtures were sonicated for 30 min at 50°C, and then were incubated at room temperature with shaking for 24 hours at 100 rpm. After filtration, the extraction solvents were evaporated by rotatory evaporator and the extracts were concentrated before drying under reduced pressure using a high vacuum pump overnight. The obtained dried extracts were stored at - 30°C.

For the cell line experiment, 20 mg of the dried extract was dissolved in one mL of DMSO. The total number of samples was 84 samples. The prepared samples were stored in the refrigerator at - 30°C before usage.

2.1.4. Cell Line Study

The prepared samples were tested for their anti-proliferative activity against human gastric cancer cell line; AGS (derived from the gastric adenocarcinoma of a 45 years old female patient). The toxicity and selectivity of the effective samples were examined by using normal gastric fibroblast (**see Appendix. Cell Line Maintenance for further information**). These cell lines were obtained from The International Research Center for Medical Sciences (IRCMS) at the School of Medicine of Kumamoto University (Kumamoto, Japan).

2.1.4.1. Screening for Anti-proliferative Activity

Briefly, seeding of 10000 cells/well in 96-well plates was performed for AGS. The seeded plate was incubated inside Incucyte® S3 Live-Cell Analysis System (Sartorius Corporation, Michigan, USA) for 24 hours. Thereafter, the prepared plant samples (100 µg/mL) was added to the seeded cells. DMSO (0.5%) and untreated cells were used as the negative control. The readings were repeated three times for samples and negative control. The seeded plates with added samples were placed inside Incucyte® S3 Live-Cell Analysis System for incubation at 37°C with 5% CO_2 for three days. Cell confluence was compared between treated and untreated cells and samples potential to inhibit cancer cell growth was examined. Concentration that cause inhibition to the growth of 50% of AGS cells (IC_{50}) was calculated for strong samples.

2.1.4.2. Toxicity Study

To examine the safety of the plant extracts and its selectivity toward gastric cancer cells, human gastric fibroblast cells were utilized for toxicity study. The cells (15000 cells/well) were seeded into 96-well plate and treated with different concentrations of the samples (100 µg/mL, 25 µg/mL, and 6.25 µg/mL). The experiment was repeated three times and the concentration that cause inhibition to the growth of 50% of fibroblast cells was calculated (IC_{50}). DMSO at concentration 0.5%, 0.25%, and 0.125% was used as the negative control.

2.1.5. Extraction and Phytochemical Investigation on *Myrtus communis*

The leaves were separated from the branches/stem. All the plant materials were made into fine powder by the mill. The leaves powder (100 g) was extracted with 1 L of EtOH two times, sonicated for 30 minutes, and incubated at room temperature with shaking at 100 rpm for 24 hours. Thereafter, the extraction solvent was evaporated using rotatory evaporator before drying the extract with a high vacuum pump under reduced pressure overnight. Similar procedure was applied to the fine powder of the branches/stem (43 g). The weight of the dried leaf and branches/stem extracts were 14.1 g, and 3.7 g, respectively.

Fractionation of the dried extract of the leaf was done using four solvents by liquid-liquid separation. Briefly, the dried extract of the leaf was suspended in 500 mL of pure water and extracted with 500 mL of hexane in the separatory funnel three times. The hexane fraction (Hex. fr.) was collected and evaporated by rotatory evaporator and dried under reduced pressure with a high vacuum pump. Next, the collected aqueous layer was extracted with 500 mL of ethyl acetate three times using the separatory funnel. The obtained ethyl acetate fraction (EA. fr.) was collected and evaporated by rotatory evaporator and dried under reduced pressure with a high vacuum pump. Finally, the remaining aqueous layer was extracted with 500 mL of 1-butanol three times. The obtained butanol fraction (BuOH fr.) and water fraction (water fr.) were evaporated and

dried. The dried weight of Hex. fr., EA. fr., BuOH fr., and water fr. were 3.3 g, 2.9 g, 4.3, and 3.5 g respectively.

Similar manner was applied to the fractionation of the branches/stem extract. The obtained dried weight of hexane fraction (Hex. fr.), ethyl acetate fraction (EA. fr.), 1-butanol fraction (BuOH fr.) and water fraction (water fr.) were 0.1 g, 0.3 g, 1.4 g, and 1.8 g, respectively.

The obtained fractions were used to prepare samples in DMSO (20 mg/mL) to investigate the activities against gastric cell line (AGS) and toxicity toward gastric fibroblast.

The crude EtOH extracts of the L and Br/St of *M. communis*, and their fractions were analyzed using Ultra High-performance Liquid Chromatography (UHPLC) connected with Corona Charged Aerosol Detector (CAD). A Dacapo DX-C18 column (2 mm × 100 mm, 2.5 µm) was used under the following conditions: elution with acetonitrile–water linear gradient (10:90 at 0 min to 100:0 at 20 min), flow rate; 0.3 mL/min, oven temperature; 40°C, sample injection; 1.0 µL, and detection by a PDA detector (200−360 nm). A 0.5% solution of the crude extracts and their fractions were prepared in HPLC graded methanol. The prepared solutions were centrifuged at 16,000 rpm for 10 minutes and the supernatant was transferred to the HPLC for analysis.

2.2. Phytochemical and Habitat Suitability Investigations of Glycyrrhiza glabra

2.2.1. Plant Materials and Study Area

In 2018, preliminary investigation was conducted in the Nizip and the Hatay regions in Turkey to select the area with highest phytochemical content from *G. glabra* root for further field investigation **(Figure 1**) (**Figure 2**).

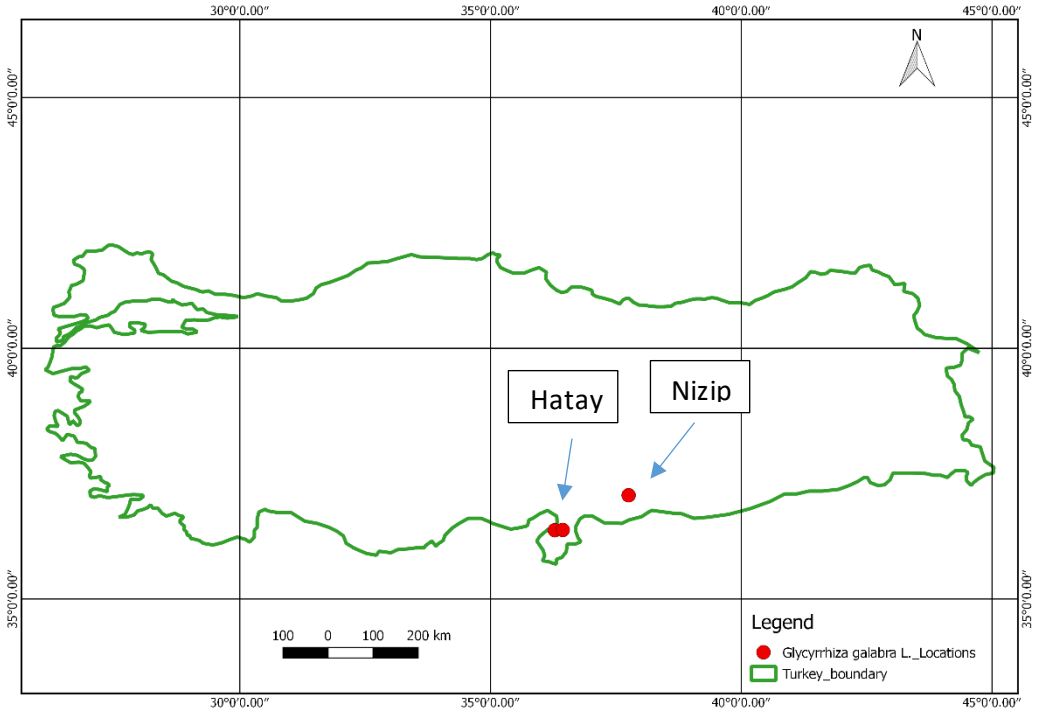


Figure 1. Map of the study areas in Turkey. [Hatay located at 36° 24' 8.36"–36° 26' 22.75" E Longitude and 36° 21' 25.76"–36° 22' 33.42" N Latitude, and Nizip lies between 37° 47' 49.0164" E longitude and 37° 0' 35.7336" N latitude.]

Figure 2. *G. glabra* collected from preliminary investigations

The preliminary analysis revealed that samples from Hatay region (TUR–1, TUR–2, and TUR–3) have higher phytochemical content than Nizip region (TUR–4, TUR–5). Furthermore, the level of bioactive content of samples from the Hatay region is very close to that from three samples obtained from Japanese companies; namely, Matsuura, Uchida, and Maruzen (JPN–1, JPN–2, and JPN–3, respectively) (**Figure 3**). The soil analysis also showed higher soil nutrients in the Hatay region compared to the Nizip region as exhibited in **Table 2**.

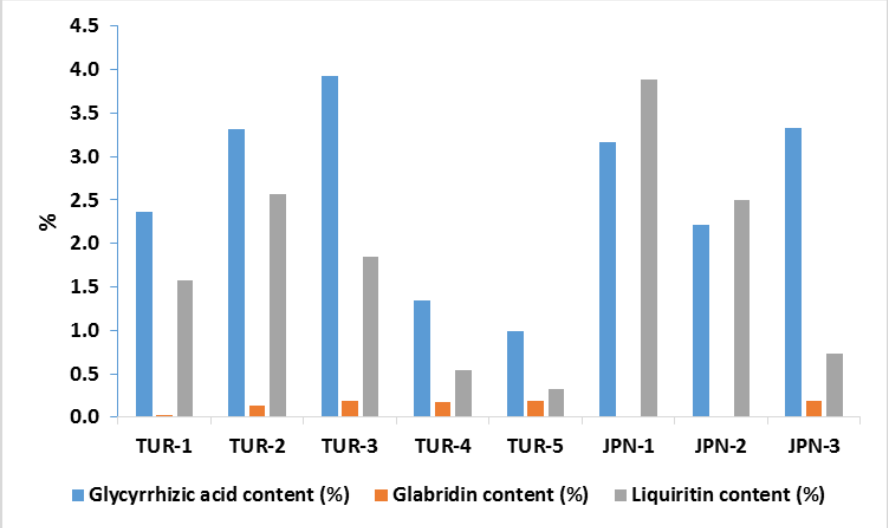


Figure 3. Percentage of glyccyrhizic acid, glabridin, and liquiritin from collected samples. TUR-1, TUR-2, and TUR-3 represent root samples from the Hatay region and TUR-4 and TUR-5 are root samples from the Nizip region. JPN-1, JPN-2, and JPN-3 represents samples purchased from Matsuura, Uchida, and Maruzen pharmaceutical companies, respectively.

Table 2. Preliminary soil analysis

Measured item	Hatay	Nizip	Japan (typical value) *
pH	8.5–8.7	8.2–8.5	**6.0–6.5**
Electrical conductivity (mS/cm)	0.3	0.2–0.3	**0.1–0.3**
Ammonium Nitrogen (mg/100 g)	5.1–7.0	3.1–5.3	**0.3–1.5**
Nitrate Nitrogen (mg/100 g)	1.6–5.3	2.1–4.6	**0.7–3.5**
Available Phosphorus (mg/100 g)	8.8–16	5–16	**20–60**
Exchangeable Potassium (mg/100 g)	63–127	37–70	**15–40**
Exchangeable Calcium (mg/100 g)	686–782	696–797	**200–400**
Exchangeable Magnesium (mg/100 g)	159–218	30–203	**35–70**
Exchangeable Manganese (mg/Kg)	4.3–4.9	3.7–5.9	**7.0–20.0**
Available Iron (mg/Kg)	1.2–3.5	1.0–2.3	**15–100**
Available Copper (mg/Kg)	0.3–0.5	0.1–0.2	**1.0–3.5**
Available Zinc (mg/Kg)	0.3–0.4	1.9–3.0	**10.0–40.0**
Boron (mg/Kg)	1.6–1.9	0.7–1.5	**0.7–2.5**

* Source: (https://n-seikaken.co.jp/soil/check-shindan.html)

The subsequent investigations were continued in the Hatay region. In 2019, 28 samples of wild *G. glabra* roots were collected from 10 locations (A–J) within the study area in the Hatay region (three root samples from each sites except location A, which had one root sample) (**Figure 4**). The diameter of the root from these samples were between 6 to 27 mm. Soil analysis was conducted on the site of collection (*in-situ*). Samples locations were indicated by using a global positioning system (GPS) (Garmin eTrex 30x, Olathe, KS, USA). The documented data of annual average temperature, precipitation (2019), and the climate classification of the plants collection sites were acquired from the Turkish Ministry of Agriculture and Forestry, General Directorate of Meteorology (2020) [54], as shown in **Table 3**. The root samples were dried for seven days at 50°C. The dried samples were ground into a fine powder for the extraction experiment.

Table 3. Geographic and environmental data of *G. glabra* samples collected in Hatay region

Location	Longitude (E)	Latitude (N)	Elevation (m)	Average Temperature (°C) [a]	Average Precipitation (mm) [a]	Climate Classification [b]
A	36° 16' 0.27"	36° 24' 21.59"	75.6	20.4	900	Semi-arid–dry sub-humid
B	36° 16' 30.41"	36° 24' 19.29"	82.5	19.9	900	Semi-arid–dry sub-humid
C	36° 19' 5.3"	36° 27' 54.3"	116.1	21.1	900	Semi-arid–dry sub-humid
D	36° 19' 5.25"	36° 27' 50.27"	125.3	21.1	900	Semi-arid–dry sub-humid
E	36° 26' 45.21"	36° 26' 3.21"	89.4	19.4	900	Semi-humid
F	36° 29' 30.94"	36° 25' 52.03"	88.1	19.4	900	Semi-humid
G	36° 27' 44.42"	36° 24' 1.09"	83.6	19.4	900	Semi-humid
H	36° 15' 52.8"	36° 8' 56.47"	328.0	18.3	900	Humid
I	36° 21' 42.68"	36° 3' 45.51"	198.8	19.4	1100	Humid
J	36° 19' 50.46"	36° 11' 2.27"	164.5	19.4	1100	Humid

[a] Record of 2019 [b] Based on Thornthwaite style for the period 1981–2010 [55] [a and b] Obtained records from the Turkish Ministry of Agriculture and Forestry, General Directorate of Meteorology (2020) [54].

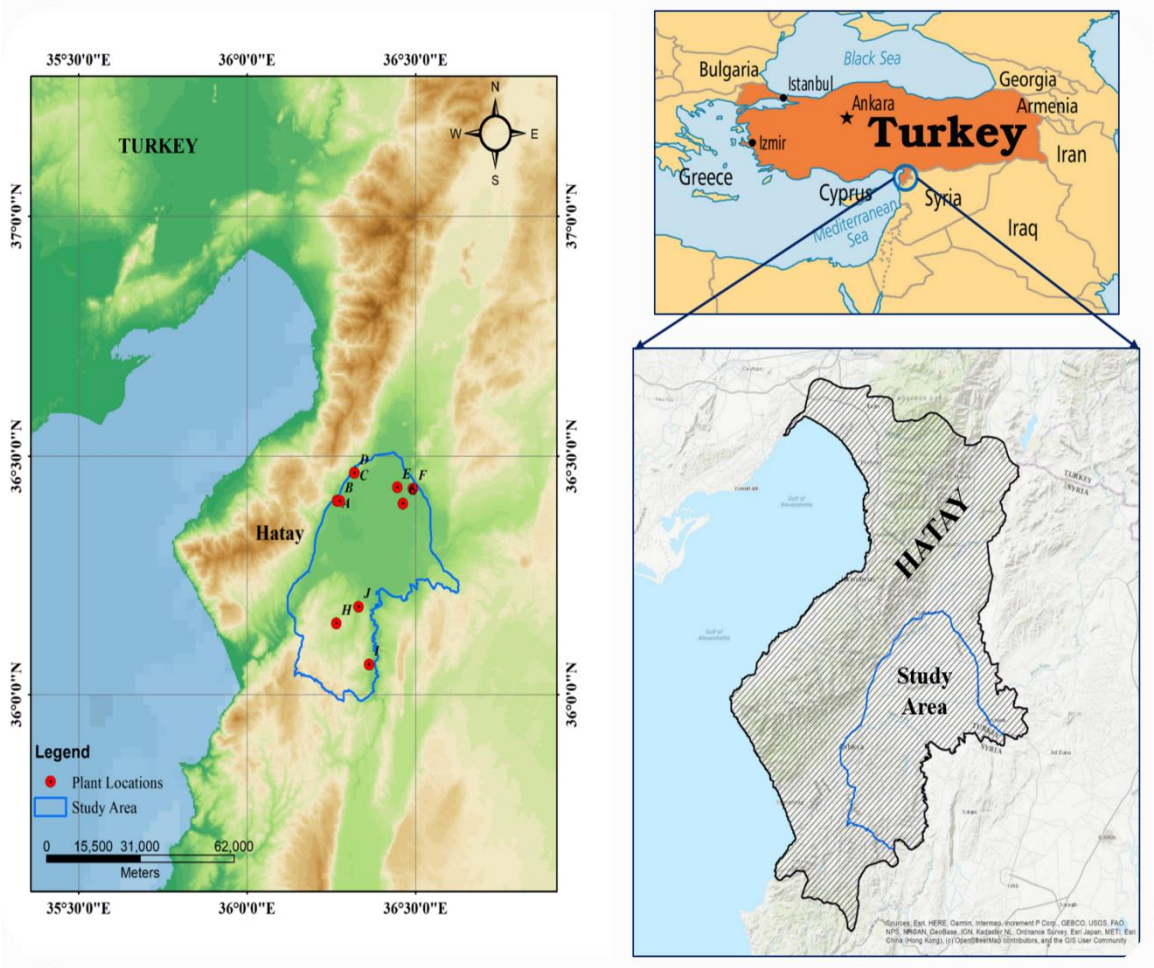


Figure 4. Maps of the study area, which covers an area of 1354 km^2 in the Hatay region of Turkey. The map on the left shows the 10 sampling sites (red dots), and was generated by using Google Earth Pro and ArcGIS version 10.5.1. (Licensed).

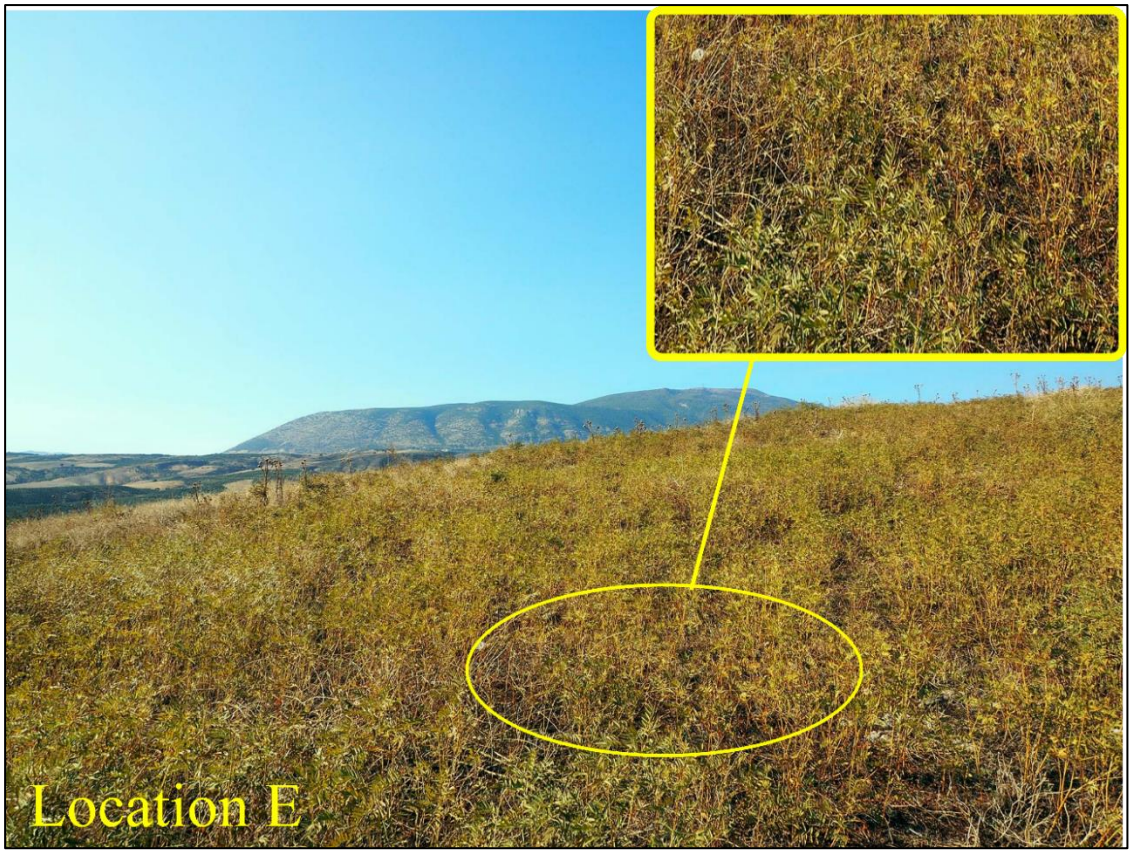
The study area in the Hatay region of Turkey is positioned at 36°0′00″–36°35′00″ E, 36°0′00″–36°30′00″ N and includes an area of 1354 km^2 (as determined by Google Earth Pro and ArcGIS 10.5.1 version (ESRI Japan Corporation, Tokyo, Japan)) (**Figure 4**). The southern region of the study area is characterized by a mountainous setting and geological distinctions. The northern and central regions have a flat surface and contain urban areas and farming grounds. Based on the digital elevation model (DEM), the altitude ranges from 75 m to 328 m above sea level with a slope angle that varies from 0° to 52°. **Figure 5** shows the localities of *G. glabra* collected in Hatay region of Turkey.


Location A

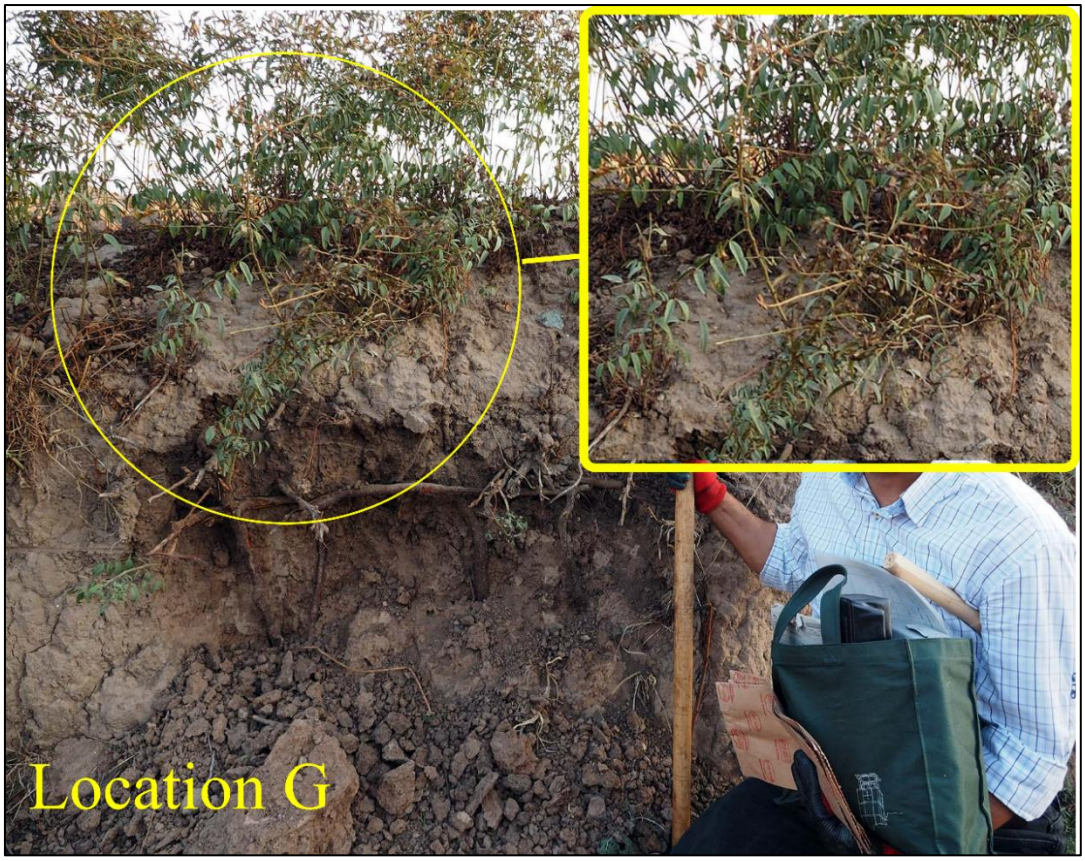

Location B


Location C


Location D


Location E


Location F


Location G


Location H

Figure 5. Localities of *G. glabra* collected in Hatay region of Turkey

2.2.2. Instruments and Chemicals

The *in-situ* soil analysis utilized a soil moisture sensor kit SM150T from Delta-T Devices (Cambridge, UK) and a Yamanaka-type soil hardness tester from Fujiwara Seisakusho, Ltd. (Tokyo, Japan). A COSMOSIL Protein-R column was obtained from Nacalai Tesque (Kyoto, Japan). A Dacapo DX-C18 column was acquired from Imtakt (Kyoto). For chromatographic purification purposes, a prominence HPLC instrument (Shimadzu, Kyoto) was utilized. For the quantitative analysis, A Nexera X2 HPLC/UHPLC system (Shimadzu, Kyoto) was used. Corona-Cad (Thermo Fisher Scientific, Tokyo) was connected to the HPLC system for the detection of oils and lipids. For chemical identification, an amaZon speed-ion trap mass spectrometer (Bruker, Billerica, MA, USA) and an AVANCE-I 600 NMR (Bruker, Billerica, MA) were used. Glycyrrhizic acid, glabridin, and the remaining chemicals were bought from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan).

2.2.3. Soil Analysis

A conventional soil pH meter and Yamanaka-type soil hardness tester were used to measure the soil pH and soil bearing capacity of the study area, respectively. The refractive index ($\sqrt{\varepsilon}$) and volumetric soil moisture content (VSMC) were established by the value of SM150T output voltage [56, 57] and Equations. (1) and (2), respectively:

$$\sqrt{\varepsilon} = 1 + 14.4396\,V - 31.2587\,V^2 + 49.0575\,V^3 - 36.5575\,V^4 + 10.7117\,V^5 \quad (1)$$

$$VSMC = (\sqrt{\varepsilon} - a_0) / a_1 \quad (2)$$

Where V is the SM150T output in volts and a_0 and a_1 are constant values decided by soil type [58], as displayed in **Table 4**.

Table 4. Soil analysis of collection sites

Location	Soil Bearing Capacity [a]	pH	SM150T output (V)	Refractive index [b]	VSMC [c]
A	4.2 ± 0.3	6.8 ± 0.4	0.2 *	2.6 ± 0.3	0.2 *
B	4.5 ± 1.2	7.0 ± 0.2	0.1 ± 0.1	2.5 ± 0.4	0.2 ± 0.1
C	4.1 ± 1.4	6.5 ± 0.2	0.2 *	3.0 ± 0.2	0.2 *
D	3.1 ± 0.7	6.7 ± 0.3	0.2 *	2.9 ± 0.1	0.2 *
E	3.9 ± 0.8	7.0 ± 0.1	0.1 *	2.4 ± 0.2	0.1 *
F	2.6 ± 0.6	6.9 ± 0.1	0.1 *	2.2 ± 0.1	0.1 *
G	3.4 ± 2.0	7.0 ± 0.1	0.1 *	2.3 ± 0.2	0.1 *
H	3.6 ± 0.4	6.8 ± 0.1	0.4 ± 0.1	3.8 ± 0.6	0.3 ± 0.1
I	3.4 ± 0.4	6.9 ± 0.3	0.2 ± 0.2	2.8 ± 0.5	0.2 ± 0.1
J	3.5 ± 0.3	6.8 ± 0.3	0.2 *	3.0 ± 0.3	0.2 *

All calculations were repeated three times, from which the mean and standard deviation (SD) were calculated. * Measured SD is ≤ 0.01. [a] determined as tons per square foot (t sf^{-1}). [b] the refractive index was calculated from the output of SM150T. [c] The percentage of water content in organic soils ($m^3\ m^{-3}$), where a_0 and a_1 values are 1.3 and 7.7, respectively.

2.2.4. HPLC Analysis of *G. glabra*

A sample of licorice root was obtained from Uchida Wakanyaku Ltd. (Tokyo, Japan) and milled into a fine powder. The obtained powder (100 g) was extracted with 50% EtOH (1 L X 2) to acquire around 15 g of dry extract. The extract was suspended in water and extracted with ethyl acetate three times. About 2.5 g of EA.fr. was obtained after drying. Using a preparative HPLC with a COSMOSIL Protein-R column (20 mm × 250 mm, 5 µm), the EA.fr. (100 mg/mL in methanol) was purified. The following condition was adopted for the purification: column temperature; 40°C, flow rate; 8 mL/min, sample injection; 1.0 mL, and elution with methanol–water linear gradient (40:60 at 0 min to 76:24 at 30 min). The peak signal was detected by a photodiode array (PDA) detector (200−360 nm). A peak detected between 14.1 min and 15.3 min was collected repetitively to obtain roughly 10 mg of the isolated compound.

The value of the isolated compound matches the values of liquiritin mentioned in relevant literature [59,60] : faintly yellow powder, ESI-MS (ion-trap) *m/z*: [M – H]− 417.3; 13C NMR (150 MHz, DMSO-d6): δ 78.23 (C-2), 44.98 (C-3), 193.22 (C-4), 129.91 (C-5), 111.89 (C-6), 165.44 (C-7), 103.91 (C-8), 166.88 (C-9), 115.08 (C-10), 134.51 (C-1′), 128.86 (C-2′, C-6′), 117.9 (C-3′, C-5′), 159.28 (C-4′), 102.28 (C-1′′), 74.96 (C-2′′), 78.07 (C-3′′), 71.45 (C-4′′), 78.22 (C-5′′), and 62.59 (C-6′′); 1H NMR (600 MHz, DMSO-d6): δ 7.64 (1H, d, J = 8.64 Hz, 5-H), 7.45 (2H, d, J = 8.76 Hz, 2′-H, 6′-H) 7.07 (2H, d, J = 8.76 Hz, 3′-H, 5′-H), 6.51 (1H, dd, J = 8.64, 2.22 Hz, 6-H), 6.35 (1H, d, J = 2.22 Hz, 8-H), 5.52 (1H, dd, J = 12.72, 2.82 Hz, 2-H), 4.89 (1H, d, J = 7.44 Hz, 1′′-H), 3.7 (1H, d, J = 11.16 Hz, 6′′-Hα), 3.46 (1H, m, J = 5.7 Hz, 6′′-Hβ), 3.13 (1H, m, J = 4.95 Hz, 3-Hα), 2.67 (1H, m, J = 4.4 Hz, 3-Hβ).

The milled *G. glabra* root samples (100 mg) were suspended in 10 mL of 50% EtOH and sonicated at 50°C for 30 min. The prepared suspensions were incubated at 25°C for 24 h with continuous shaking at 100 rpm. After centrifugation at 16,000 rpm

for 10 min, the supernatant was used for HPLC analysis. The contents of three bioactive compounds (glycyrrhizic acid, glabridin, and isolated liquiritin) were analyzed with a Dacapo DX-C18 column (2 mm × 100 mm, 2.5 µm) under the following settings: column temperature; 40°C, flow rate; 0.3 mL/min, sample injection; 1.0 µL, elution with acetonitrile–water linear gradient (10:90 at 0 min to 100:0 at 20 min) containing 0.1% phosphoric acid, and detection by a PDA detector (200–360 nm).

To prepare HPLC standard curve equations with $R^2 > 0.999$, glycyrrhizic acid and glabridin were dissolved in 50% EtOH at 50 µg/mL, 100 µg/mL, 200 µg/mL, and 300 µg/mL while liquiritin was dissolved in methanol at 50 µg/mL, 100 µg/mL, 300 µg/mL, and 500 µg/mL. **Figure 6** shows the standard curve of glycyrrhizic acid (A), glabridin (B), and liquiritin (C).

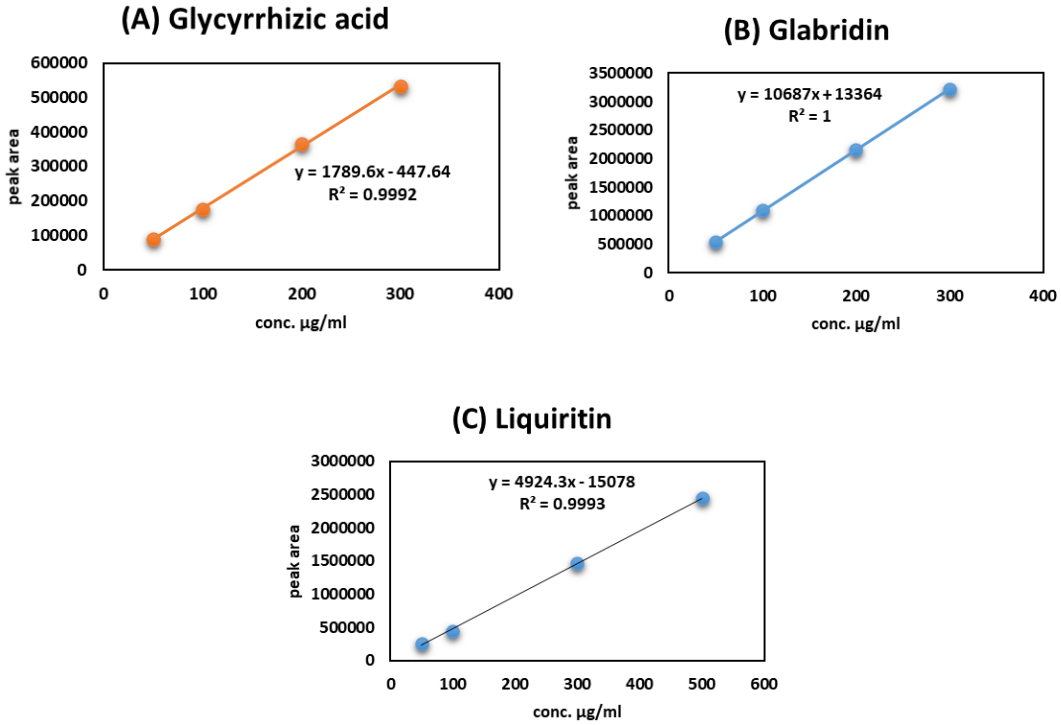


Figure 6. standard curves

2.2.5. Dataset Arrangement for Habitat Suitability Zones

Selecting and identifying appropriate habitat zones with suitable GIS-based models require diversity in the topographical, climatic, and soil datasets of the study area [61,62]. In the current study, the topographical features (aspect, curvature, elevation, hillshade, and slope), soil states (soil bearing capacity, soil moisture content, and pH,), and meteorological statistics (annual mean temperature and annual mean precipitation) were examined. Shuttle Radar Topography Mission–digital elevation model (SRTM–DEM) with 90 m × 90 m resolution was used to extract the topographical features (**Figure 7A**–**6J**). The illustrations were acquired from Jarvis et al. [63]. The slope range (0–52°) was classified into seven classes (**Figure 7A**). The

slope aspect orientation map displays the direction and degree of a slope for a given surface. The slope aspect of the area was classified into nine classes (**Figure 7B**). The south, southwest, and west facing slopes occupied a larger area and had the high probability of getting a large amount of sunlight and rainfall. The rate of change of a slope or an aspect in a specific direction was characterized by the curvature. The curvature adjusts the hydrological behavior of the soil and preserves more water in convex slopes after rainfall. The curvature map was classified into three classes: concave (< −0.001), flat (−0.001 to +0.001), and convex (> 0.001) (**Figure 7C**). The hillshade map was also prepared from the Shuttle Radar Topography Mission–digital elevation model, and shows topographical forms of highlands by utilizing a color scale classified into seven classes (**Figure 7D**). The elevation map was divided into five classes ranging from 0 m to 400 m (**Figure 7E**).

Variables like the annual mean temperature, maximum temperature of the warmest month, minimum temperature of the coldest month, annual precipitation, precipitation of the wettest month, and precipitation of the driest month (BIO1, BIO5, BIO6, BIO12, BIO13, and BIO14, respectively) are known as the bioclimatic variables [64]. These variables were acquired for the period 2010–2018 and used during the creation of the habitat suitability map **(see Appendix Table A2. Various environmental variables of *G. glabra* collection sites)**.

Thematic maps of meteorological data and soil elements were prepared for the 10 plant locations of the study area. The inverse distance weighted (IDW) spatial analyst technique in ArcGIS was applied to create the interpolation of thematic maps (**Figure 7F**–**7J**). Lastly, all these maps were converted into a raster format with the same resolution of 85 m for further studies. The habitat suitability zone map was designed using the frequency ratio (FR) model and overlay analysis of every thematic map in a GIS environment.

The Frequency Ratio (FR) is a generally used statistical technique for the detection and mapping of potential zones. The FR method was used in this study to create habitat suitability zones from multiple thematic spatial datasets. The FR described as the ratio of the probabilities of occurrence to nonoccurrence for a certain plant training subclass. It is also specified as the ratio of the area of plant locations to the total study area (Equation (3))[61,65].

$$\text{FR} = (\text{points in each subclass} / \text{total points}) / (\text{class area} / \text{total area}) \quad (3)$$

In the GIS setting, all thematic maps were reclassified by their subclasses. For each thematic layer's subclass, the FR values were calculated, hence obtaining the relative frequency (RF) (Equation (4)).

$$\text{RF} = \text{class}_{\text{FR}} / \text{sum of classes}_{\text{FR}} \quad (4)$$

The prediction ratio (PR) of each thematic map was determined with the geographic coverage of every subclass in the study area using equation (5).

$$PR = (RF_{max.} - RF_{min.}) / (RF_{max.} - RF_{min.})_{min.} \quad (5)$$

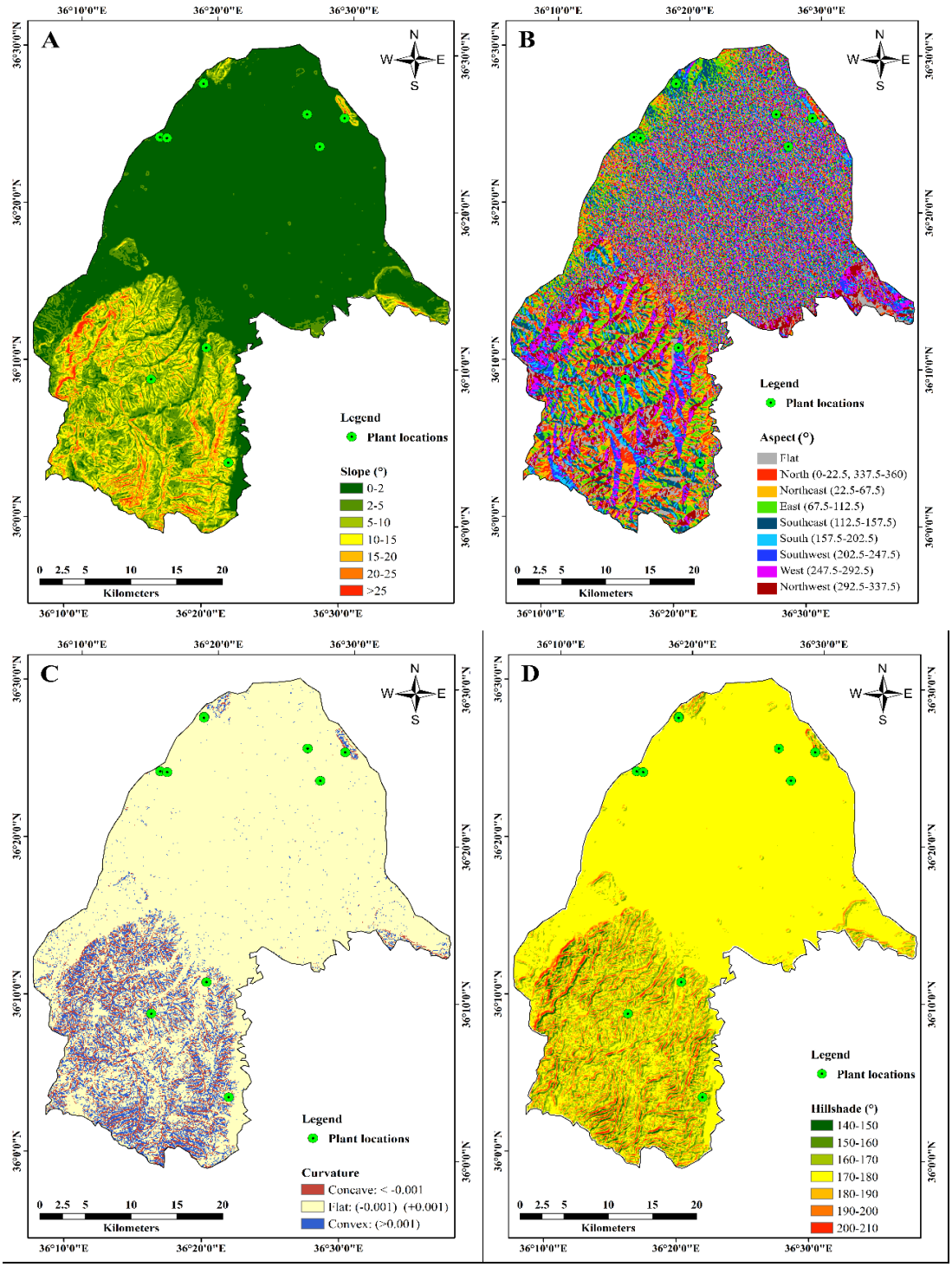
To predict the Frequency Ratio of the thematic maps, Microsoft Excel and ArcGIS 10.5.1 were employed **(see Appendix Table A3. Data used for spatial modeling)**.

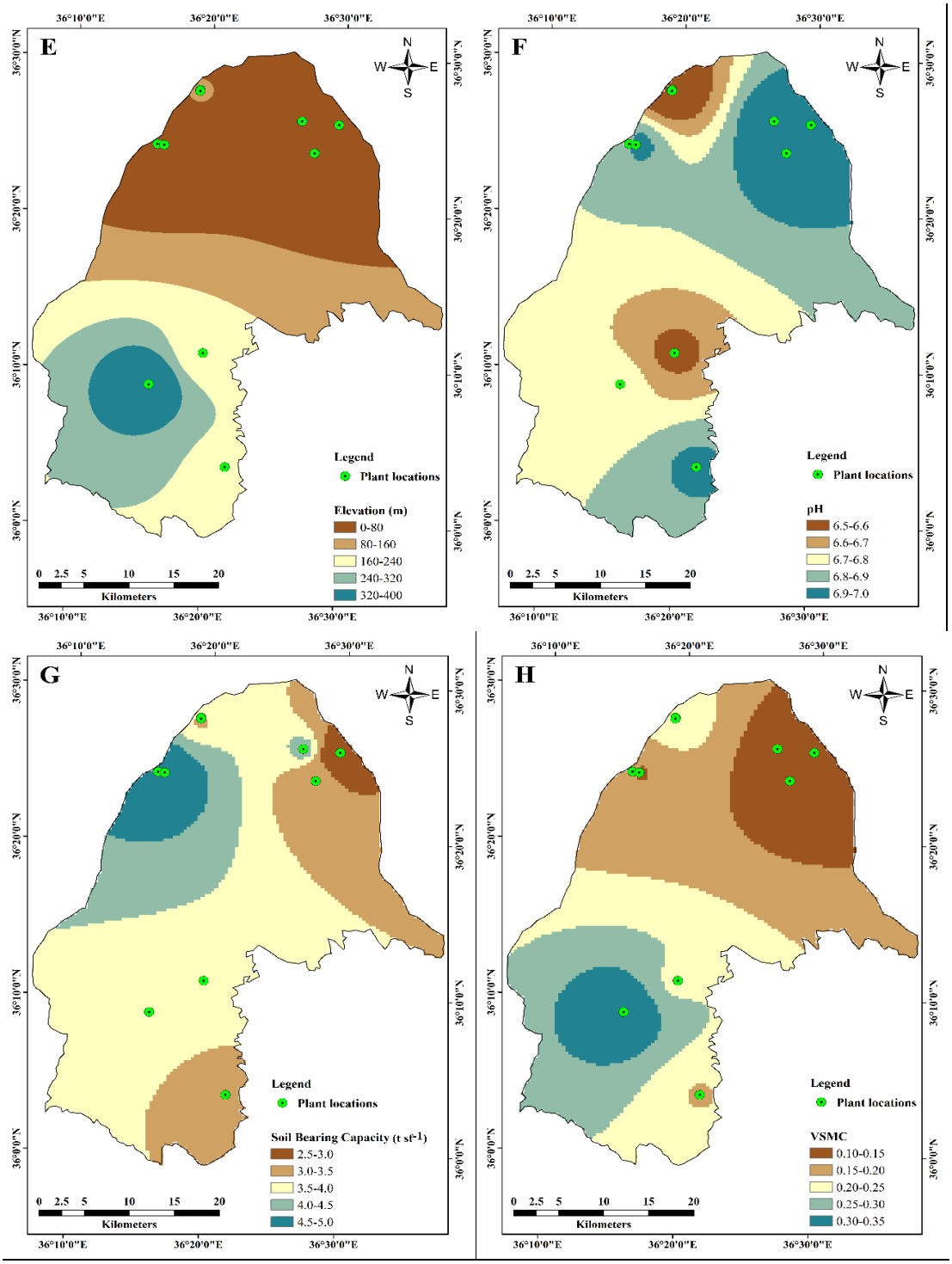
Lastly, the habitat suitability zone map (HSZM) was prepared by adding all of the prediction ratio values for each thematic layer, as presented in Equation (6)

$$\begin{aligned} HSZM = {} & (1.86 \times aspect) + (1.64 \times curvature) + (11.11 \times elevation) + (9.33 \times soil\ bearing\ capacity) + (1 \times hillshade) + (12.86 \times pH) + (11.25 \times precipitation) + (13.13 \times temperature) + \\ & (11.93 \times VSMC) + (1.64 \times slope) + (14.43 \times BIO1) + (4.18 \times BIO5) + (6.29 \times BIO6) + (7.89 \times BIO12) + (5.97 \times BIO13) + (7.68 \times BIO14) \end{aligned} \quad (6)$$

The HSZM signifies the relative environmental potential zones for plant conservation, cultivation, and future investigations. Using a natural break classification method, HSZM was reclassified into four main suitability zones (very high, high, moderate, and low).

The receiver operator characteristic (ROC) curve and the area under the curve (AUC) were used to measure the accuracy of the final habitat suitability map. These measurements characterizes the prediction accuracy of the FR model. The ROC curve can be described as the graphical depiction of the tradeoff relating the false-positive rate on the X-axis and the true-positive rate on the Y-axis for every possible cutoff value [66,67].


A
36°10'0"E
36°20'0"E
36°30'0"E
36°30'0"N
36°20'0"N
36°10'0"N
36°0'0"N
N
W
E
S
Legend
Plant locations
Slope (°)
0-2
2-5
5-10
10-15
15-20
20-25
>25
0 2.5 5 10 15 20
Kilometers
B
Legend
Plant locations
Aspect (°)
Flat
North (0-22.5, 337.5-360)
Northeast (22.5-67.5)
East (67.5-112.5)
Southeast (112.5-157.5)
South (157.5-202.5)
Southwest (202.5-247.5)
West (247.5-292.5)
Northwest (292.5-337.5)
C
Legend
Plant locations
Curvature
Concave: < -0.001
Flat: (-0.001) (+0.001)
Convex: (>0.001)
D
Legend
Plant locations
Hillshade (°)
140-150
150-160
160-170
170-180
180-190
190-200
200-210


E
36°10'0"E
36°20'0"E
36°30'0"E
36°30'0"N
36°20'0"N
36°10'0"N
36°0'0"N
N
W
E
S
Legend
Plant locations
Elevation (m)
0-80
80-160
160-240
240-320
320-400
0 2.5 5 10 15 20
Kilometers
F
Legend
Plant locations
pH
6.5-6.6
6.6-6.7
6.7-6.8
6.8-6.9
6.9-7.0
G
Legend
Plant locations
Soil Bearing Capacity (t sf-1)
2.5-3.0
3.0-3.5
3.5-4.0
4.0-4.5
4.5-5.0
H
Legend
Plant locations
VSMC
0.10-0.15
0.15-0.20
0.20-0.25
0.25-0.30
0.30-0.35

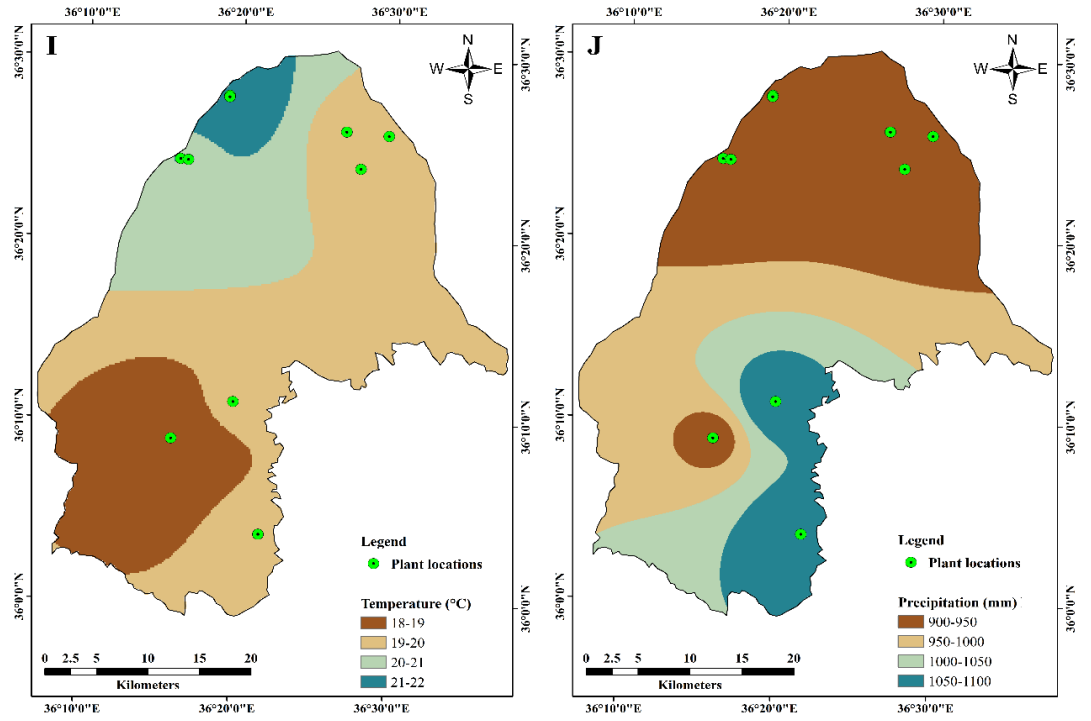


Figure 7. Thematic maps of the study area: (A) slope, (B) aspect, (C) curvature, (D) hillshade, (E) elevation, (F) pH, (G) soil bearing capacity, (H) volumetric soil moisture content (VSMC), (I) temperature, and (J) precipitation.

2.2.6. Statistical Analysis

All measurement were repeated three times from which the mean and standard deviation were calculated. For HPLC standard curve measurement, linear fittings of the standard curves were arranged using Microsoft Excel 2013 by drawing the peak area versus concentration. The Pearson's correlation, one-way analysis of variance (one-way ANOVA), and multivariate analysis of variance (MANOVA) were estimated using XLSTAT statistical and data analysis solution (Addinsoft (, 2020), New York, NY, USA) in order to evaluate the influences of soil, meteorological data, and topographical information on the plant's chemical contents. XLSTAT was also applied to measure the prediction accuracy of the FR model using the receiver operator characteristic (ROC) curve. Any correlation with a p-value of < 0.05 was considered to be significant.

Chapter 3. Results and discussion

3.1. Ant-proliferative Activities of Turkish plants against Gastric Cancer

3.1. 1. Screening of selected Turkish plants

For screening purposes, all samples were examined for their activities against the growth of cancer cells at a concentration of 100 µg/mL.

Table. 5 shows the inhibition rate of the samples in AGS cell line whereas DMSO (0.5%) and medium were used as the negative control as they did not affect cells growth rate.

Table. 5 Inhibition rate in AGS at sample concentration of 100 µg/mL.

Samples	Examined parts	Extraction solvent	Inhibition rate in AGS cell line
Alchemilla mollis	Aerial part	95% EtOH	83.3 ± 0.3
		50% EtOH	N/I
		Water	N/I
Ammi majus	Aerial part	95% EtOH	8.3 ± 4.3
		50% EtOH	N/I
		Water	N/I
Anastatica hierochuntica	Seed	95% EtOH	6.6 ± 0.1
		50% EtOH	6.3 ± 0.1
		Water	N/I
Capparis spinosa	Leaf	95% EtOH	21.1 ± 4.4
		50% EtOH	29.0 ± 3.1
		Water	N/I
	Branch/stem & Thorn	95% EtOH	39.7 ± 2.5
		50% EtOH	30.7 ± 2.1
		Water	N/I
Clematis vitalba	Aerial part	95% EtOH	20.9 ± 0.1
		50% EtOH	42.0 ± 0.1
		Water	29.9 ± 0.1

Samples	Examined parts	Extraction solvent	Inhibition rate in AGS cell line
Dioscorea communis	Aerial part	95% EtOH	16.7 ± 0.1
		50% EtOH	17.3 ± 0.1
		Water	N/I
Echium plantagineum	Aerial part	95% EtOH	42.7 ± 1.8
		50% EtOH	44.6 ± 3.4
		Water	N / I
Erica manipuliflora	Aerial part	95% EtOH	40.6 ± 0.4
		50% EtOH	24.1 ± 0.3
		Water	14.8 ± 0.1
Gentiana asclepiadea	Aerial part	95% EtOH	10.4 ± 0.5
		50% EtOH	29.6 ± 0.1
		Water	25.3 ± 0.1
	Root	95% EtOH	28.6 ± 0.1
		50% EtOH	28.8 ± 0.1
		Water	N/I
Jacobaea aquatica	Aerial part	95% EtOH	N/I
		50% EtOH	12.9 ± 0.2
		Water	N/I
Myrtus communis	Leaf	95% EtOH	55.4 ± 2.1
		50% EtOH	53.8 ± 3.1
		Water	4.4 ± 3.5
	Branch/stem	95% EtOH	58.1 ± 1.1
		50% EtOH	55.4 ± 2.9
		Water	N/I
Nigella sativa	Seed	95% EtOH	14.1 ± 0.1
		50% EtOH	17.2 ± 0.8
		Water	25.5 ± 0.1
Pistacia terebinthus	Leaf	95% EtOH	22.8 ± 0.6
		50% EtOH	32.6 ± 4.4
		Water	N/I

Samples	Examined parts	Extraction solvent	Inhibition rate in AGS cell line
Pistacia terebinthus	Stem	95% EtOH	23.6 ± 2.0
		50% EtOH	18.3 ± 1.7
		Water	N/I
	Fruit	95% EtOH	21.5 ± 7.1
		50% EtOH	20.8 ± 3.6
		Water	N/I
Polygonatum multiflorum	Leaf & Stem	95% EtOH	N/I
		50% EtOH	28.5 ± 0.1
		Water	11.7 ± 0.1
	Root	95% EtOH	N/I
		50% EtOH	31.2 ± 0.1
		Water	22.5 ± 0.1
	Fruit	95% EtOH	N/I
		50% EtOH	23.6 ± 0.1
		Water	24.6 ± 0.1
Quercus coccifera	Leaf	95% EtOH	22.3 ± 1.4
		50% EtOH	32.6 ± 2.3
		Water	32.1 ± 0.1
	Branch/stem	95% EtOH	56.0 ± 1.6
		50% EtOH	33.8 ± 2.5
		Water	7.8 ± 4.3
Rhus coriaria	Fruit	95% EtOH	29.7 ± 2.2
		50% EtOH	44.3 ± 2.4
		Water	27.3 ± 0.1
Rubus sanctus	Aerial part	95% EtOH	12.1 ± 0.4
		50% EtOH	23.7 ± 0.2
		Water	32.0 ± 0.1
Tanacetum macrophyllum	Aerial part	95% EtOH	59.2 ± 7.3
		50% EtOH	33.3 ± 2.7
		Water	N/I

Samples	Examined parts	Extraction solvent	Inhibition rate in AGS cell line
Tilia platyphyllos	Aerial part	95% EtOH	11. 6 ± 0.1
		50% EtOH	8.4 ± 0.2
		Water	N/I
Trigonella foenum-graecum	Seed	95% EtOH	53.6 ± 1.8
		50% EtOH	59.4 ± 1.3
		Water	N/D

All calculations were repeated three times, from which the mean and standard deviation (SD) were calculated. N/I refers to no inhibition. N/M refers to not determined.

Out of 84 plant extracts that used in this study, nine plant extracts have shown high activity against gastric cancer. These samples include the 95% EtOH extract of the aerial part of *A. mollis*, both the 95% EtOH and 50% EtOH extracts of the leaf and branches/stem of *M. communis*, 95% EtOH extract of the branches/stem of *Q. coccifera*, 95% EtOH extract of the aerial part of *T. macrophyllum*, and 95% EtOH as well as 50% EtOH extracts of the seeds of *T. foenum-graecum*. The concentration that cause inhibition of the growth of 50% of cancer cells (IC_{50}) was examined for samples that have high inhibition rate against cancer cells growth. Furthermore, toxicity study was conducted to validate the safety of *A. mollis*, *M. communis*, and *T. foenum-graecum* by comparing their IC_{50} in AGS and IC_{50} normal gastric fibroblast cells. **Table 6** shows the IC_{50} in AGS and normal gastric fibroblast cells of samples with high inhibition toward AGS.

Table 6. The IC_{50} in AGS and normal gastric fibroblast cell lines for samples with strong anti-proliferative activities

Samples	Plant part	Extraction solvent	IC_{50} (µg/mL)	
			In AGS	In normal gastric fibroblast
A. mollis	Aerial part	95% EtOH	60.0 ± 6.9	280.8 ± 1.2
M. communis	Leaf	95% EtOH	82.2 ± 2.2	18.0 ± 4.7
	Branch/stem	95% EtOH	78.0 ± 6.4	110.7 ± 6.3
T. foenum-graecum	Seed	50% EtOH	28.6 ± 3.0	2.4 ± 0.8

The experiments were repeated three times, in which the mean and standard deviation (SD) were calculated.

The sample with the strongest anti-proliferative activity toward AGS was the 50% EtOH extract of the seed of *T. foenum-graecum*. However, it showed to be toxic toward normal gastric fibroblast cells at the effective concentration. Another potent sample was the 95% EtOH extract of the aerial parts of *A. mollis* which showed strong inhibition of AGS as well as the lowest toxicity toward normal fibroblast cells compared to other potent samples.

From 84 plant extracts that used in this study, only nine plant extracts showed strong inhibition against AGS. One of these plant is *A. mollis* which showed good safety at the effective concentration. This suggests that *A. mollis has* great selectivity to cancer cells. There are multiple researches that indicate this plant potential to treat and manage influenza [68], diabetes, liver diseases [69], inflammation, and bacterial infection [70]. This investigation shows the potential to use *A. mollis* as a prophylaxis, in the treatment, and/or the drug development of stomach cancer medicine. *M. communis* also showed strong inhibition to the AGS cell growth. The branches/stem extracts showed to be less toxic than leaf extracts at the effective concentration. This plant has long history of usage in traditional medicine and folklore [71]. Many recent researches showed the therapeutic application of this plant and its bioactive compounds. It has activities against fungus infection [72], inflammation [73], diarrhea [74], ulcer [75], and cancer [71]. *Q. coccifera* and *T. macrophyllum* showed also anticancer potential. *Q. coccifera* was reported to have the potential to treat diabetes, skin diseases [76] while *T. macrophyllum* was researched for its antioxidant and insecticidal effects [77]. These plants has little and/or no previous reports for their potential to treat gastric cancer which make them a great target for future drug discovery and development researches.

On the other hand, *T. foenum-graecum* showed non selective inhibition to the growth of gastric cancer and normal fibroblast cells. This plant, which known as fenugreek (Koroba in Japanese), is a widely known spice/flavor in the Mediterranean diet. It has numerous health benefit such as lowering blood glucose and cholesterol level [53]. Some of its properties are attributed to an isolated saponin known as diosgenin which has anticancer effect [78]. However, this plant was reported to have serious side effects such as diarrhea if ingested in high amount by children and pregnant women [79]. More studies are needed to identify the mechanisms by which the phytochemical compounds of *T. foenum-graecum* exhibit the toxic effect on normal fibroblast cells.

3.1.2. Determination of Bioactive Fraction from *M. communis*

3.1.2.1. Plant Information

Figure 8. *Myrtus communis* used for the extraction process

One of the plants that showed high activity against gastric cell line was *M. communis* (**Figure 8**). This plant belong to Myrtaceae family and has been used in Turkey and other countries to treat various diseases. In Turkey, the fruit and leaves of *M. communis* have been used to treat diarrhea, gastric ulcer, rheumatism, hemorrhoid, skin disease, and as antiseptics among other usage. The boiled leaves with tea are usually drunk on daily basis in some parts of Turkey for mental strain and anxiety [80]. The fume of *M. communis* also has various applications as flavoring agent to meat and sauce. In addition to that, alcoholic drinks made from *M. communis* leaves and fruits have been gaining popularity in Italy and other parts of the world. This plant is rich in essential oil such as, α-pinene, β-pinene, myrcene, α-terpinene, limonene, 1.8-cineole, linalool, myrtenol, and nerol [81].

This plant has been investigated for its various biological activities. For instance, the leaf, flower, and stem have shown high antioxidant activities in different analysis methods [82]. The plant is also shown ant-inflammatory [83], ant-allergic [84], and antitumor activities [85]. *M. communis* exhibited antimicrobial activities against *Staphylococcus aureus* [86], *Escherichia coli*, *Klebsiella pneumonia*, *Enterococcus faecalis*, and *Bacillus cereus* [87]. It also reported to have the potential to be applied for the treatment of diabetes through the inhibition of glycosidase enzyme [88].

Most of the studies related to the chemical isolation from *M. communis* focused on the essential oil with only few researches dealing with the isolation and identification of the remaining secondary metabolites [89,90,91].

3.1.2.2. Anti-proliferative Activity against Gastric Cancer

The activity of the plant extracts and their fractions at 100 µg/mL against gastric cancer in AGS as well as the IC_{50} in normal gastric fibroblast cell line are shown in **table 7**. For the leaf, the inhibition strength in AGS was as follows; EtOH extract > water fr. ≥ BuOH fr. > Hex. fr. > EA. fr. While the branches/stem showed the following; EtOH extract ≥ BuOH fr. > Hex. fr. > water fr. > EA. fr.. The leaf extract and its fractions exhibited non selective inhibition to the growth of both AGS cancer cells and normal gastric fibroblast cells which proposed lower safety. Meanwhile, the Branch/stem extract and its fractions showed to have more specific inhibition toward AGS than normal gastric fibroblast cells.

These findings suggest the usefulness and safety of the branches and stems of *M. communis* in comparison to its leaf.

There are multiple reports that indicate the bioactivities of the leaves extracts of *M. communis* and the isolated bioactive compounds from the leaves. However there is no enough studies on the toxicity and/or selectivity of these extracts and compounds [92]. On the other hand, studies focusing on the branches and stem of *M. communis* are very limited even though they showed promising activity and selectivity against gastric cancer cell growth in the setting of cell line in this study.

Table 7. The inhibition rate in AGS and IC_{50} in normal gastric fibroblast cell line for the leaf and Br/St extracts of *M. communis* and their fractions.

Sample	The inhibition rate in AGS at a concentration of 100 µg/mL	IC_{50} in normal gastric fibroblast cell line
EtOH extract of the Leaf	82.2 ± 2.2	18.0 ± 4.6
Hex. fr.	11.6 ± 10.0	3.7 ± 0.5
EA. fr.	1.1 ± 3.7	140.2 ± 2.5
BuOH fr.	51.2 ± 8.6	N/D [b]
water fr.	55.1 ± 7.1	52.3 ± 2.0
EtOH extract of the Br/st	78.0 ± 6.4	110.7 ± 6.3
Hex. fr.	38.0 ± 1.2	119.5 ± 0.6
EA. fr.	19.0 ± 2.1	73.1 ± 1.6
BuOH fr.	59.0 ± 0.9	176.5 ± 5.6
water fr.	33.0 ± 2.9	72.4 ± 2.6

The experiments were repeated three times, from which the mean and standard deviation (SD) were calculated. [a] measured in µg/mL. [b] not determined.

3.1.2.3. HPLC Analysis

The chromatograms of HPLC and Corona-CAD showed the presence of essential oils in the EtOH extracts of the leaf and Branches/stem extracts of *M. communis* (**Figure 9**).

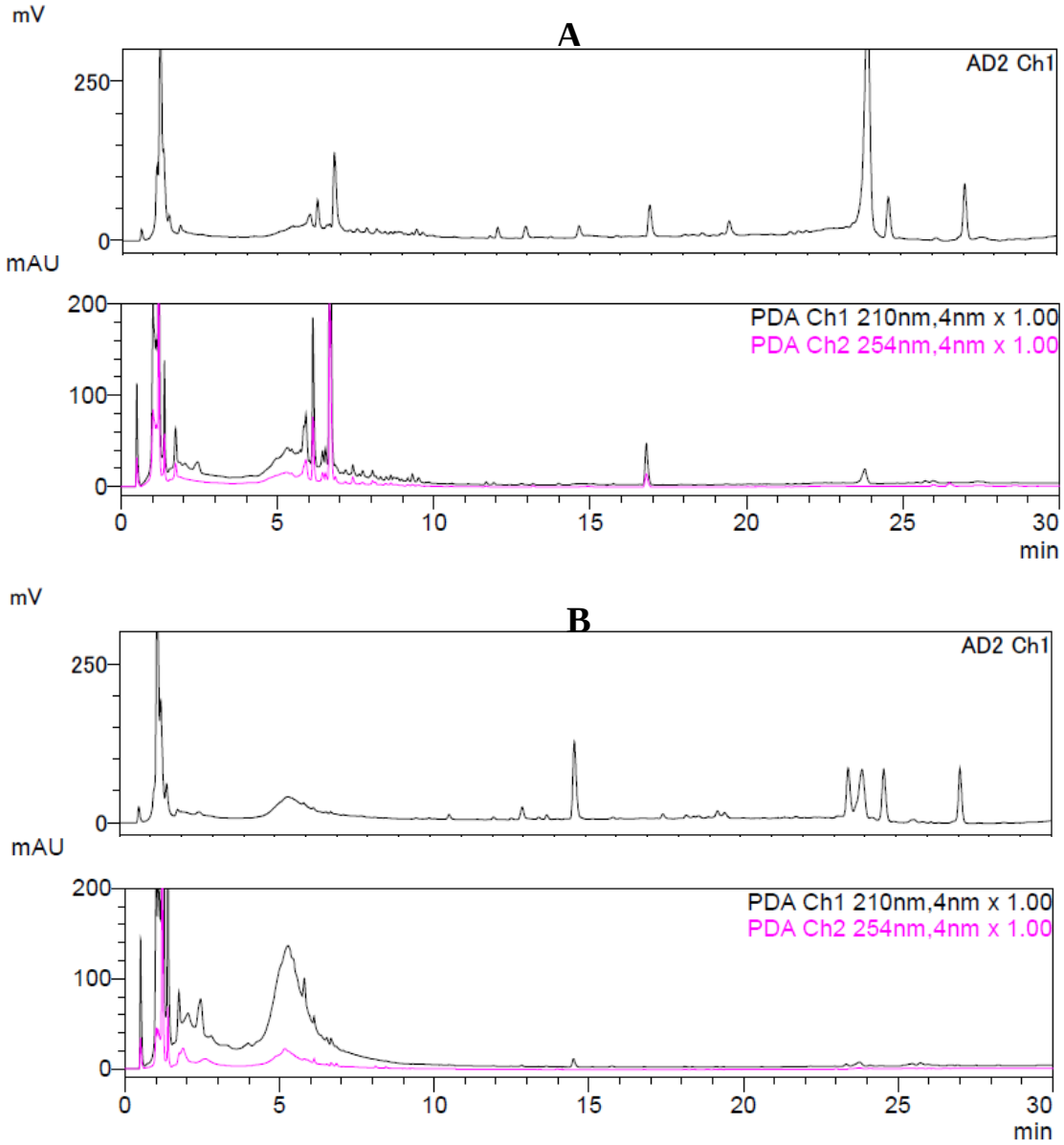


Figure 9. Corona-CAD and HPLC chromatograms of EtOH extract *M. communis*; (**A**) the chromatograms of the Leaf extract, (**B**) the chromatograms of the Branches/stem extract

For the leaves part, most of compounds were concentrated in the EA. fr. even though no significant activity was observed for this fraction in cell line (**Figure 10**).

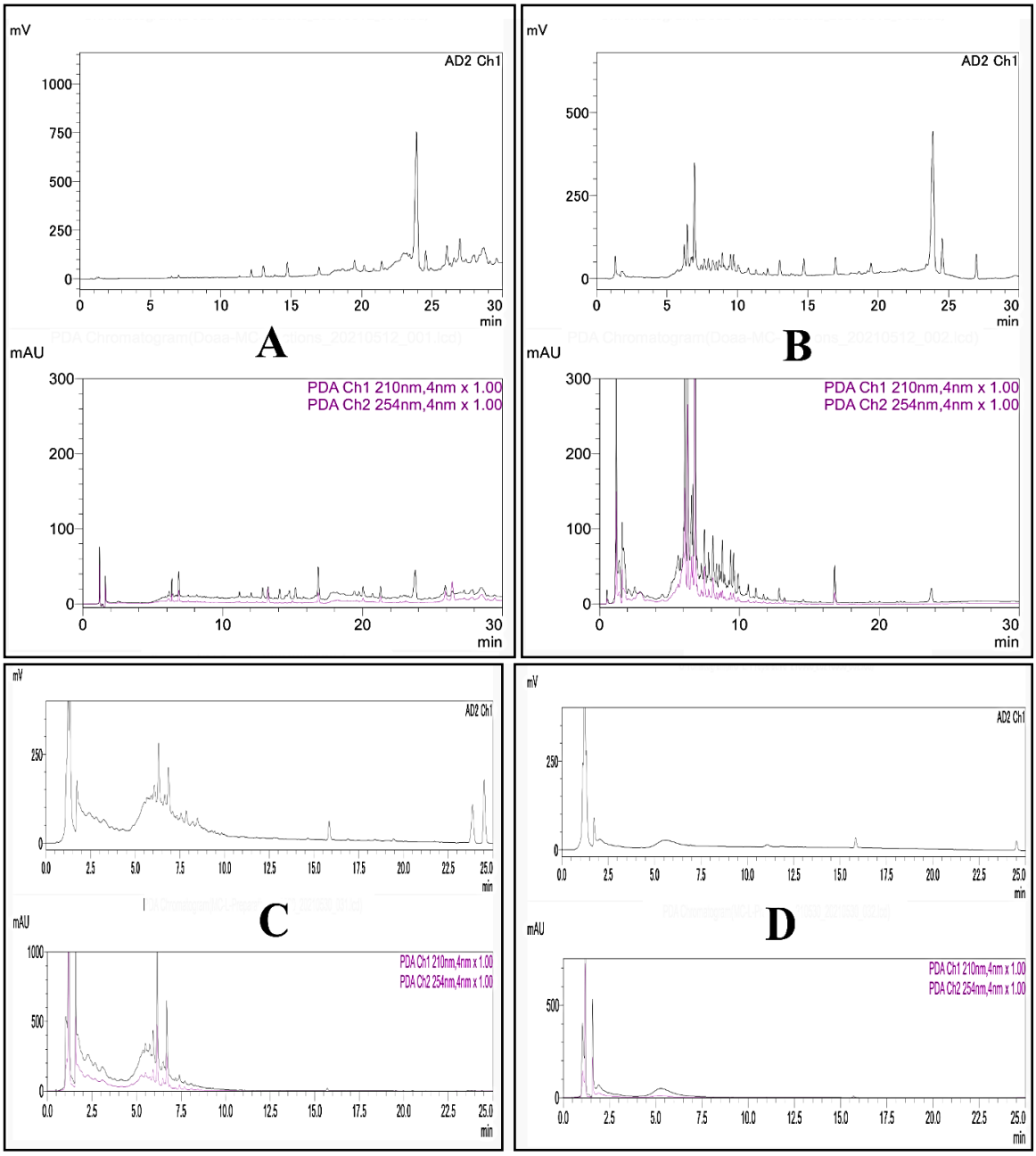


Figure 10. HPLC chromatogram of the fractionation of EtOH extract of *M. communis* leaf. (A) Hexane fraction. (B) Ethyl acetate fraction. (C) Butanol fraction. (D) Water fraction

On the other hand, the BuOH fr. and water fr. of the Branches/stem extract showed the highest activities against gastric cancer cells and the highest number of chemical compounds compared to the other fractions from the Branches/stem of *M. communis* (**Figure 11**).

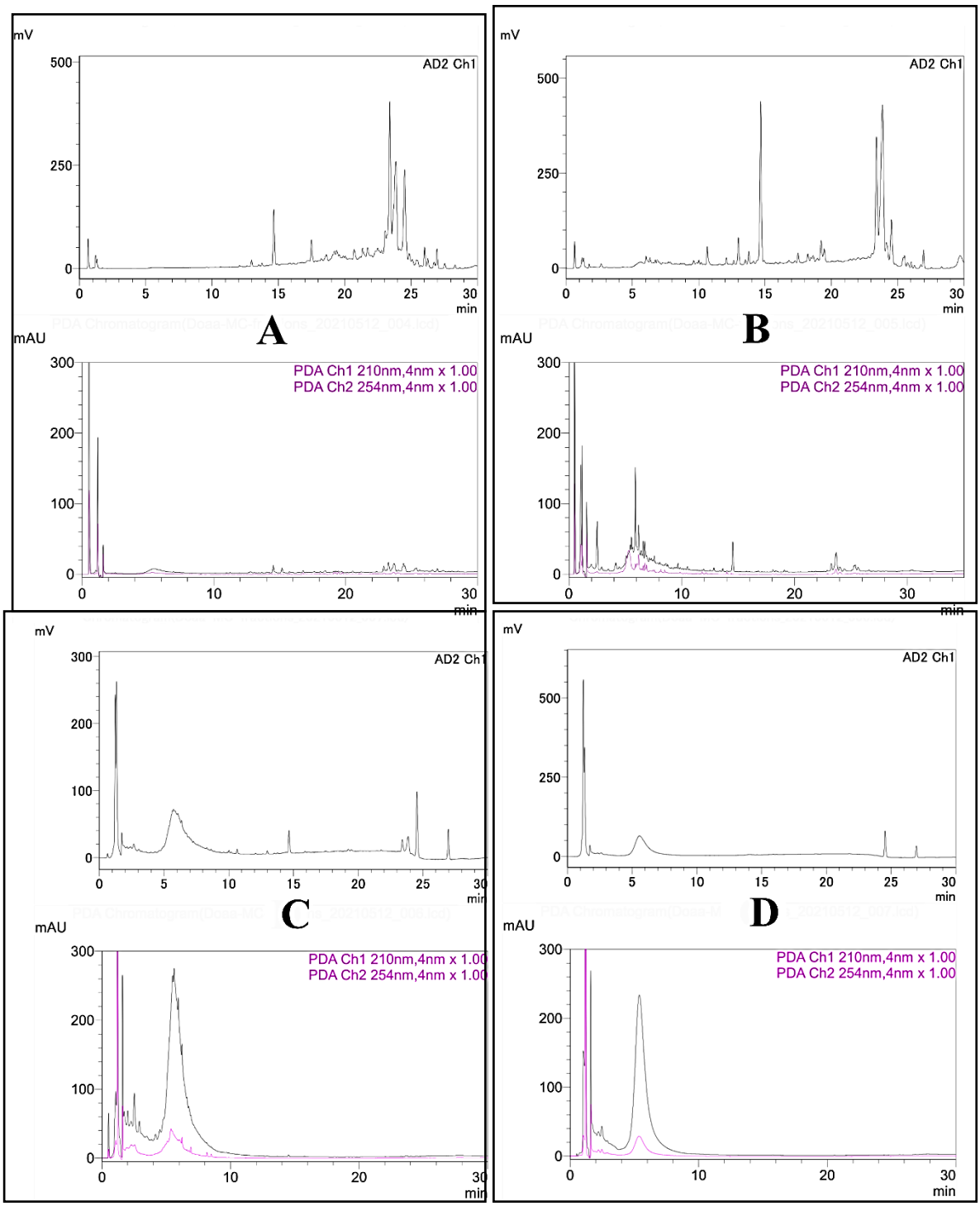


Figure 11. HPLC chromatogram of the fractionation of EtOH extract of *M. communis* Branches/stem. (A) Hexane fraction. (B) Ethyl acetate fraction. (C) Water fraction. (D) Butanol fraction.

3.2. Determination of Factors Effecting G. glabra Distribution and Phytochemical Content

Peaks of glabridin and liquiritin were identified at UV 210 nm, whereas glycyrrhizic acid peak was detected at 254 nm. **Figure 12** displayed a typical HPLC chromatogram for the 50% EtOH extract of *G. glabra roots*.

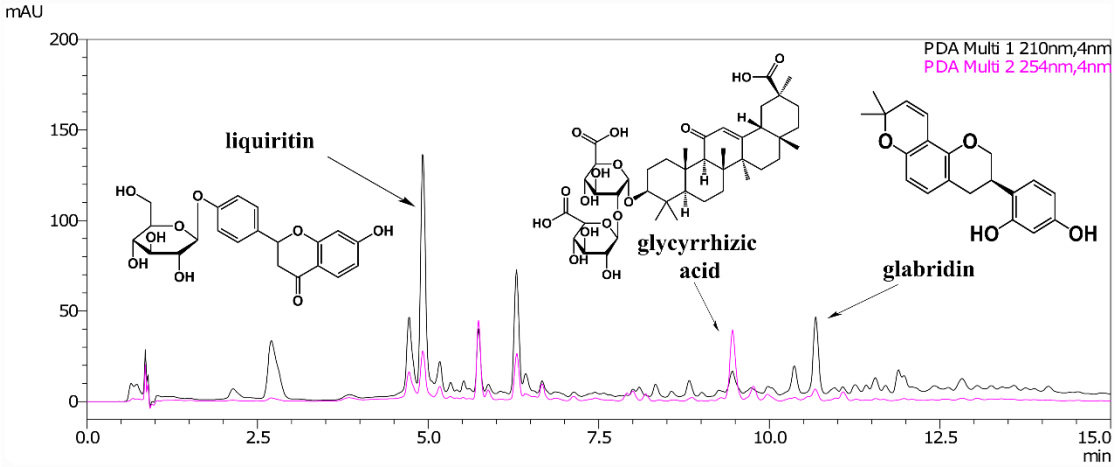


Figure 12. HPLC chromatogram for the 50% EtOH extract of *G. glabra* roots

Considerable variations of the content were observed, even at the same locality. The ranges of the tested bioactive compound were 0.54% to 2.40%, for glycyrrhizic acid, 0.02% to 0.31%, for glabridin, and 0.18% to 1.85% for liquiritin contents. Samples E-1, F-2, and F-3 met the Japanese Pharmacopoeia requirement of glycyrrhizic acid content ≥ 2% while samples F-1 and J-3 were very close to these requirements (**Table 8**). A weak correlation was observed between glycyrrhizic acid and liquiritin contents ($r = 0.6$, $p < 0.001$).

In the study area, the soils were neutral to slightly acidic in nature and dry to slightly wet. The volumetric soil moisture content (VSMC) was between (0.12–0.33). A soil bearing capacity ranged from 2.61 t/sf to 4.50 t/sf with different percentages of sand, silt, loam, and clay was observed in the study area.

Table 8. Chemical contents of *G. glabra* analyzed with HPLC.

Sample Information		Bioactive Contents		
ID[a]	Root Diameter (mm)	Glycyrrhizic Acid (%) [b]	Glabridin (%) [b]	Liquiritin (%) [b]
A-1	8	1.3 ± 0.1	0.2 *	0.5 *
B-1	17	1.0 ± 0.2	0.2 ± 0.1	0.9 ± 0.1
B-2	16	0.7 *	0.1 *	0.5 *
B-3	10	0.9 ± 0.1	0.1 *	0.7 ± 0.1
C-1	11	0.8 ± 0.2	≤ 0.05	0.6 ± 0.2
C-2	13	1.2 ± 0.1	0.1 *	1.1 ± 0.1
C-3	13	1.1 ± 0.3	0.1 *	0.7 ± 0.2
D-1	11	0.6 ± 0.1	≤ 0.05	0.5 ± 0.1
D-2	10	0.7 ± 0.2	0.1 *	0.7 ± 0.1
D-3	6	0.5 ± 0.1	≤ 0.05	0.6 ± 0.1
E-1	17	2.2 ± 0.6	0.2 ± 0.1	0.9 ± 0.2
E-2	17	0.9 ± 0.1	0.1 *	0.3 *
E-3	20	1.2 ± 0.2	0.1 *	0.4 ± 0.1
F-1	16	2.0 ± 0.5	0.1 *	1.1 ± 0.2
F-2	11	2.1 ± 0.4	0.1 *	1.3 ± 0.3
F-3	20	2.4 ± 0.4	0.1 *	1.9 ± 0.2
G-1	15	1.2 ± 0.4	0.1 *	1.0 ± 0.4
G-2	12	1.4 ± 0.3	0.1 *	0.8 ± 0.1
G-3	27	1.1 ± 0.4	≤ 0.05	1.2 ± 0.5
H-1	7	0.7 ± 0.2	0.1 *	0.2 *
H-2	10	1.7 ± 0.4	0.2 *	0.5 ± 0.1
H-3	12	1.5 ± 0.3	0.1 *	0.4 ± 0.1
I-1	8	0.6 ± 0.3	0.2 *	0.6 ± 0.3
I-2	16	1.3 ± 0.3	0.1 *	1.7 ± 0.3
I-3	11	1.2 ± 0.2	0.2 *	1.3 ± 0.2
J-1	15	0.9 ± 0.2	0.1 *	0.5 ± 0.1
J-2	14	1.4 ± 0.2	0.1 *	1.0 ± 0.1
J-3	15	2.0 ± 0.2	0.3 *	1.3 ± 0.1

The experiments were repeated three times, in which the mean and standard deviation (± SD) were calculated. [a] Uppercase letters in the sample ID point to the sample location. [b] the ratio of content represents the % of dry weight. * indicate that SD ≤ 0.05.

An increase in the volumetric soil moisture content (VSMC) values was observed when the attitude ($r = 0.9$, $p < 0.001$) and slope degree ($r = 0.8$, $p < 0.001$) increased, which was the result of high level of rainfall. Moreover, the multivariate analysis of variance MANOVA (using Wilks' test) and analysis of variance ANOVA shown that the glycyrrhizic acid and liquiritin contents of *G. glabra* were significantly affected by the aspect, curvature, elevation, slope, soil bearing capacity, and VSMC of the study area. In contrast, the meteorological variables and soil pH showed no effect with the bioactive contents. On the other hand, any correlation between the glabridin content and tested variables was not observed (**Table 9**).

Table 9. Statistical effects of tested variables on the bioactive contents of *G. glabra*.

Variable	MANOVA Analysis (Wilks' Test)		ANOVA Analysis					
			Glycyrrhizic Acid Content		Glabridin Content		Liquiritin Content	
	Wilks' Lambda	F Value	R^2	F Value	R^2	F Value	R^2	F Value
Elevation	0.03***	4.15	0.64**	3.62	0.36	1.15	0.63*	3.38
Curvature	0.04***	4.88	0.57**	3.78	0.27	1.03	0.61**	4.42
Hillshade	0.18***	4.20	0.06	0.39	0.22	1.60	0.31	2.61
Aspect	0.03***	4.15	0.64**	3.62	0.36	1.15	0.63*	3.38
Slope	0.06***	3.39	0.47	2.07	0.36	1.35	0.57*	2.47
Soil bearing capacity	0.1***	3.86	0.54**	4.06	0.07	0.25	0.49*	3.38
Soil pH	0.34	1.79	0.21	1.15	0.28	1.68	0.37	2.60
VSMC	0.03***	4.63	0.61**	3.77	0.27	0.86	0.61**	3.49
Average annual temperature	0.41	1.87	0.29	2.37	0.22	1.65	0.32	2.69
Average annual precipitation	0.59**	5.67	0.00	0.01	0.16	5.02	0.11	3.05
Climate	0.50**	3.17	0.34	6.35	0.18	2.71	0.10	1.37

* p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001.

The final map with the characterized suitability zones is displayed in **Figure 13**. Sites C, D, F, H, and J, where *G. glabra* samples were collected, were in the very high suitability zones. Locations A, B, E, G, and I were in the high suitability zones. None of the collected plants were within the moderate and low suitability zones. From the habitat suitability zone map, the ratio of each zone was; very high (15.1%), high (31.5%), moderate (40.3%), and low suitability (13.1%). Further investigations will target the very high and high suitability zones of the study area.

From the curvature map in **Figure 7C**, location C and D were positioned in flat areas while F and J were located at foothills. Location H was in a concave–convex region. These characteristics of the very high suitability zone provide rich soil with enough amount of rainfall. Similarly, the high suitability zone showed some features that facilitate plant growth and distribution. Flat areas characterized Sites A, B, E, and G while multifaceted concave–flat–convex area defined location I of the study area (**Figure 7J**). According to this study, suitable zones for *G. glabra* cultivation should have soil with a pH ranges between 6.5–7.0, a low soil bearing capacity (< 4.0 mm), and a low soil moisture content (< 0.24).

Overall, it was declared that most of the study area is suitable for *G. glabra* cultivation, growth, and distribution. The northern region of the study area has is consisting farming land, while the central region include urban cities, and the southern region has hilly area with geographical structures. Two of the collection sites, location I and J, were near the roads. Nevertheless, the influence of pollutant from fuel combustion on the chemical composition was ignored since these streets were mostly not busy with traffic. Furthermore, the temperature and precipitation of the study area were within a range that could boost cultivation activities and plant growth. Additionally, samples from high suitability zones showed to have relatively decent levels of bioactive compounds, particularly F-3, which had the highest glycyrrhizic acid and liquiritin contents.

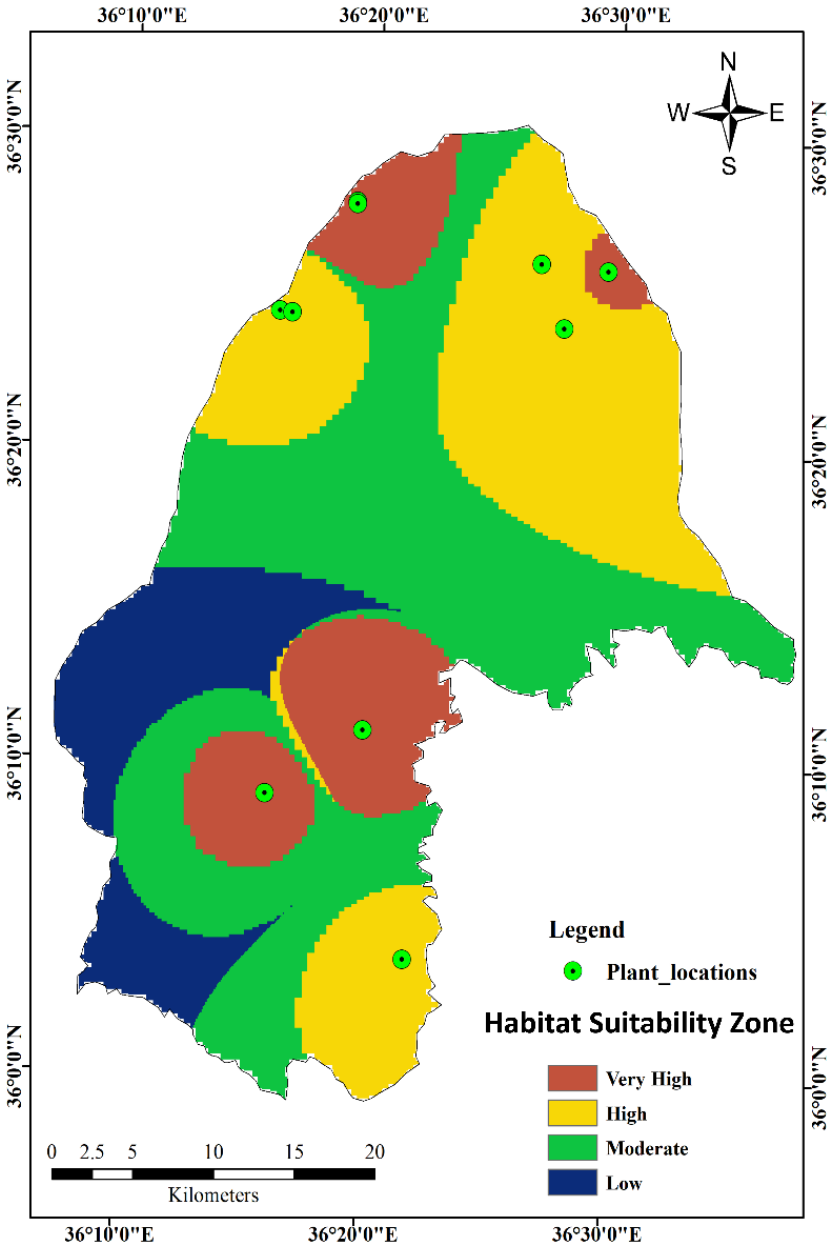


Figure 13. Habitat suitability zone map of *G. glabra* based on the FR model

The results revealed that the frequency ratio (FR) model had an excellent presentation with an AUC value of 0.905 (**Figure 14**). Therefore, a logical and acceptable output with a good precision for predicting the habitat suitability of *G. glabra* in the study area was achieved by using the FR model.

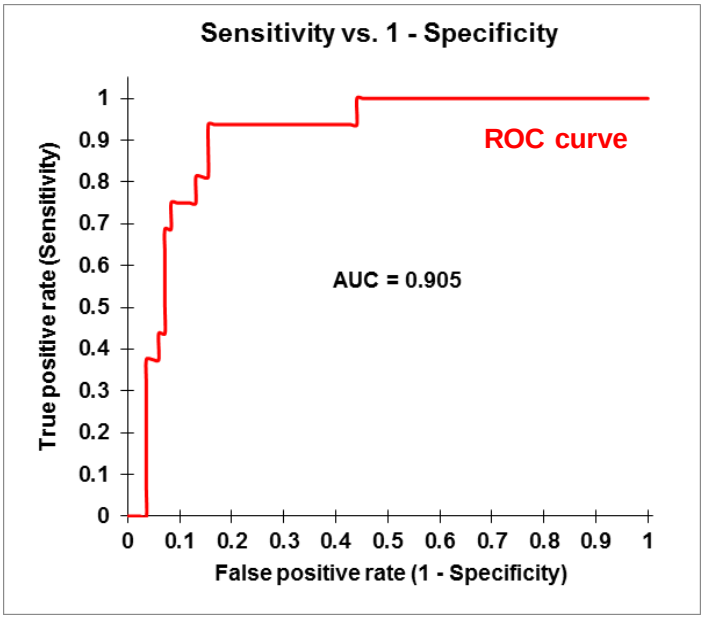


Figure 14. The receiver operator characteristic (ROC) curve for the habitat suitability map of *G. glabra* produced by the frequency ratio model

Differences in genetics [93,94] as well as environmental and/or soil parameters [29] have been reported before to effects the glycyrrhizic acid, glabridin, and liquiritin contents of *G. glabra*. In this study, glabridin content was not affected by tested variables. These coincide with similar results of Esmaeili *et al.* [94], who concluded that the glabridin content of *G. glabra* was influenced by the genetic diversity.

Glycyrrhiza glabra seems to adopt soils that have less soil bearing capacity and moisture content. These characteristics make the soils less vulnerable to fissuring, disruption, , and submersion [95].

The limited size of the study area could explain the minor impact of the climate on the contents of bioactive compounds. Moreover, The Hatay region of Turkey has been reported before to have high levels of potassium, calcium, and magnesium [29]. This indicate the presence of sufficient micronutrients in the soil which have the ability to maintain the pH within a certain range causing less variation.

Chapter 4. Conclusion

The usage of plant to treat various aliment is an old custom that still practiced to this day in Turkey. The emergent of new diseases and complication has resulted in the further utilization of the plant materials in the research and development of medicine. One of the conditions where plants can be used as medicine and/or supplement is gastric cancer. Late diagnosis has resulted in advance stage of this cancer in most of the patients in which treatment is not sufficient to handle the situation. The usage of plant materials that have anti gastric cancer activity as a food and/or supplement may offer a solution for this issue. From this study, *A. mollis*, *M. communis*, *Q. coccifera*, *T. macrophyllum*, and *T. foenum-graecum* have shown remarkable inhibition to the growth of gastric cancer cells in the cell line study. Furthermore, the 95% EtOH extract of the aerial parts of *A. mollis* and the 95% EtOH Br/St extract of *M. communis* and its butanol fraction have shown higher selectivity towards cancer cells than normal gastric fibroblast cells. Some of these findings are new which requires further attentions to the importance of the plant materials in gastric cancer treatment. These plants can be used as a source for research and development of cancer drugs. Utilization of these plants may help in averting cancer and/or finding a new lead compound that can be used in cancer treatment. Further researches are needed to isolate and identify the bioactive compounds and elucidate their mechanism of action against cancer cells.

Furthermore, this study also investigated the factors that affect the phytochemical content of *G. glabra* as well as its distribution and growth. This investigation considers as the first GIS-based study on the distribution of *G. glabra* in Turkey.

Various effects were observed on the bioactive compounds of *G. glabra* from the influence of environmental and soil parameters. Topographical factors (aspect, curvature, elevation, and slope) and soil parameters (soil bearing capacity and volumetric soil moisture content) of the study area were effecting the glycyrrhizic acid and liquiritin contents significantly. On the other hand, the environmental factors and/or soil conditions did not affect the glabridin content, which proposes the influence of other factors. The introduced GIS-based model suggested the high potential *G. glabra* growth and distribution in the Hatay region. Simulating a parallel condition for cultivation purposes of *G. glabra* might demonstrate beneficial outcome, especially if supplemented with a system to control soil aeration and moisture level while sustaining suitable amounts of soil micronutrients. Data obtained from this study will be used as a reference while conducting similar investigations on licorice distribution in the Hatay and nearby regions.

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Appendix

Cell Line Maintenance

After thawing the stock of AGS in water bath for three minutes, the cancer cells were diluted 10X with the cell line medium (contain 10% FBS) before centrifuging at 4°C and 1000 rpm for three minutes. The medium was aspirated and the cell pallet was re-dispersed in 10 ml of the medium. The cell mixture transferred to a 10 cm dish for incubation at 37°C with 5% CO_2 for two days. Thereafter, the sub-cultured process begins, the medium was aspirated and the cells were washed with 10 mL of PBS. Next, the PBS was aspirated and 2 ml of trypsin was added. The dish was incubated for 5–10 minutes at 37°C with 5% CO_2. Then it was examined under the microscope to confirm cell detachment. Medium (8 mL) was added to the cell-trypsin mixture and centrifuged for three minutes at 4°C and 1000 rpm. Afterward, the medium was aspirated and the cell pallet was re-dispersed in 10 mL of the medium. The cell mixture transferred to a 10 cm dish for incubation at 37°C with 5% CO_2 for two days. The sub-cultured process was repeated every 2–3 days. The screening experiment start after 2–3 passages.

Similar procedure was conducted for the sub-culturing of normal human gastric fibroblast cells except the trypsin was replace with Accutase and the cells were cultured into 20 cm dish once every week.

Table A1. Plants collection sites in Turkey

Scientific name	ID	Collection sites	Floral zone
Alchemilla mollis	TUR(KU)170907-0949	Anzer	Europe-Siberian zone
Ammi majus	TUR(KU)170906-1159	Rize~Anzer	Europe-Siberian zone
Anastatica hierochuntica	TUR(KU)170905-1	Trabzon market	Europe-Siberian zone
Capparis spinosa	TUR(KU)191103-1440	Hatay	Mediterranean zone
Clematis vitalba	TUR(KU)170909-1122	Yason	Europe-Siberian zone
Dioscorea communis	TUR(KU)170908-1412	Hidirnebi	Europe-Siberian zone
Echium plantagineum	TUR(KU)170906-1207	Rize~Anzer	Europe-Siberian zone
Erica manipuliflora	TUR(KU)191103-1500	Hatay	Mediterranean zone
Gentiana asclepiadea	TUR(KU)170907-1548	Ayder	Europe-Siberian zone
Jacobaea aquatica	TUR(KU)170909-1100	Yason	Europe-Siberian zone
Myrtus communis	TUR(KU)191103-1425	Hatay	Mediterranean zone
Nigella sativa	TUR(KU)180317-01	Hatay market	Mediterranean zone
Pistacia terebinthus	TUR(KU)191103-1420	Kilis market	Mediterranean zone
Polygonatum multiflorum	TUR(KU)170908-1350	Hidirnebi	Europe-Siberian zone
Quercus coccifera	TUR(KU)191103-1405	Hatay	Mediterranean zone
Rhus coriaria	TUR(KU)170210-6	Hatay market	Mediterranean zone
Rubus sanctus Schreber	TUR(KU)170909-1112	Hatay market	Mediterranean zone
Tanacetum macrophyllum	TUR(KU)170906-1408	Rize~Anzer	Europe-Siberian zone
Tilia platyphyllos	TUR(KU)160924-1	Ordu market	Europe-Siberian zone
Trigonella foenum-graecum	TUR(KU)180317-02	Hatay market	Mediterranean zone

Table A2. Various environmental variables of *G. glabra* collection sites

Variables	Location									
	A	B	C	D	E	F	G	H	I	J
Elevation (m)	75.6	82.5	116.1	125.3	89.4	88.1	83.6	328.0	198.8	164.5
Curvature	0.11	−0.78	2.22	−0.11	−0.11	−0.11	0.22	0.67	0	−0.22
Hillshade (°)	0	0	0	0	92	0	0	112	110	86
Aspect (°)	135.0	213.7	127.9	187.6	255.9	225	153.4	6.5	7.2	253.7
Slope (°)	0.7	0.9	2.9	8.5	4.8	1.9	1.9	21	15.7	13.4
Average temp. (°C)*	20.4	18.3	19.4	19.4	19.9	21.1	21.1	19.4	19.4	19.4
Maximum temp. (°C)*	37.1	37.1	37.1	37.1	37.1	37.1	37.1	39.6	39.6	37.1
Minimum temp. (°C)*	2.5	2.5	2.5	2.5	2.5	2.5	2.5	−2.0	−2.0	−2.0
Maximum daily rainfall (mm)*	62.5	62.5	12.5	12.5	12.5	12.5	12.5	62.5	62.5	62.5
Average areal precipitation (mm)*	900	900	1100	1100	900	900	900	900	900	900
Precipitation efficiency index**	−9.5	−9.5	−9.5	−9.5	10.5	10.5	10.5	30.5	30.5	30.5
Average annual temp. (°C)***	19.4	19.4	19.5	19.5	19.5	19.4	19.5	18.0	19.1	19.2
Average max. temp. of warmest month (°C) ***	32.2	32.2	33.0	33.0	32.6	32.9	32.8	32.3	33.8	32.9
Average min. temp. of coldest month (°C)***	6.1	4.1	5.0	5.5	6.1	6.0	6.0	5.5	5.3	5.4
Average annual precipitation (mm)***	855	855	813	813	731	702	720	890	778	836
Average precipitation of wettest month (mm)***	139	151	139	132	132	123	120	122	172	145
Average precipitation of driest	9	9	8	8	5	4	5	5	4	7

month (mm)*	

* Recorded in 2019 (Turkish Ministry of Agriculture and Forestry, General Directorate of Meteorology. https://www.mgm.gov.tr/. (Accessed on Jan.11th 2020))

** Climate classification based on Thronthwaite method for the period 1981–2010 (Thornthwaite 1948)

***Bioclimatic variables for the period 2010-2018 (WorldClim, Bioclimatic variables. https://www.worldclim.org/data/bioclim.html. (Accessed on May 1st 2020))

Table A3. Data used for spatial modeling

Variables	Class	Value	Class Pixel	% Subclass Pixel	Plant Pixels			FR [a]	RF [b]	RF (%)	RF (INT)	Min (RF)	Max (RF)	PR [c] Value
					Area	Pixels	% Pixels							
Aspect (°)	(−)1–40	1	259477	12.83	996300	1107	14.08	1.10	0.12	12.28	12			
	40–80	2	231529	11.45	415800	462	5.87	0.51	0.06	5.74	5			
	80–120	3	234080	11.57	574200	638	8.11	0.70	0.08	7.84	7			
	120–160	4	258309	12.77	1125900	1251	15.91	1.25	0.14	13.93	13			
	160–200	5	247161	12.22	1442700	1603	20.38	1.67	0.19	18.66	18			
	200–240	6	190569	9.42	721800	802	10.20	1.08	0.12	12.11	12			
	240–280	7	171593	8.48	700200	778	9.89	1.17	0.13	13.05	13			
	280–320	8	222734	11.01	562500	625	7.95	0.72	0.08	8.07	8			
	320–360	9	207199	10.24	539100	599	7.62	0.74	0.08	8.32	8			
Total			**2022651**			**7865**		**8.94**	**1.00**	**100.00**		**0.06**	**0.19**	**1.86**
Curvature	(−)25–(−)1.5	1	139370	6.90	351000	390	4.96	0.72	0.27	26.62	26			
	(−)1.5–1.5	2	1741293	86.15	6256800	6952	88.39	1.03	0.38	37.97	37			
	1.5–25	3	140482	6.95	470700	523	6.65	0.96	0.35	35.41	35			
Total			**2021145**			**7865**		**2.70**	**1.00**	**100.00**		**0.27**	**0.38**	**1.64**
Elevation (m)	75–130	1	1178213	58.25	4721400	5246	66.70	1.15	0.11	10.84	10			
	130–185	2	322827	15.96	788400	876	11.14	0.70	0.07	6.61	6			
	185–240	3	381501	18.86	785700	873	11.10	0.59	0.06	5.57	5			
	240–295	4	112594	5.57	0	0	0.00	0.00	0.00	0.00	0			
	295–350	5	27516	1.36	783000	870	11.06	8.13	0.77	76.98	76			
Total			**2022651**			**7865**		**10.56**	**1.00**	**100.00**		**0.00**	**0.77**	**11.11**
Soil bearing capacity (t sf^{-1})	2.5–3.0	1	21852	1.08	785700	873	11.10	10.27	0.68	68.47	68			
	3.0–3.5	2	392009	19.38	1900800	2112	26.85	1.39	0.09	9.23	9			
	3.5–4.0	3	1458332	72.10	2933100	3259	41.44	0.57	0.04	3.83	3			

	4.0–4.5	4	150458	7.44	1458900	1621	20.61	2.77	0.18	18.47	18			
Total			**2022651**			**7865**		**15.01**	**1.00**	**100.00**		**0.04**	**0.68**	**9.33**
	0–23	1	1142838	56.50	4176000	4640	59.00	1.04	0.21	20.68	20			
	23–68	2	131839	6.52	504000	560	7.12	1.09	0.22	21.64	21			
Hillshade (°)	68–111	3	154800	7.65	605700	673	8.56	1.12	0.22	22.14	22			
	111–152	4	219283	10.84	787500	875	11.13	1.03	0.20	20.32	20			
	152–181	5	373891	18.49	1005300	1117	14.20	0.77	0.15	15.22	15			
Total			**2022651**			**7865**		**5.05**	**1.00**	**100.00**		**0.15**	**0.22**	**1**
	6.5–6.6	1	390	0.02	336600	374	4.76	246.58	0.89	89.19	89			
	6.6–6.7	2	54997	2.72	569700	633	8.05	2.96	0.01	1.07	1			
pH	6.7–6.8	3	248041	12.27	788400	876	11.14	0.91	0.00	0.33	0			
	6.8–6.9	4	1263009	62.45	1460700	1623	20.64	0.33	0.00	0.12	0			
	6.9–7.0	5	446397	22.07	3138300	3487	44.34	2.01	0.01	0.73	0			
	7.0–7.1	6	9475	0.47	784800	872	11.09	23.66	0.09	8.56	8			
Total			**2022309**			**7865**		**276.45**	**1.00**	**100.00**		**0.00**	**0.89**	**12.86**
	900–940	1	1221090	60.37	5504400	6116	77.76	1.29	0.22	22.04	22			
	940–980	2	453773	22.43	0	0	0.00	0.00	0.00	0.00	0			
Average areal precipitation (mm)*	980–1020	3	133972	6.62	0	0	0.00	0.00	0.00	0.00	0			
	1020–1060	4	115083	5.69	0	0	0.00	0.00	0.00	0.00	0			
	1060–1100	5	98733	4.88	1574100	1749	22.24	4.56	0.78	77.96	77			
Total			**2022651**			**7865**		**5.84**	**1.00**	**100.00**		**0.00**	**0.78**	**11.25**
	18–19	1	164026	8.11	783000	870	11.06	1.36	0.04	3.54	3			
Average temp. (°C)*	19–20	2	1440412	71.21	4518000	5020	63.83	0.90	0.02	2.33	2			
	20–21	3	410943	20.32	871200	968	12.31	0.61	0.02	1.57	1			
	21–22	4	7270	0.36	906300	1007	12.80	35.62	0.93	92.55	92			
Total			**2022651**			**7865**		**38.49**	**1.00**	**100.00**		**0.02**	**0.93**	**13.13**
	0.10–0.18	1	1071748	52.99	3815100	4239	53.90	1.02	0.09	8.84	8			

VSMC (m^3 m^{-3})	0.18–0.25	2	727666	35.98	2480400	2756	35.04	0.97	0.08	8.47	8			
	0.25–0.33	3	199709	9.87	0	0	0.00	0.00	0.00	0.00	0			
	0.33–0.40	4	23528	1.16	783000	870	11.06	9.51	0.83	82.69	82			
Total			**2022651**			**7865**		**11.50**	**1.00**	**100.00**		**0.00**	**0.83**	**11.93**
Slope (°)	0–3	1	1268826	62.73	4706100	5229	66.48	1.06	0.18	18.32	18			
	3–7	2	231927	11.47	691200	768	9.76	0.85	0.15	14.72	14			
	7–12	3	196895	9.73	670500	745	9.47	0.97	0.17	16.82	16			
	12–17	4	150517	7.44	568800	632	8.04	1.08	0.19	18.66	18			
	17–24	5	109658	5.42	324000	360	4.58	0.84	0.15	14.59	14			
	24–32	6	47809	2.36	92700	103	1.31	0.55	0.10	9.58	9			
	32–64	7	17019	0.84	25200	28	0.36	0.42	0.07	7.31	7			
Total			**2022651**			**7865**		**5.79**	**1.00**	**100.00**		**0.07**	**0.19**	**1.64**
Average annual mean temperature (°C)**	12–14	1	1647	0.08	0	0	0.00	0.00	0.00	0.00	0			
	14–16	2	11990	0.59	0	0	0.00	0.00	0.00	0.00	0			
	16–18	3	269896	13.34	0	0	0.00	0.00	0.00	0.00	0			
	18–20	4	1739118	85.98	7078500	7865	100.00	1.16	1.00	100.00	100			
Total			**2022651**			**7865**		**1.16**	**1.00**	**100.00**		**0.00**	**1.00**	**14.43**
Average max. temperature of warmest month (°C)**	29.5–31.8	1	106336	5.26	0	0	0.00	0.00	0.00	0.00	0			
	31.8–32.4	2	575022	28.43	1836000	2040	25.94	0.91	0.22	22.15	22			
	32.4–32.99	3	591537	29.25	3550500	3945	50.16	1.72	0.42	41.63	41			
	32.99–33.9	4	544024	26.90	993600	1104	14.04	0.52	0.13	12.67	12			
	33.9–34.8	5	205732	10.17	698400	776	9.87	0.97	0.24	23.55	23			
Total			**2022651**			**7865**		**4.12**	**1.00**	**100.00**		**0.13**	**0.42**	**4.18**
Average min. temperature of coldest	(−)1.6–0.2	1	3941	0.19	0	0	0.00	0.00	0.00	0.00	0			
	0.2–1.3	2	2708	0.13	0	0	0.00	0.00	0.00	0.00	0			
	1.3–2.2	3	11450	0.57	0	0	0.00	0.00	0.00	0.00	0			
	2.2–2.9	4	36359	1.80	0	0	0.00	0.00	0.00	0.00	0			
	2.9–3.6	5	103930	5.14	0	0	0.00	0.00	0.00	0.00	0			

month (°C)**	3.6–4.3	6	201169	9.95	377100	419	5.33	0.54	0.16	15.89	15			
	4.3–4.9	7	270508	13.37	483300	537	6.83	0.51	0.15	15.15	15			
	4.9–5.4	8	439229	21.72	1314000	1460	18.56	0.85	0.25	25.36	25			
	5.4–6.2	9	953357	47.13	4904100	5449	69.28	1.47	0.44	43.61	43			
Total			**2022651**	**.**		**7865**		**3.37**	**1.00**	**100.00**		**0.00**	**0.44**	**6.29**
Average annual precipitation (mm)**	550–650	1	179515	8.88	0	0	0.00	0.00	0.00	0.00	0			
	650–750	2	497152	24.58	0	0	0.00	0.00	0.00	0.00	0			
	750–850	3	571596	28.26	2356200	2618	33.29	1.18	0.21	21.03	21			
	850–950	4	600861	29.71	2862900	3181	40.45	1.36	0.24	24.31	24			
	950–1050	5	173527	8.58	1859400	2066	26.27	3.06	0.55	54.66	54			
Total			**2022651**			**7865**		**5.60**	**1.00**	**100.00**		**0.00**	**0.55**	**7.89**
Average precipitation of wettest month (mm)**	100–125	1	597932	29.56	2356200	2618	33.29	1.13	0.34	34.20	34			
	125–150	2	661037	32.68	3150900	3501	44.51	1.36	0.41	41.37	41			
	150–175	3	558164	27.60	1571400	1746	22.20	0.80	0.24	24.43	24			
	175–200	4	193456	9.56	0	0	0.00	0.00	0.00	0.00	0			
	200–225	5	12062	0.60	0	0	0.00	0.00	0.00	0.00	0			
Total			**2022651**			**7865**		**3.29**	**1.00**	**100.00**		**0.00**	**0.41**	**5.97**
Average precipitation of driest month (mm)**	1–3	1	134516	6.65	0	0	0.00	0.00	0.00	0.00	0			
	3–6	2	1144927	56.61	4407300	4897	62.26	1.10	0.47	46.79	46			
	6–9	3	610285	30.17	2671200	2968	37.74	1.25	0.53	53.21	53			
	9–12	4	132923	6.57	0	0	0.00	0.00	0.00	0.00	0			
Total			**2022651**			**7865**		**2.35**	**1.00**	**100.00**		**0.00**	**0.53**	**7.68**

* Recorded in 2019 (Turkish Ministry of Agriculture and Forestry, General Directorate of Meteorology. https://www.mgm.gov.tr/. (Accessed on Jan.11th 2020))

**Bioclimatic variables for the period 2010-2018 (WorldClim, Bioclimatic variables. https://www.worldclim.org/data/bioclim.html. (Accessed on May 1st 2020))

[a] Frequency ratio. [b] relative frequency. [c] prediction ratio.