T.C.

YEDİTEPE UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

DEPARTMENT OF PHYTOTHERAPY

IN VITRO BIOAVAILABILITY STUDIES ON PHYTOCHEMICAL PROFILE AND ANTIOXIDANT ACTIVITY POTENTIAL OF *Clematis viticella* L.

MASTER THESIS

ESRA ACAR ŞAH, Pharm.

İstanbul, 2018

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SUPERVISOR

Assist. Prof. Dr. Engin CELEP

İstanbul, 2018

THESIS APPROVAL

Institute : Yeditepe University Institute of Health Sciences

Programme : Phytotherapy

Title of the Thesis : *In vitro* Bioavailability Studies on Phytochemical Profile and Antioxidant Activity Potential of *Clematis viticella* L.

Owner of the Thesis : Esra Acar Şah

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This study have approved as a Master/Doctorate Thesis in regard to content and quality by the Jury.

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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated 22.06.2018.... and numbered 2018/11-30

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Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgement has been made in the text.

19.04.2018

Esra ACAR ŞAH

I dedicate this to my "Dear Husband" and "Supervisor"...

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LIST OF SYMBOLS AND ABBREVIATIONS

AAE:	Ascorbic Acid Equivalent
ABS:	Absorbance
BAcI:	Bioaccessibility Index
BAvI:	Bioavailability Index
BHT:	Butylated Hydroxy Toluene
CAE:	Caffeic Acid Equivalents
Cat:	Catalase
COX:	Cyclooxygenase
CUPRAC:	Cupric Reducing Antioxidant Capacity
DMPD:	N, N-dimethyl-p-phenylendiamine
DPPH:	2,2-Diphenyl-1-picrylhydrazyl
ECE:	(-)-Epicatechin Equivalents
EDTA:	Ethylenediaminetetraacetic Acid
EE:	Escin Equivalents
FCA:	Freund's Complete Adjuvant
FCR:	Folin Ciocalteu Reagent
FRAP:	Ferric Reducing Antioxidant Power
GAE:	Gallic Acid Equivalents
GI:	Gastrointestinal
GSH-Px:	Glutathione Peroxidase
HIV:	Human Immunodeficiency Virus
IL-1:	Interleukin-1

IN:	Serum Available
LDH:	Lactate Dehydrogenase
LDL:	Low Density Lipoprotein
LPS:	Lipopolysaccharide
MeOH:	Methanol
MIA:	Monosodium Iodoacetate
MTT:	Modified Microculture Tetrazolium
ND:	Non-digested
NO:	Nitric Oxide
OUT:	Colon Available
PG:	Postgastric
PGE2:	Prostaglandin-E2
QE:	Quercetin Equivalents
RNS:	Reactive Nitrogen Species
ROS:	Reactive Oxygen Species
S.D.:	Standard Deviation
SNP:	Sodium Nitroprusside
SOD:	Superoxide Dismutase
TE:	Trolox Equivalent
TNF-α:	Tumor Necrosis Factor-α
TOAC:	Total Antioxidant Capacity
TPTZ:	2,4,6-tripyridyl-s-triazine
WHO:	World Health Organization
YEF:	Yeditepe University Faculty of Pharmacy Herbarium

ABSTRACT

Acar Şah, E. (2018). *In vitro* Bioavailability Studies on Phytochemical Profile and Antioxidant Activity Potential of *Clematis viticella* L. Yeditepe University, Institute of Health Science, Department of Phytotherapy, MSc Thesis, İstanbul.

Oxidative stress is a major problem for living organisms and might lead to the formation of reactive oxygen species which may cause cellular damage. It is also known for playing an important role in the formation of various degenerative diseases, such as rheumatoid arthritis, diabetes, cancer and cardiovascular diseases. Although the human body has a number of enzymatic and non-enzymatic antioxidant mechanisms, it should be supplemented by consumption of antioxidant-rich foods. Since biotransformation processes generally affect the biological activities of antioxidant compounds, bioavailability is the first step in determining the potency of the activity. Previous studies have shown that *in vitro* antioxidant capacity of phytochemicals cannot reflect real health benefits. *Clematis* species (Ranunculaceae) are used to eliminate the symptoms of many diseases including particularly in the treatment of rheumatic diseases in Turkey, but the number of studies conducted is limited. In the light of this information, the total phytochemical profile of the aerial parts of *C. viticella* prepared with 80% methanolic and the gastrointestinal digestion products and the antioxidant activity profile were evaluated by subjecting it to the *in vitro* simulation model of the human digestive tract in the study. It was observed that both total phytochemical content and antioxidant activity decreased after simulated human digestion. Moreover, this study showed potential oral bioaccessibility and bioavailability of total phenol, phenolic acid, flavonoid, and saponin content found in the aerial parts of *Clematis viticella*. Consequently, these indicate that it resulted from the complicated interaction of compounds in the plant matrix in an *in vitro* simulated human digestion.

Key words: *Clematis viticella* L., Ranunculaceae, Phenolic Profile, Antioxidant Activity, *In vitro* Digestion Simulation Model, Bioavailability

ÖZET

Acar Şah, E. (2018). *Clematis viticella* L. Bitkisinin Fitokimyasal Profili ve Antioksidan Aktivite Potansiyeli Üzerine *in vitro* Biyoyararlanım Çalışmaları. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Fitoterapi ABD., Master Tezi, İstanbul.

Oksidatif stres, canlı organizmalar için önemli bir sorundur ve hücresel hasara neden olabilen reaktif oksijen türlerinin oluşmasına yol açabilir. Bu durumun inflamasyon, romatoid artrit, diyabet, kanser ve kardiyovasküler hastalıklar gibi çeşitli dejeneratif hastalıkların oluşumunda da önemli bir rol oynadığı bilinmektedir. İnsan vücudunda enzimatik ve non-enzimatik antioksidan mekanizmalar bulunmasına rağmen, antioksidan bakımından zengin gıdaların tüketimi ile desteklenmelidir. Biyotransformasyon işlemleri genellikle antioksidan bileşiklerin biyolojik aktivitelerini etkilediği için biyoyararlanım, aktivite potansiyelini belirlenmesinde ilk adımdır. Çünkü önceki çalışmalar, fitokimyasalların *in vitro* antioksidan kapasitesinin gerçek sağlık yararlarını yansıtmadığını göstermiştir. *Clematis* türleri (Ranunculaceae), Türkiye'de özellikle romatizmal hastalıkların tedavisi dahil olmak üzere birçok hastalığın semptomlarını iyileştirmek için kullanılmaktadır, ancak bu türler üzerine yapılan çok az çalışma bulunmaktadır. Bu bilgiler ışığında, bu çalışmada *C. viticella*'nın %80 metanol ile hazırlanan ekstre ve gastrointestinal sindirim ürünlerinin toplam fitokimyasal profili ve antioksidan aktivite potansiyeli insan sindirim sisteminin *in vitro* simülasyon modeline tabi tutularak değerlendirilmiştir. İnsan sindirim simülasyon sürecinden sonra hem toplam fitokimyasal hem de antioksidan aktivitenin azaldığı gözlemlenmiştir. Ayrıca bu çalışma, *Clematis viticella*'nın topraküstü kısımlarında bulunan fenol, fenolik asit, flavonoid ve saponin içeriğinin potansiyel oral biyoyararlanımı ve biyoerişilebilirliğini göstermiştir. Sonuç olarak, bu sonuçlar, *in vitro* simüle edilmiş insan sindiriminde, bitki matriksindeki bileşiklerin karmaşık etkileşimlerinden kaynaklandığını göstermektedir.

Anahtar kelimeler: *Clematis viticella*, Ranunculaceae, Fenolik Profil, Antioksidan Aktivite, *In vitro* Sindirim Simülasyon Modeli, Biyoyararlanım

1. INTRODUCTION & AIM

Human beings have benefited from nature to sustain their life, they have developed various methods of using plants as both protective and therapeutic against diseases (1). According to archaeological finding from the first ages, people had benefited from plants to resolve health problems and to get nutrients. These informations previously obtained through trial and error. They have also reached up to these days with some changes and developments in the manner of use throughout the ages (1, 2).

In 1957, 60 thousand-year-old ruins in Şanidar Cave in northern Iraq were accompanied by dead people with yarrow, groundsel, hollyhock, purple hyacinth, cornflower, mallow and ephedra. This shows the presence of belief that after death it will turn into life again, and that these plants begin to separate as edible and herbaceous plants. Today, these plants are still used as medicinal plants (3, 4).

During the 19th and 20th centuries, the fast development of chemistry and especially biochemistry has enabled the pharmaceutical industry to develop rapidly. A huge number of drugs have been developed, resulting in analytical, toxicological, pharmacological and clinical studies which were taken in to consideration efficacy, harmlessness and quality. One in four of the existing drugs are of herbal origin and most of them are produced by synthesizing the active compound desired to be obtained from the plants in the laboratory. Herbal treatment has become popular again due to many factors, such as the medical and economic problems caused by serious side effects that have come to the fore with synthetic drugs in recent years, ecological approaches and movements strengthened by environmental pollution, and the threat posed by many chronic illnesses for which curative treatments have not sufficient to cure yet (5).

Currently, it can be stated that the most important factors of traditional treatment methods are medicinal plants. According to World Health Organization (WHO), 3.3 billion people benefit from medicinal herbs for treatment. Turkey has not only one of the world's richest floras with its almost ten thousand species of plants but also it has a deep-rooted culture (1).

There are radical or non-radical reactive species in the living organisms. Reactive nitrogen species (RNS) and reactive oxygen species (ROS) are the most crucial of them (6). There are various defense systems against reactive oxygen and nitrogen species. These systems are generally grouped under the heading ‘‘Antioxidant System’’.

Oxidants are constantly produced in the organism while the antioxidant system prevents these oxidants and their adverse effects. This is a constant balance. If this balance is broken in favor of the oxidants, it is named ‘‘Oxidative Stress’’. In the case of oxidative stress, the amount of reactive species increases, and many systems of the organism, especially lipids, proteins and nucleic acids, are damaged. Affected systems affect other related systems, and this continues as a chain. These chain reactions are terminated by antioxidant defense systems. If antioxidant defenses are inadequate, these reactive species causes the death of cells directly or indirectly (7, 8). In addition, oxidative stress has critical matter in formation of various degenerative diseases, for instance, inflammation, cardiovascular diseases, accelerated aging, atherosclerosis, cancer, central nervous system disorder, rheumatoid arthritis, diabetes, liver diseases and AIDS (9, 10).

Plants and foods have wide phenolic compound derivatives such as simple phenols, phenyl propanoides, benzoic acid derivatives, flavonoids, stylbens, tannins, lignans and lignins. In 1947, Boland and Ten Have performed the first detailed kinetic study of antioxidant activity of phenolic compounds. Phenolic antioxidants interfere with lipid oxidation by easily donating electrons to lipid radicals. In addition, they are perfect hydrogen donors. The resulting radical intermediates are comparatively stable since they have resonance delocalization and their molecular oxygen cannot attack phenolic antioxidants the reason is that there are no suitable parts to connect (11-13).

The correlation between phenolic compounds and antioxidant activity was supported by a lot of works using different methods. Nevertheless, with the advance of science, it has been shown that *in vitro* antioxidant capacity of phytochemicals cannot reflect real health benefits. Because of the fact that biotransformation processes generally affect the biological activities of antioxidant compounds. For an exact evaluation of bioavailability of phytochemicals, information is needed concerning their pharmacokinetic properties for instance absorption, metabolism, distribution, and excretion from the organism. To paraphrase, molecules are needed to be bioaccessible so as to demonstrate their biotransformation and bioactivity. In addition, the conducting *in vivo* and clinic investigation is complicated, technically difficult, expensive, and can appear ethic conditions, as well. Thus, GI digestion is expressed as a significant inceptive step in the analysis of the action potential of compounds (14).

Clematis species (Ranunculaceae) are used to eliminate the symptoms of many diseases including particularly in the treatment of rheumatic diseases in Turkey. Among these species, *Clematis viticella* L. aerial parts have been widely used traditionally in rheumatic complaints in Kocaeli and its vicinity, but the number of studies conducted is limited (15). In the light of this information, the total phenolic and saponin profile of the aerial parts of *C. viticella* prepared with 80% methanolic and the gastrointestinal digestion products and the antioxidant activity profile are going to be evaluated in this study by subjecting it to the *in vitro* simulation model of the human digestive tract. The total phenol, flavonoid, phenolic acid, proanthocyanidin and saponin profile of the extract and gastrointestinal digestion products and the antioxidant activity profile demonstrated by different mechanisms are going to be appraised.

2. GENERAL DESCRIPTION

2.1. *Clematis* L.

2.1.1. Botanical Information

2.1.1.1. Ranunculaceae

The family Ranunculaceae has 59 genus and about 2500 species in the world. There are 17 genus and 216 species in our country. Endemism rate of the family in Turkey is 23.5%. It is a plant that grows in the moist, temperate and cold parts of the Northern Hemisphere, annual or perennial, mostly herbaceous, some woody or climbers. Leaves are usually alternate, occasionally opposit or all collected on the basal; the lamina is full, little or multipart, pennate or palmate vascular. Flowers are actinomorph or zygomorph, hermaphrodite, hypogynous, fragments are acyclic. The periant is either calix-corolla or calix petaloid; the segments are usually free, those of the inner whorl often nectariferous, entemogamous. Receptaculum is convex and prolonged. The stamens are usually numerous, spirally arranged, centripetal, anthers extrorse. Ovaries are one or many carpels and apocrine, ovules are singular or plural. In this family, there are different kinds of fruit; follicle (*Helleborus*), nuks (*Anemone*), capsule (*Nigella*), baccate (*Actea*), achene (*Clematis*). Flowers formula is in the form a/z K_5 C_5 A_∞ G_∞. Some of the plants contain alkaloids, some are heterosides, some have volatile lactones (16).

1. Leaves opposite; plant climbing **11. *Clematis***
1. Leaves alternate or all basal; plant not climbing
2. Fruit a group of follicles, or baccate
3. Flowers actinomorphic
4. Inflorescence a raceme: fruit baccate **5. *Actaea***
4. Inflorescence a panicle, or cymose, or flowers solitary: fruit a group of follicles
5. Perianth biseriate, the segments of the inner series nectariferous; leaves ± compound
6. Annuals; leaves 2-3 x pinnatisect with elongated segments **3. *Nigella***
6. Perennials; leaves ternately or palmately compound, digitate or pedate, segment rarely elongated
7. Perianth segments of the inner whorl spurred; leaves ternately compound

16. ***Aquilegia***

7. Perianth segments of the inner whorl not spurred; leaves not ternately compound

8. Stem leaves forming a whorled involucre subtending the flower **2.** ***Eranthis***

8. Stem leaves absent, or not forming a whorled involucre subtending the flower

9. Follicles completely free; outer perianth segments yellow, decidious **4.** ***Trollius***

9. Follicles united, at least at the base; outer perianth segments greenish, tipped or sometimes fading purple, persistent **1.** ***Helleborus***

5. Perianth uniseriate; nectar secreted by the carpels; leaves simple, cordate **6.** ***Caltha***

3. Flowers zygomorphic

10. Follicles 3-5; inner perianth segments free

11. Upper outer perianth segment hooded; 2 upper, inner perianth segments crozier-shaped, standing beneath the hood **7.** ***Aconitum***

11. Upper outer perianth segment spurred; inner perianth segments laminate, the 2 upper spurred and contained within the sepal spur **8.** ***Delphinium***

10. Follicle solitary; inner perianth segments united, with a single spur **9.** ***Consolida***

2. Fruit a group of achenes

12. Perianth uniseriate, all the segments similar

13. Leaves palmately or pinnately divided; stem leaves in a single, often involucrate whorl **10.** ***Anemone***

13. Leaves ternately divided; stem leaves never ivolucrate or in a single whorl **17.** ***Thalictrum***

12. Perianth biseriate, the segments in 2 dissimilar whorls

14. Leaves all radical

15. Leaves simple; outer perianth segments minutely spurred, deciduous **15.** ***Myosurus***

15. Leaves palmatisect; outer perianth segments not spurred, persistent **14.** ***Ceratocephalus***

14. Cauline leaves present

16. Perianth segments of inner whorl usually more than 5, without a nectary pit; leaves pinnatisectly divided or decompound **12. *Adonis***

16. Peiranth segments of the inner whorl often 5, with a nectary pit near the base often protected by a scale; leaves often palmately divided **13. *Ranunculus*** (17)

2.1.1.2. *Clematis* species

The genus *Clematis* is extensive genus within Dicotyledons and has about 300 species worldwide (16).

Woody climbers, climbing by means of the petioles. Leaves opposite, simple or compound. Flowers solitary or in fascicles or panicles, actinomorphic. Perianth uniseriate, the segments (tepals) petaloid, 4, aestivation induplicate. Stamens and carpels numerous. Achenes with long persistent styles, frequently plumose (18).

5 species are grown in Turkey. The identification key for discrimination of these species is as follows.

1. Flowers subtended by 2 large connate bracteoles borne just beneath the flower; leaves usually simple **5. *cirrhosa***

1. Bracteoles absent; leaves compound

2. Flowers borne singly, purple; perianth segments 2·5-4·5 cm **4. *viticella***

2. Flowers borne in panicles or dichasia, White or yellowish; perianth segments shorter

3. Filaments pilose; perianth segments acute **3. *orientalis***

3. Filaments glabrous; perianth segments obtuse

4. Anthers much shorter than the filaments; internodes hairly **1. *vitalba***

4. Anthers often as long as the filaments; internodes glabrous **2. *flammula*** (18)

2.1.1.3. *Clematis viticella* L.

Stems weakly striate, shortly pilose. Leaves pinnate with ternate segments. Ultimate segments usually with a strongly marked reticulate venation, often deeply trifid, adpressed pilose beneath. Flowers borne singly, terminal on long peduncles. Perianth segments dark purple, 22 – 38 mm, sericeous on the outside. Anthers about equalling the glabrous filaments. Styles not plumose. Achenes silky – pilose when young, later glabrescent (18).

Flowering season: 6th - 7th months

Habitat: Scrub, etc.

Altitude: Sea level - 900 m.

Distribution in Turkey: Regions of Marmara, Aegean, Black Sea and Anatolia Interior.

Worldwide distribution: Romania, Balkans, Georgia, Northwestern Iran, West Syria.

Kingdom: Plantae

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Order: Ranunculales

Family: Ranunculaceae

Genus: *Clematis* sp.

Species: *Clematis viticella* L. (19).

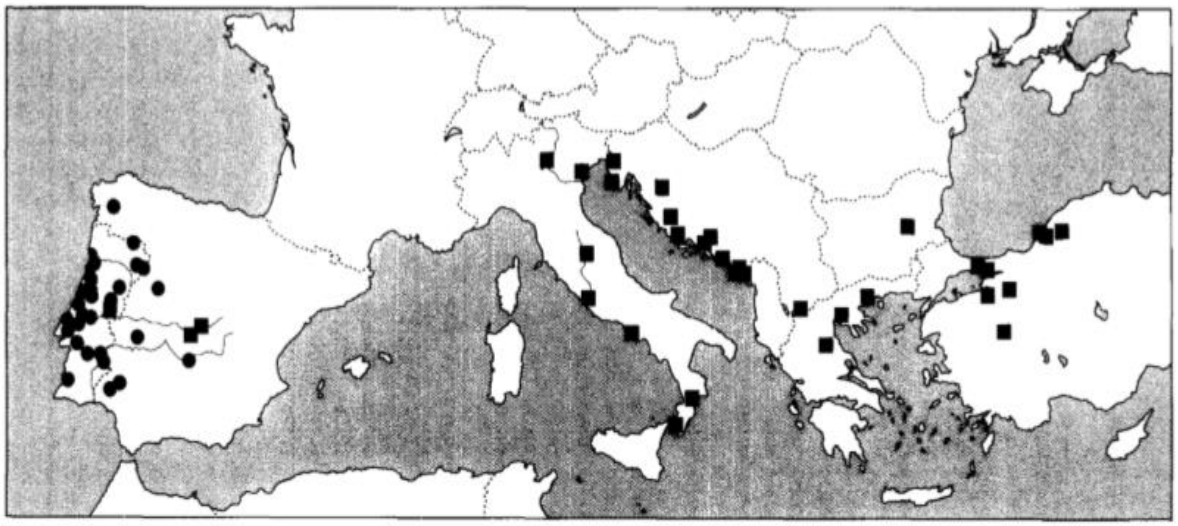


Figure 1: Natural distribution of *Clematis viticella* L. in Europe. (●) *Clematis viticella* subsp. *campaniflora* (■) *Clematis viticella* subsp. *viticella* (18)

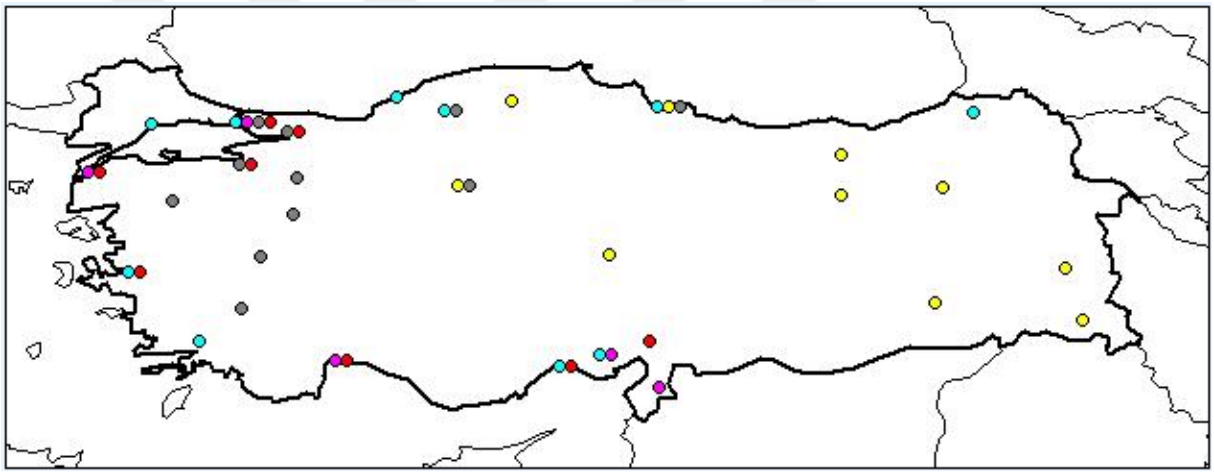


Figure 2: Natural distributions of *Clematis* species in Turkey. O *C. vitalba*, O *C. flammula*, O *C. orientalis*, O *C. viticella* and O *C. cirrhosa* (20)

2.1.1.4. Local names of *Clematis* species

In Turkish: yakmuk, delibağ, karanane, yakıotu, akçabağ, yaban asması, Meryem asması, akasma, filbahr, filbahri, fukaraotu, peçek, manzaotu, acıtevek, köpek tuzağı, muşurbazotu, mısırbazotu (21).

In Other Languages: Blue clematis, Virgin's bower, Traveller's joy, Ladies' bower, Love vine, White vine, Bind-with, Hedge vine, Climbers, Traveller's ivy, Headache vine, Whorl-leaved, Italian leather flower (English), Wei-ling-xian, Mu-Mong-na-bao (Chinese) (22).

2.1.1.5. Traditional Usage of *Clematis* Species

5 *Clematis* species (*Clematis viticella, C. orientalis, C. flammula, C. vitalba* and *C. cirrhosa*) are grown in Turkey. The leaves of *C. vitalba* are used as congested against rheumatism pain. *Clematis viticella* leaves are also used by pulping in knee pain (23).

Clematis cirrhosa and *Clematis flammula* aerial parts are used in the treatment of the edematous joint in North Anatolia. After, the joint is drilled and is discharged by irritating the skin. Thus, it is reported that alleviating joint pain. Fresh leaves of *Plantago major* ssp. *major* plant are hugged for the treatment of the wound (24).

Aerial parts of *Clematis orientalis* are applied onto the legs for 24 hours in empty pericarp of walnut during rheumatism treatment in East Anatolia. It is also recorded that the same plant has also been used to mature the abscess in the inflammation conditions. It is also stated that the application period should generally be kept short time, otherwise; problems may arise due to excessive irritation (21).

Clematis vitalba branches are used for the removal of toothache by smoking them like a cigarette (25).

Approximately 26 of the *Clematis* species have traditionally been used as a preventive and/or therapeutic agent in many diseases, such as nervous diseases, syphilis, gout, malaria, dysentery, rheumatism, asthma, beriberi, since they are extensively distributed in the Northern hemisphere. They are also used as an antidote in insect bites, in eye infections, as analgesics, anti-inflammatory, diuretic, diaphoretic, antitumour, antibacterial and anticancer. Furthermore, they are used in the treatment of chronic dermatological disorders, rheumatic fever, pain, gout, varicosity, gout, eye infections, gonorrheal symptoms, blisters, ulcers and bone illnesses in Europe and East Asia. These uses may be similar or different from those used in our country as mentioned above (22, 26).

It is known that the extract prepared from leaves of *C. glycinoides* (Traveler's joy or Headache vine) is used against rheumatism, headaches and colds by inhalation in Australia. The plants in the same region are used as therapeutics in inflammatory diseases. *C. glycinoides, C. pickeringii* and *C. microphylla* have traditionally been used as therapeutics in inflammatory diseases like headache, pain, rheumatism, infections and common colds in the same region (22, 26).

Clematis species also have important uses in Traditional Chinese Medicine. The aqueous decoctions prepared from the roots of the *Clematis tangutica*, which is known as ‘‘Mu-mong-na-bao’’ and situated abundantly in North and Southwest China, are used against skin diseases and dyspepsia. It is known that extract prepared from roots of the *Clematis chinensis* plant, which is registered in Chinese Pharmacopoeia and known as ‘‘Wei-Ling-Xian’’, is used as analgesic in rheumatism, toothache and traumatic pain, in skin and breast infections, anti-inflammatory, diuretic, antitumour and insecticide and to treat rheumatic arthritis, tonsillitis and laryngitis (22, 26). *Clematis parviloba* is traditionally used in the treatment of various diseases such as rheumatism, arthralgia, emmenagogue, galactopoietic and hydropsy in the Southwest China (26).

The leaves of *Clematis vitalba* are used as antibacterial and antimycotic in Europe and are safely consumed as food in Italy after briefly boiled (26). It is also seen that the same plant is used in migraine and for genital disorders in men. The crushed roots and crusts of the plant are boiled with water for a few minutes, and also its stored form in oil can be used against itch. This condition is thought to be induced by flavonoid which contain in the plant and also called as clematine (22).

In addition to the medical uses of *Clematis* species as described above, it is also known that the species are consumed as tea. However, it is reported that the protoanemonin (β-angelica lactone derivative), which is found in young tissues of these species and is believed to be toxic, should be used after it has been disintegrated by boiling in water (22).

The aerial parts of *Clematis apiifolia* have been utilized as a therapeutic in asthma, beriberi, urinary diseases and travail for centuries. *C. apiifolia*, *C. florida* and *C. heracleifolia* have traditionally been used by indigenous healers as analgesic, diuretic, antitumor and anti-inflammatory agents in Korea and China (26).

In Traditional Chinese Medicine, *Clematis montana* is known as ‘‘Mu tong’’. It is reported in the literature that dermatological diseases are treat by *C. montana* leaves and its seeds are used against constipation in the Himalayas (22). *Clematis montana* (and triterpenoid glycosides) and *Clematis armandii* (flavonoids) aerial parts are also used to reduce fever, increase lactation and menstrual irregularities (26).

Clematis erecta is called Upright Virgin's Bower in Europe and is used as remedy in the treatment of many diseases in homoeopathic medicine. Examples of these diseases include insomnia, severe ulcers, neuralgic and rheumatic headache, rheumatic pains, gout, gonorrhea, cancer, neurosis, varicose veins and memory deficits. Form of infusion or extract of *Clematis montana* leaves is used as diuretic and diaphoretic, antisyphilis, antiulcer and anticancer by utilizing externally or internally (26).

2.1.2. Bioactivity Studies on *Clematis* Species

2.1.2.1. Studies on Analgesic Activity

In a study conducted by Yeşilada and Küpeli in 2007, the analgesic effect of 90% ethanolic extract and its fractions of *Clematis vitalba* aerial parts were investigated by using p-benzoquinone-induced abdominal constriction test in mice. The extracts and fractions were administered orally to mice at different doses (150, 300, 500, and 800 mg/kg) and results were compared with aspirin (100 and 200 mg/kg). The ulcer rate was also assessed. As a result, *Clematis vitalba* showed dose dependent analgesic effect in doses 150 to 500 mg/kg and did not ulcer. However, the activity decreased at 800 mg/kg (15).

Aqueous extract of *Clematis brachiata* leaves (100, 200, 400 mg/kg) was investigated for *in vivo* analgesic effect by using different test models by Mostafa et al. in 2010. When the results were compared with indomethacin (10 mg/kg), *Clematis brachiata* showed significant depending on the dose analgesic effect. It even showed higher activity than the standard at a concentration of 400 mg/kg. The tail immersion model was used to detect acute pain. In this study, analgesic and antinociceptive activity of the plant was displayed by measuring the reaction times of the rats against the pain composed (27).

2.1.2.2. Studies on Anticancer Activity

The study performed by Youn et al. was about anticancer activity of the methanolic extract of aerial parts of *Clematis apiifolia*. Adriamycin (2 μg/mL) was used as a positive control. Hederagenic acid, ursolic acid and oleanolic acid from these plants was first isolated and their cytotoxic effects were compared against different tumour cell pathways (L1210, HL-60, SK-OV-3). It is thought that these 3 compounds are quite effective for the cytotoxic activity of the plant and IC_{50} values ranged from 7.7 to 25.6 μg/mL (26).

In a former study, the anticancer effect of ethanolic extract of *Clematis chinensis* roots was investigated against EAC, S180 A and HepA tumour cells by using *in vitro* microculture method. However, no significant results were obtained (26).

The cytotoxic effect of flavonoids found in the aerial parts of *Clematis aethusifolia*, which was used in Traditional Mongolian Medicine, was studied by Chang et al. in 2016. Apigenin and its derivatives indicated moderate cytotoxicity against a panel of various human solid tumour cell lines (28).

In another study, 4 triterpenoid saponins were isolated from 70% ethanolic extract of *Clematis ganpiniana* aerial parts by bioassay-guided fractionation technique. After that apoptosis rates and IC_{50} values of these compounds and adriamycin (2 μg/mL) used as a control group were evaluated against MCF-7 and MDA-MB-231 tumour cells. α-hederin was shown stronger activity and higher apoptosis rates than other compounds and even adriamycin. As a result, all compounds showed significant anticancer activity depending on the dose and time in human breast cancer cell lines (29).

Triterpene glycosides (montanoside A, montanoside B) were isolated from methanolic extract of *Clematis montana* aerial parts by Jangwan and Dobhal in 2008. They reported that these compounds had *in vitro* cytotoxic activity against HGF and HSC-2 cells at a concentration of 50 or 100 μg/mL. After one year, Peng and colleagues isolated lectin from the methanolic extract of *Clematis montana* stems with NaAc-HAC buffer. Lectin was found to be cytotoxic against L929, MCF-7, HeLa and HepG2 cells via MTT. It has also been shown that carbohydrate binding systems are effective in the antineoplastic mechanism, and when these regions are inhibited, the antitumour activity is abruptly reduced (26, 30).

Methanolic extracts of *Clematis ligustifolia* and *Akebia trifoliate*, used in traditional Chinese and Japan medicine, were prepared and then cytotoxic compounds were isolated from the ethyl acetate fractions against porcine renal cell line LLC-PKI by Pawar et al. Three of these compounds have been found to increase apoptosis in caspase 3/7 assay. This effect is thought to be related to the structural properties of the oleanane skeletons of the compounds and to structure of saccharide linkages (31).

In 2009, α-hederin and clematoside S was isolated from 95% ethanolic extract of *Clematis parviloba* stems, and the effect of these compounds was investigated against different cell lines using *in vitro* MTT assay (32).

2.1.2.3. Studies on Anti-inflammatory Activity

Antiinflammatory activity of extracts, fractions and subfractions of *Clematis vitalba* aerial parts were also investigated with the same study by Yeşilada and Küpeli. Different experimental methods were applied to assess acute, subacute and chronic anti-inflammatory activity of the plant. The ethyl acetate fraction of the plant showed the highest antiinflammatory activity and vitalboside, 4'-*O*-coumaroyl-isovitexin, was isolated as the active compound from this fraction. It did not develop gastric lesions, as well (15).

Mostafa et al. also researched the antiinflammatory activity of aqueous extract of leaves of *Clematis brachiata* using histamine-induced and carrageenan-induced edema rat paw edema. The results were compared with indomethacin (10 mg/kg). The extract importantly decreased dose dependent to the edema paw volumes induced by carrageenan and histamine. The highest concentration of extract showed equal activity, when compared to the control group (27).

Fu and colleagues isolated 7 new triterpene saponins which were derived hederagenin and oleanolic acid and 17 known saponins from 50% ethanolic extract of *Clematis chinensis* roots and rhizomes. Then, *in vitro* antiinflammatory activity of all compounds was investigated against COX-1 and COX-2 enzymes. Although all compounds showed potential antiinflammatory activity, 6 of the newly isolated compounds (Clematochinenoside A, C, D, E, F and G) displayed the highest activity (33).

SKI306X® is a hydro-alcoholic extract of roots prepared as a combination of *Prunella vulgaris* stems and flower, *Clematis mandshurica*, and *Trichosanthes kirilowii* roots mixed in mass ratio 1:2:1 and is used to treat osteoarthritis and alleviate joint pain. The antiinflammatory effect of this preparation was investigated against human peripheral blood mononuclear cells (PBMC) and bovine cartilage explants were stimulated with lipopolysaccharide (LPS) and IL-1β. Results demonstrated that SKI306X® has antiinflammatory and cartilage protective activity (34).

2.1.2.4. Studies on Arthritis, Osteoarthritis and Adjuvant Arthritis

The antiinflammatory activity of ethanolic extracts and fractions of three *Clematis* species (*C. glycinoides, C. microphylla* and *C. pickeringii*) was investigated using the different methods in HepG2 cells. While these three extracts showed antiinflammatory effect at different concentrations, *Clematis pickeringii* stems showed the highest activity in all experimental pathways (35).

In two different studies in 2006, it was observed that both aqueous extract and ethanolic extract of *Clematis mandshurica* roots have dose-dependent antiinflammatory effect by determinate of tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), and measure of nitric oxide (NO) and prostaglandin-E2 (PGE2) content (36, 37).

The efficacy of the saponin-rich fraction of 70% ethanolic extract of *Clematis chinensis* roots in the treatment of osteoarthritis was studied both *in vitro* and *in vivo*. Monosodium iodoacetate (MIA) was intraarticularly injected into knee joints of rats and they were provided to have osteoarthritis. The extract and diclofenac were then administered orally once daily for 28 days. Monosodium iodoacetate (MIA)- and sodium nitroprusside (SNP)-stimulated rabbit chondrocytes were examined *in vitro* using MTT studies to detect chondrocyte injury. *Clematis chinensis* extract significantly improved dose dependent (50, 100 or 200 mg/kg) osteoarthritis. It was discovered that this was accomplished by inhibiting extracellular matrix degradation and chondrocyte damage. Diclofenac (4 mg/kg) was only slightly effective (38). In another study, the antiarthritic effect of different solvent-extracted *Clematis chinensis* roots was still evaluated *in vivo* and *in vitro*. Acetone extract, having the highest saponin content, showed the highest antiinflammatory and antiarthritic activity (39).

5 distintc compounds including anemonin were isolated from different fractions of methanolic extract of *Clematis crassifolia* aerial parts and the antiinflammatory effect of these compounds was investigated by Lee et al. in 2008. Anemonin showed the highest activity. In addition, the anemonin showed selective iNOS inhibitor effect in Male Sprague Dawley rats, leading to the notion it had significantly antiinflammatory and antiarthritic activity (40).

It was observed that SKI306X® significantly inhibited staurosporin-induced apoptosis in rat chondrocyte cell line RCJ3.1C.18 cells by Lee et al. However, it was emphasized that only *Clematis manshurica* which from the plants found in this

preparation is responsible for this effect. *Clematis mandshurica* showed activity by destroying the effects of staurosporine, which is a negative control, in different pathways. Thus, this study suggested that SKI306X®, especially *Clematis mandshurica*, might maintain osteoarthritis by preventing chondrocytes from apoptosis (41).

2.1.2.5. Studies on Antimicrobial Activity

The antimicrobial activity of the aqueous extract of fresh fruits of *Clematis apiifolia* was investigated using *in vitro* disc diffusion method against various bacterial (*Staphylococcus aureus, Escherichia coli, Enterobacter aerogenes, Lactobacillus plantanum* and *Pediococcus pentosaceus*) and fungal (*Saccharomyces cerevisiae, Candida utilis, Candida albicans* and *Zygosaccharomyces rouxii*) strains. Garlic was used as a positive control. It showed more potent antimicrobial activity compared with garlic with very low MIC values against fungi and bacteria except lactic acid bacteria. It is believed that this effective compound is protoanemonin. It was also understood that the dried fruit of the plant did not have antimicrobial activity (42). Previous studies have also found that the aqueous extract of fresh aerial parts of *Clematis dioscoreifolia* is antimicrobial against various bacteria and fungi (43).

Antimicrobial activity of the 70% ethanolic extract of *Clematis ganpiniana* aerial parts examined under the heading anticancer studies was also investigated. Among the four triterpenoid saponins isolated from this plant, only the α-hederin showed a weak activity against almost all tested bacteria, while the other three compounds showed nearly no activity. Thus α-hederin was thought to be a potential wide-spectrum antibiotic (29).

In another study, antimicrobial activity of volatile components of *Clematis hirsuta* whole plant was investigated against various bacterial and fungal strains. Dose-dependent effect was observed in a range from 0.5 to 20 μg/mL (26).

The effect of methanolic extract of stems and leaves of *Nauclea obversifolia* and *Clematis papuasica* leaves and stem, together with barks of leaves, stem and root barks was explored against various bacterial (*Bacillus, Lactobacillus, Micrococcus, Staphylococcus, Streptococcus, Agrobacterium, Citrobacter, Enterobacter, Escherichia, Klebsiella, Neisseria, Proteus, Pseudomonas, Salmonella* and *Serratia*) fungal (*Aspergillus, Candida* and *Trichophyton*) and protozoan (*Trichomonas*) strains. All of *Clematis papuasica* and *Nauclea obversifolia* extracts showed broad antibacterial

activity, while ethyl acetate, ether and dichloromethane fractions showed higher activity respectively. However, none of the extracts showed antifungal activity (44).

Two compounds (β-magnoflorine and α-magnoflorine) were isolated from ethanolic extract of *Clematis parviloba* aerial parts. The two compounds showed antifungal activity against *Penicillium avellaneum*, and β-magnoflorine also effected against *P. oryzae*. Amphotericin B (0.04 µg/disc) was used as positive control (45).

Buzzini and Pieroni tested antimycotic effect of 70% EtOH extract of *Clematis vitalba* young shoots and its ether, ethyl acetate and methanolic fractions on series of strain. Agar diffusion well bioassay (ADWB) method was used. The crude extract of *Clematis vitalba* showed antimycotic activity against almost all fungal strains. The crude extract of *Clematis vitalba* showed to be potential a wide spectrum antimycotic by showed activity against almost all fungal strains. Methanolic fraction presented little effect while ethyl acetate and ether fractions did not (46).

Ethanolic extract of *Clematis tangutica* aerial parts used in Tibetan herbal medicine was found to be antifungal activity against some fungal strains. Compounds which are responsible for this effect were shown to be triterpene saponin derivatives (47).

2.1.2.6. Studies on Antioxidant Activity

Zemaphyte®, consisting of ten different plant mixtures in which *Clematis armandii* is also found, has a common use as tea in atopic eczema treatment in Traditional Chinese Medicine. Antioxidant activity of this tea mixture, of each plant and of placebo was investigated by NBT (superoxide scavenging assay) and DPPH (diphenyl picrylhydrazyl) assays. When compared with placebo, the highest antioxidant activity exhibited by the preparation. *Clematis armandii* also showed antioxidant activity close to the tea mixture (48).

Fraxinus angustifolia barks, *Pistacia lentiscus* leaves and *Clematis flammula* leaves, which has a widespread use in traditional Algerian Medicine for inflammation diseases, were evaluated in terms of antioxidant activity and phenolic components. Antioxidant activity was calculated by measuring inhibition of linoleic acid peroxidation and scavenging capacity on DPPH radical and H_2O_2 using *in vitro* spectrophotometric methods. *Clematis flammula* showed substantial inhibition of lipid

peroxidation despite being in the third place in DPPH and H_2O_2 scavenging activity. It has also been underlined that phenolic compounds are associated with antioxidant activity (49).

Two known and a new compounds were isolated from ethanolic extract of *Clematis rehderiana* aerial parts. Antioxidant activity was performed by using MTT and DPPH assays. The newly discovered isovitexin 6″-*O*-*E*-*p*-coumarate showed dose dependent powerful activities against H_2O_2-induced impairment in PC12 cells, whereas isoorientin showed mighty DPPH scavenging activity (50).

In a different study, antioxidant activity of methanolic and aqueous extracts of *Clematis trichotoma* roots was investigated by using DPPH radical scavenger method, liposome peroxidation, linoleic acid emulsion and mitochondrial lipid peroxidation assays in 2005. Among the isolated compounds, luteolin and quercetin showed significant antioxidant activity in all the model systems (51).

2.1.2.7. Studies on Antipyretic Activity

Antipyretic activity of ethanolic extract, fractions and subfractions of *Clematis vitalba* aerial parts were also investigated by Yeşilada and Küpeli. In the chronic inflammation model generated by FCA (Freund's Complete Adjuvant), temperature exchange between the edema and non-edema feets of rats was observed. It was found that the 4'-*O*-coumaroyl-isovitexin which was known to have pronounced antiinflammatory effect had strong dose-dependent antipyretic activity, as well (15).

Antipyretic activity of the aqueous extract of the *Clematis brachiata* leaves was also measured using Brewer's yeast-induced hyperthermia model by Mostafa et al. It was shown that the extract (400 mg/kg) had a much higher antipyretic effect compared with the indomethacin (10 mg/kg), used as a positive control. The antipyretic effect of *Clematis brachiata* was thought to be associated with flavonoids (27).

2.1.2.8. Studies on Antiviral Activity

Lectin was isolated and purified from stem of *Clematis montana* plant by Peng et al. in 2009. Later, the antiviral activity of *Clematis montana* lectin (CML) was investigated against several viruses. No significant results were obtained when compared with control groups against *Influenzae H1N1*, *Influenzae H3N2* and *Influenza*

B viruses. However, it showed higher antiviral activity than control groups against *Influenza* virus and other different viruses (30).

2.1.2.9. Studies on Diuretic Activity

Alvarez et al. investigated diuretic activity of infusions prepared from *Clematis montevidensis* roots and aerial parts. It showed moderate diuretic activity compared with furosemide (10 mg/kg), used as the control group. The compound associated with this effect is thought to be oleanolic acid (52).

2.1.2.10. Studies on Hepatoprotective Activity

In a study conducted by Saleh et al., ethanolic extract of *Clematis hirsuta* aerial parts and some other extracts were compared liver protective effects with silymarin (10 mg/kg). Although propolis (250 and 500 mg/kg) against CCl_4, a hepatotoxic agent, showed the best activity, *Cucumis prophetarum* and *Aloe vera* showed very little protective effect. Hepatoprotective activity was not observed in *Clematis hirsuta* (53). However, methanolic extract of *Clematis chinensis* aerial parts was shown to be hepatoprotective using the same methods in a previous study (54).

2.1.2.11. Studies on Hypotensive Activity

In a study of Ho et al., *in vitro* and *in vivo* hypotensive activity of *Clematis chinensis* roots and *Desmodium styracifolium* whole plants was investigated in 1989. Both plants showed significant hypotensive effects with different mechanisms (55).

2.1.2.12. Studies on Antiobesity Activity

Marrelli et al. investigated correlation between α-amylase and pancreatic lipase enzymes and total phenolic composition of various plants used by folk including *Clematis pycnocephalus* and *Clematis vitalba*. The strongest enzyme inhibition was observed *in Clematis vitalba* in two different mechanisms. This effect is thought to be derived from chlorogenic acid, (±)-catechin and caffeic acid in the plant (56).

2.1.2.13. Studies on HIV-1 Protease Inhibitor Activity

In previous studies, *Clematis* species containing oleanolic acid derivatives were shown to be important HIV (Human Immunodeficiency Virus) protease inhibitors (57, 58). In another study, methanolic extracts of 49 Korean plants, including *Clematis heracleifolia* were assessed for inhibitor activities against HIV-1 protease, HIV-1

reverse transcriptase and anti-HIV-1 activities by Min et al. The inhibitor effect was also supported by this study (59).

2.1.2.14. Studies on Antiangiogenic Activity

In 2009, Different compounds (flavonoid, coumarin, lignan) were acquired from obtained ethyl acetate fraction from 70% aqueous acetone extract of *C. delavayi* var. *spinescens* aerial parts by Li et al. These compounds were then tested for antiangiogenic activity by *in vitro* Zebrafish method. No significant activity has been observed (IC_{50} > 45 μg/ml) (60).

2.1.2.15. Studies on Antigonorrhoeal Activity

The antigonorrhoeal activity of 46 plants, widely used in Guatemala, was investigated against *Neisseria gonorrhoeae* by *in vitro* disk diffusion method. *Clematis dioica* was found to be among the ten plants with high activity (61).

2.1.3. Side Effects and Intoxications in *Clematis* species

No cases related to the use of *Clematis* species are found in the literature. However, excessive use of the plant may cause side effects and intoxications since the fresh plant contains the rich protoanemonin. It is reported that using the plant at high doses cause nosebleeds, skin and mucous membrane irritations, difficulty in breathing, colitis, diarrhea and nephritis. In the case of poisoning, it is stated that gastric lavage should be done using activated charcoal for treatment in the literature (22).

2.1.4. Compounds Isolated from *Clematis* Species

2.1.4.1. Saponin Compounds

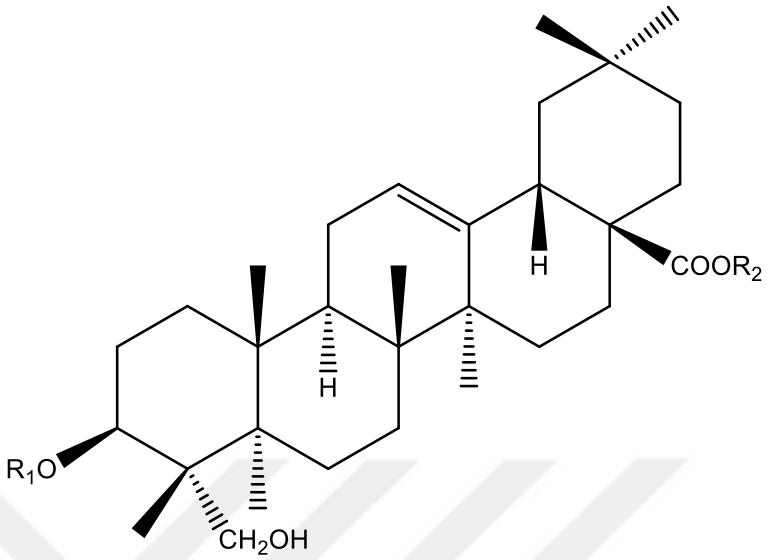


Table 1: Saponins isolated from *Clematis* species

Compound	R_1 R_2	Species	Ref.
Hederagenic acid	R_1=H R_2=H	*apiifolia*	(62)
Clematichinenoside B	R_1=Ara (2→1) Rha- (3→1) Rib- (4→1) Glc R_2=Glc (6→1) Glc (4→1) Rha	*chinensis*	(63)
Prosaponin CP_6	R_1=Rib (1→3) Rha (1→2) Ara R_2=H	*chinensis* *tibetana*	(63, 64) (65)
α-hederin	R_1=Ara (2→1) Rha R_2=H	*ganpiniana* *parviloba*	(29) (32)
Orientaloside F	R_1=Rha (1→2) Ara, R_2=H	*orientalis*	(66)
Vitalboside B	R_1=Glc R_2=H	*tangutica*	(59)
Dipsacoside B	R_1=Rha (1→2) Ara R_2=Glc (1→6) Glc	*tibetana*	(65)
Clematibetoside C	R_1=Rib R_2=Rha (1→4) Glc (1→6) Glc	*tibetana*	(65)

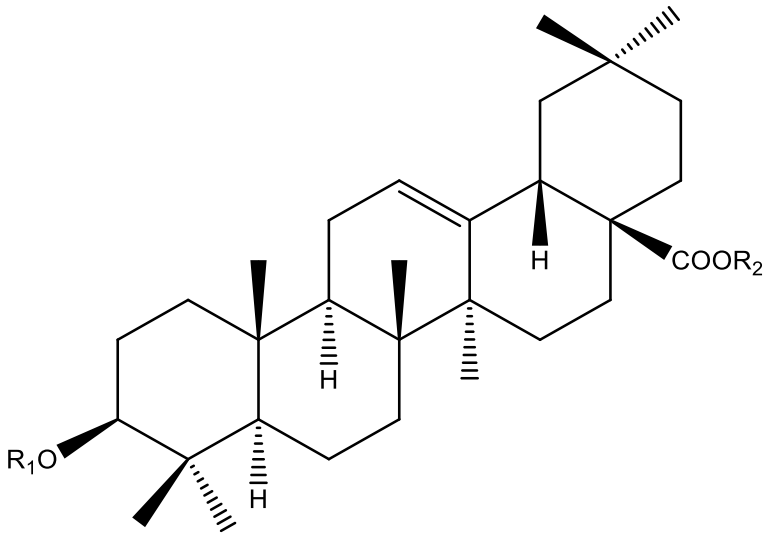


Table 2: Saponins isolated from *Clematis* species

Compound	R_1 R_2	Species	Ref.
Oleanolic acid	R_1=H R_2=H	*apiifolia* *chinensis*	(62) (63)
Huzhongoside B	R_1=Ara (2→1) Rha- (3→1) Rib R_2=Glc (6→1) Glc (4→1) Rha	*chinensis* *ternifolia*	(63) (58)
Clematomandshurica saponin D	R_1=Rha (1→6) Glc (1→4) Glc (1→4) Rha (1→2) Ara R_2=Rha (1→4) Glc (1→6) Glc	*mandshurica*	(63)
Clemontanoside F	R_1=Glu R_2=Gal (1→6) Rha (1→2) Glc	*montana*	(67)
Sieboldianoside B	R_1=Xyl (1→3) Rha (1→2) Ara R_2=Glc (1→6) Glc (1→4) Rha	*stans*	(57)

2.1.4.2. Alkaloid Compounds

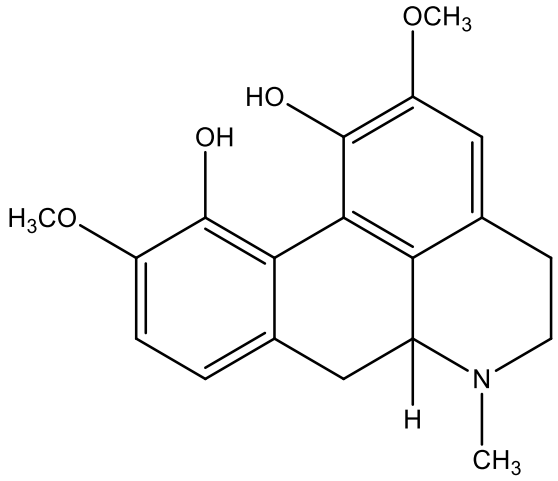


Table 3: Alkaloids isolated from *Clematis* species

Compound	Species	Ref.
Corytuberine	*recta*	(68)

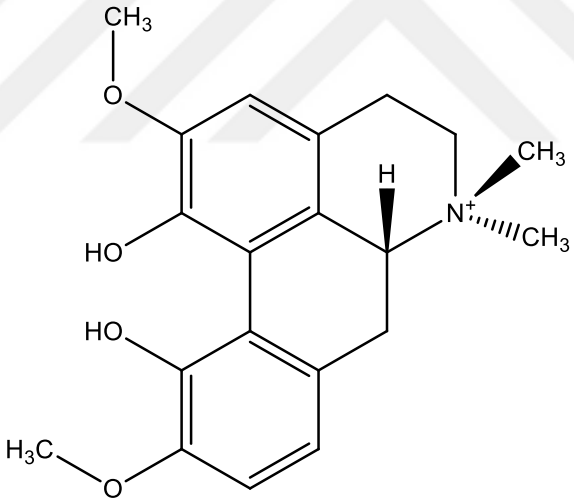


Table 4: Alkaloids isolated from *Clematis* species

Compound	Species	Ref.
β-magnoflorine	*recta*	(68)
	parviloba	(45)

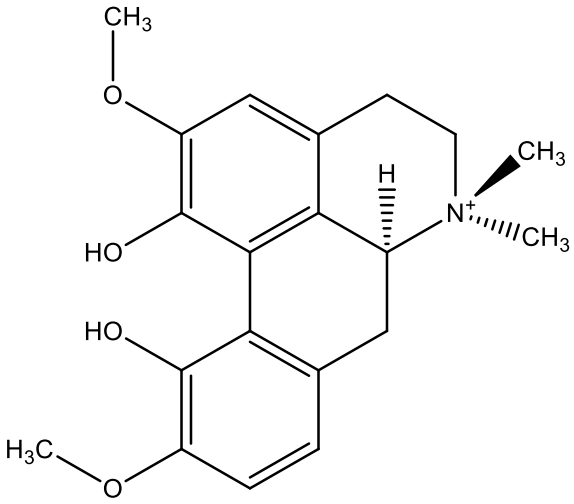


Table 5: Alkaloids isolated from *Clematis* species

Compound	Species	Ref.
α-magnoflorine	*manshurica*	(69)
	parviloba	(45)
	recta	(68)

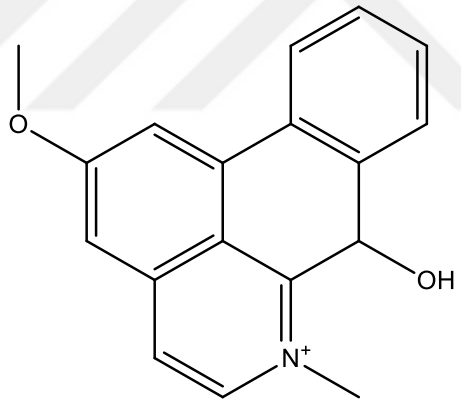


Table 6: Alkaloids isolated from *Clematis* species

Compound	Species	Ref.
Clemaine	*purpurea*	(26)

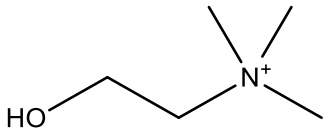


Table 7: Alkaloids isolated from *Clematis* species

Compound	Species	Ref.
Choline	*purpurea*	(26)

2.1.4.3. Simple Phenolic Compounds

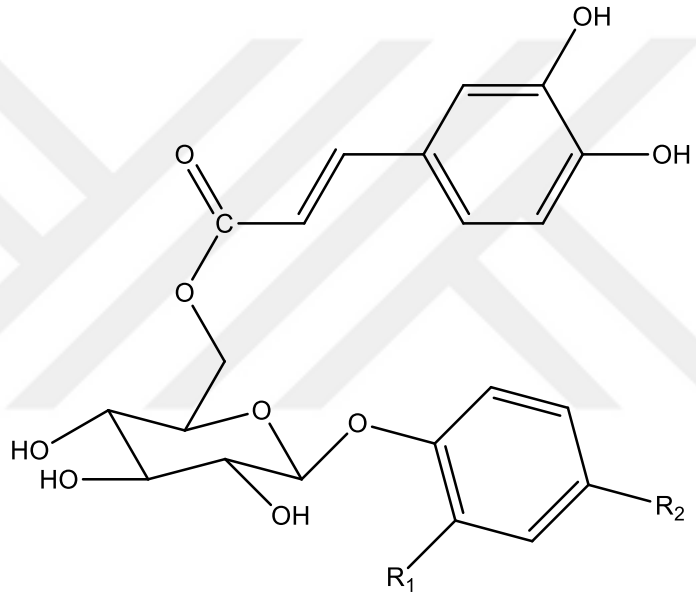


Table 8: Simple phenolic compounds isolated from *Clematis* species

Compound	R_1	R_2	Species	Ref.
Ibotanolide B	OH	CH_2CH_2OH	*crassifolia*	(40)
Calceolarioside B	H	OH	*crassifolia*	(40)

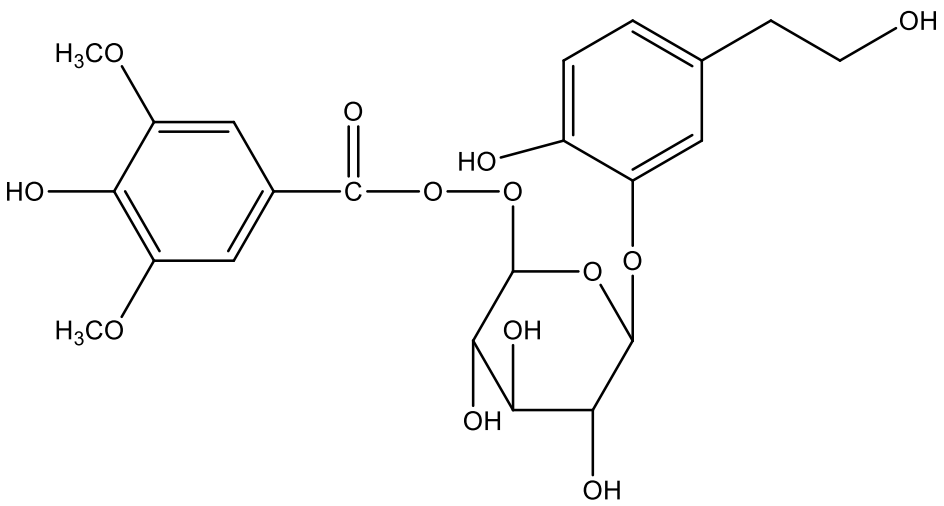


Table 9: Simple phenolic compounds isolated from *Clematis* species

Compound	Species	Ref.
Clemomandshuricoside A	*mandshurica*	(70)

2.1.4.4. Flavonoid Compounds

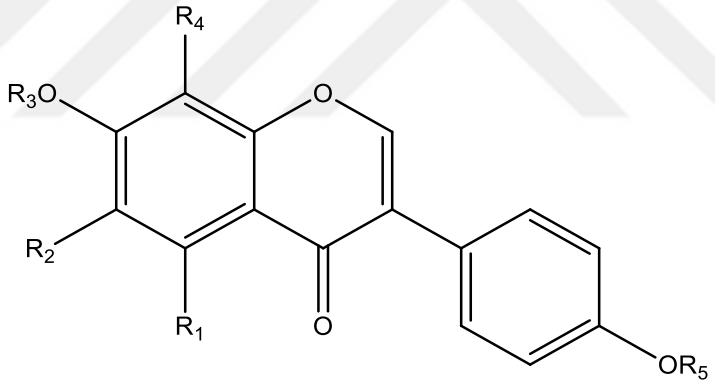


Table 10: Flavonoids isolated from *Clematis* species

Compound	R_1	R_2	R_3	R_4	R_5	Species	Ref.
Daidzein	H	H	H	H	H	*hexepetala*	(71)
Formononetin	H	H	H	H	Me	*hexepetala*	(71)
Genistein	OH	H	H	H	H	*hexepetala*	(71)
6-hydroxybiochain A	OH	OH	H	H	Me	*hexepetala*	(71)

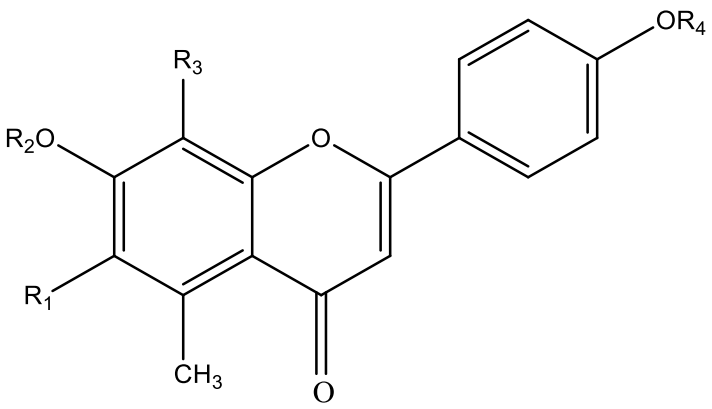


Table 11: Flavonoids and their glycosides isolated from *Clematis* species

Compound	R_1	R_2	R_3	R_4	Species	Ref.
Vitalboside	Glu	H	H	CM	*vitalba*	(15)
					viornae	(72)
Apigenin	H	H	H	H	*trichotoma*	(51)
					terniflora	(26)
Linarin	H	Rha (1→6)-Glu	H	Me	*hexepetala*	(71)
Isovitexine	Glu	H	H	H	*viornae*	(72)
Terniflorin	H	CM (1→6) -Glu	H	H	*terniflora*	(73)

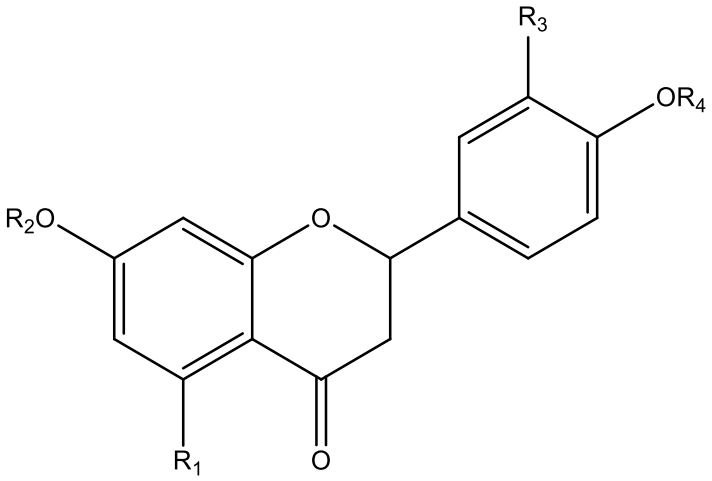


Table 12: Flavonoids and their glycosides isolated from *Clematis* species

Compound	R_1	R_2	R_3	R_4	Species	Ref.
Hesperetin	OH	H	OH	Me	*hexepetala*	(71)
Liquiritigenin	H	H	H	H	*hexepetala*	(71)
Naringenin	OH	H	H	H	*hexepetala*	(71)
Clematine	OH	Rha (1→6) -Glu	OMe	H	*armandii*	(74)

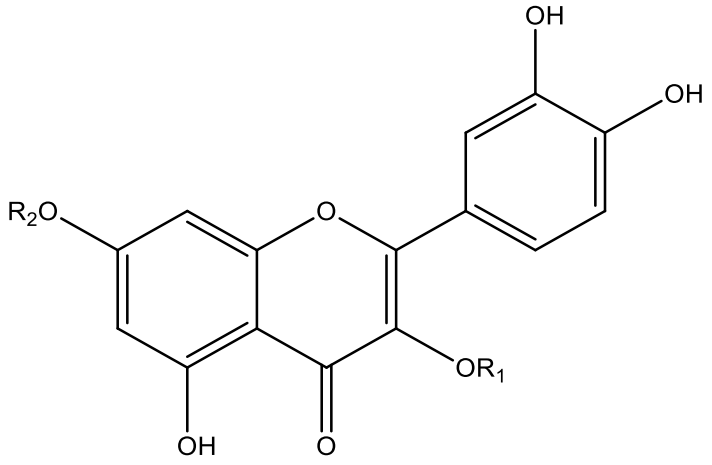


Table 13: Flavonoids and their glycosides isolated from *Clematis* species

Compound	R_1	R_2	Species	Ref.
Quercetin	H	H	*intricata* *purpurea* *trichotoma*	(75) (26) (51)
Isoquercetin	Glu	H	*stans*	(57)

Rutin	Rha (1→6) -Glu	H	*stans*	(57)
Hirsutrin	Glu	H	*purpurea*	(26)
Hyperin	Gal	H	*trichotoma*	(51)

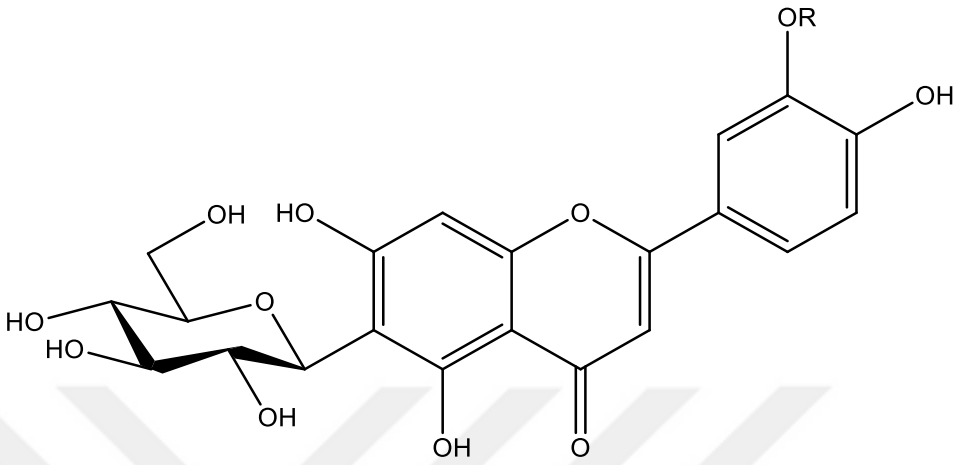


Table 14: Flavonoid glycosides isolated from *Clematis* species

Compound	R	Species	Ref.
Isoorientin	H	*rehderiana*	(50)
		viticella	(76)
Isoorientin 3′-*O*-methyl ether	CH_3	*viticella*	(76)

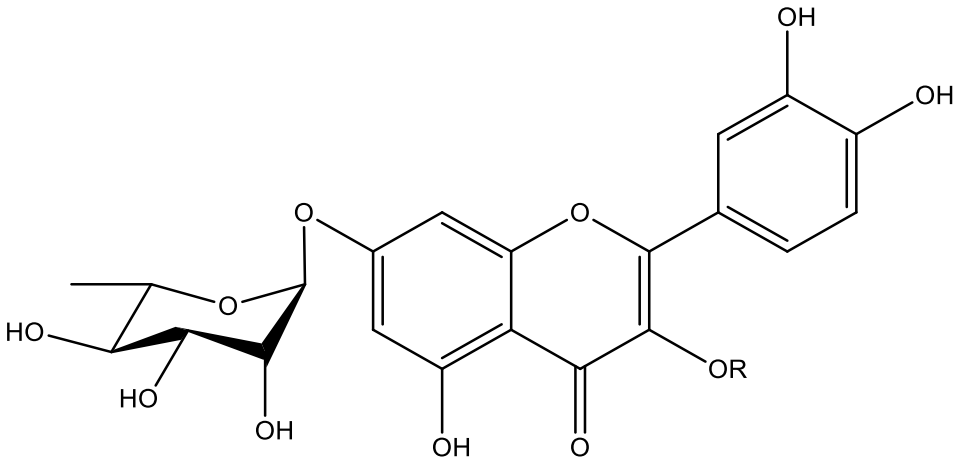


Table 15: Flavonoid glycosides isolated from *Clematis* species

Compound	R	Species	Ref.
Quercetin 7-*O*-α-L-rhamnopyranoside	H	*viticella*	(76)
Quercetin 3,7-di-*O*-α-L-rhamnopyranoside	α-L-Rha	*viticella*	(76)

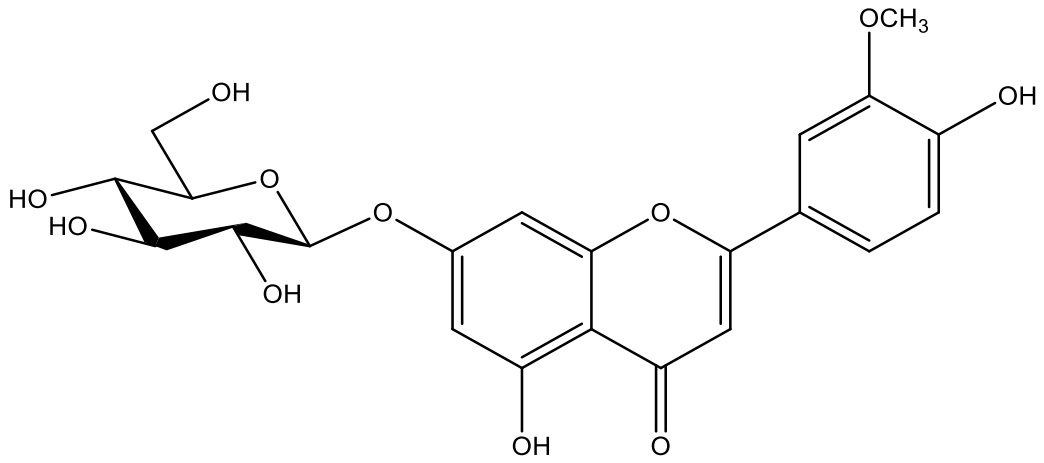


Table 16: Flavonoid glycosides isolated from *Clematis* species

Compound	Species	Ref.
Chrysoeriol 7-*O*-β-Dglucopyranoside	*viticella*	(76)

2.1.4.5. Coumarin Compounds

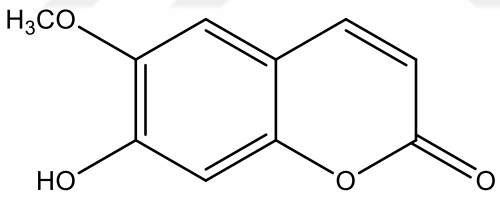


Table 17: Coumarins isolated from *Clematis* species

Compound	Species	Ref.
Scopoletin	*intricata*	(75)

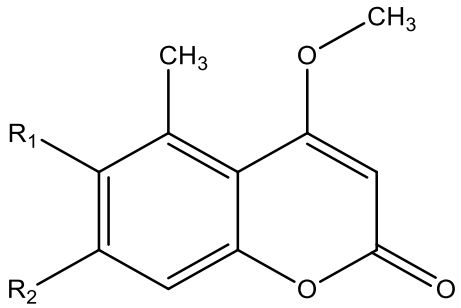


Table 18: Coumarins isolated from *Clematis* species

Compound	R_1	R_2	Species	Ref.
4,7-dimethoxy-5-methyl-coumarin	H	OCH_3	*delavayi* var. *spinescens*	(60)
4,6,7-trimethoxy-5-methyl-coumarin	OCH_3	OCH_3	*delavayi* var. *spinescens*	(60)
7-hydroxy-4,6-dimethoxy-5-methyl coumarin	OCH_3	OH	*delavayi* var. *spinescens*	(60)

2.1.4.6. Lignan Compounds

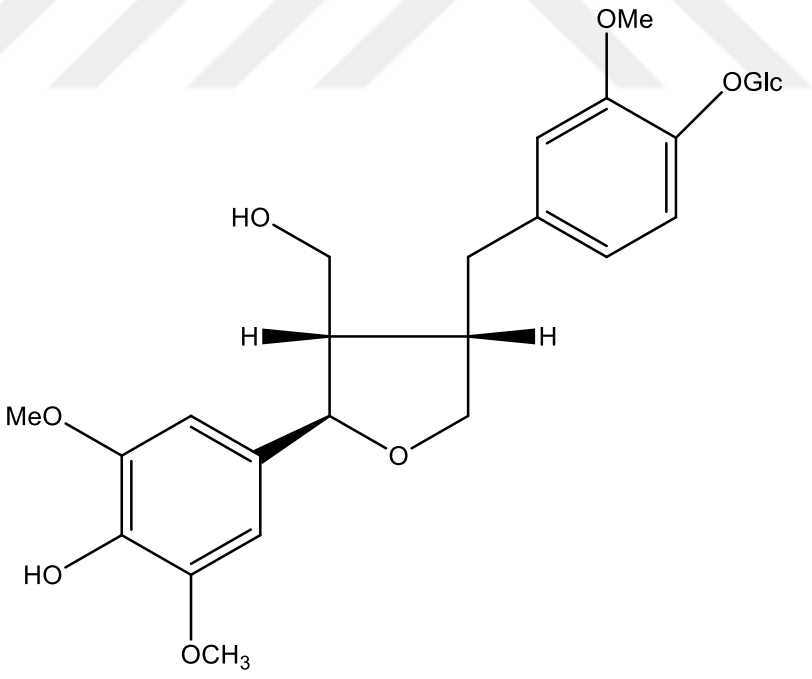


Table 19: Lignans isolated from *Clematis* species

Compound	Species	Ref.
Armandiside	*armandii*	(26)

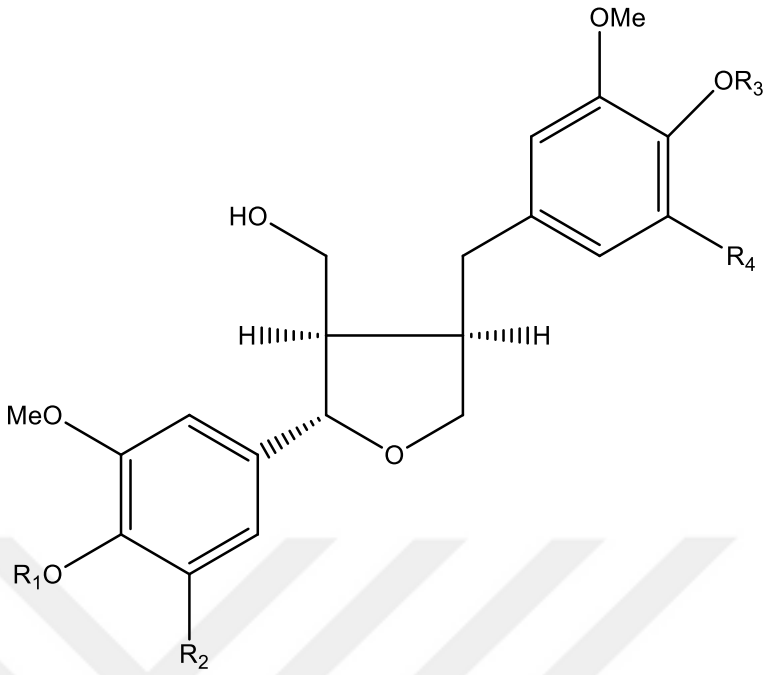


Table 20: Lignans isolated from *Clematis* species

Compound	R_1	R_2	R_3	R_4	Species	Ref.
Salvadoraside	Glc	OMe	Glc	OMe	*armandii*	(26)
Clemastanin	Glc	H	Glc	H	*stans*	(57)

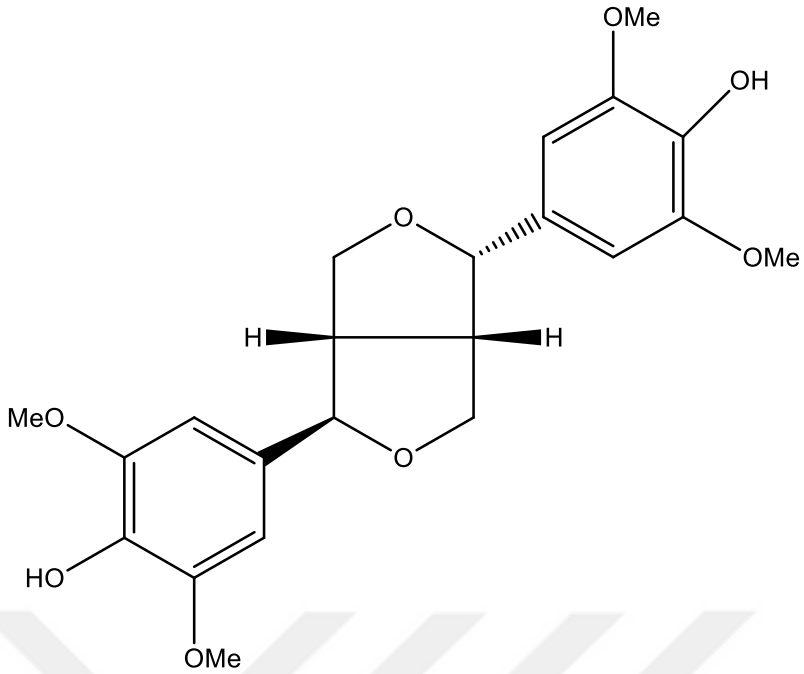


Table 21: Lignans isolated from *Clematis* species

Compound	Species	Ref.
(-)-episyringaresinol	*parviloba*	(77)

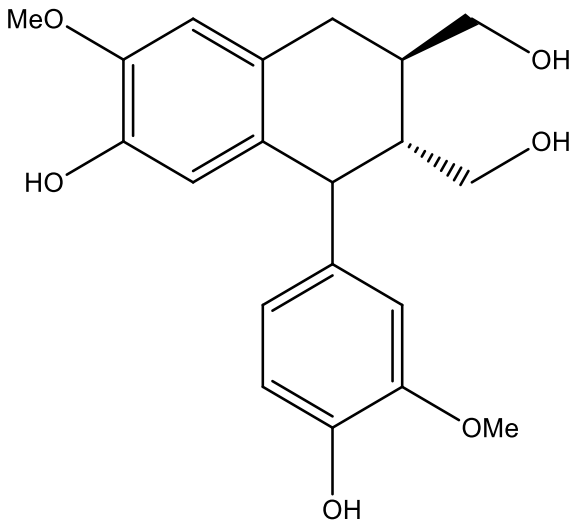


Table 22: Lignans isolated from *Clematis* species

Compound	Species	Ref.
Isolariciresinol	*hexapetala*	(71)
	stans	(57)

2.1.4.7. Macrocyclic Compounds

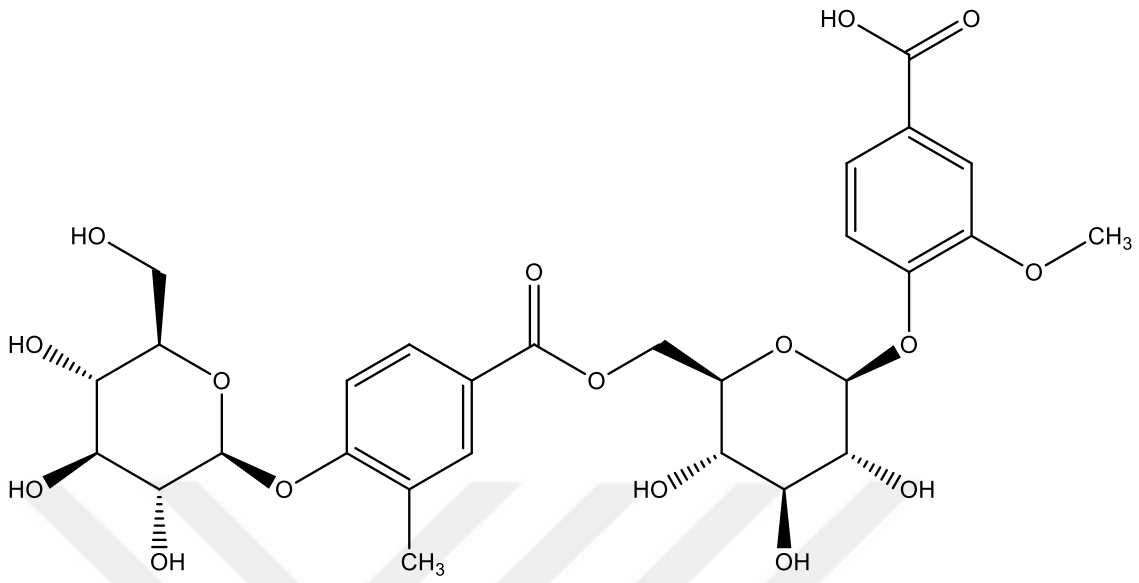


Table 23: Macrocyclic compounds isolated from *Clematis* species

Compound	Species	Ref.
Bercholine	*armandii*	(78)

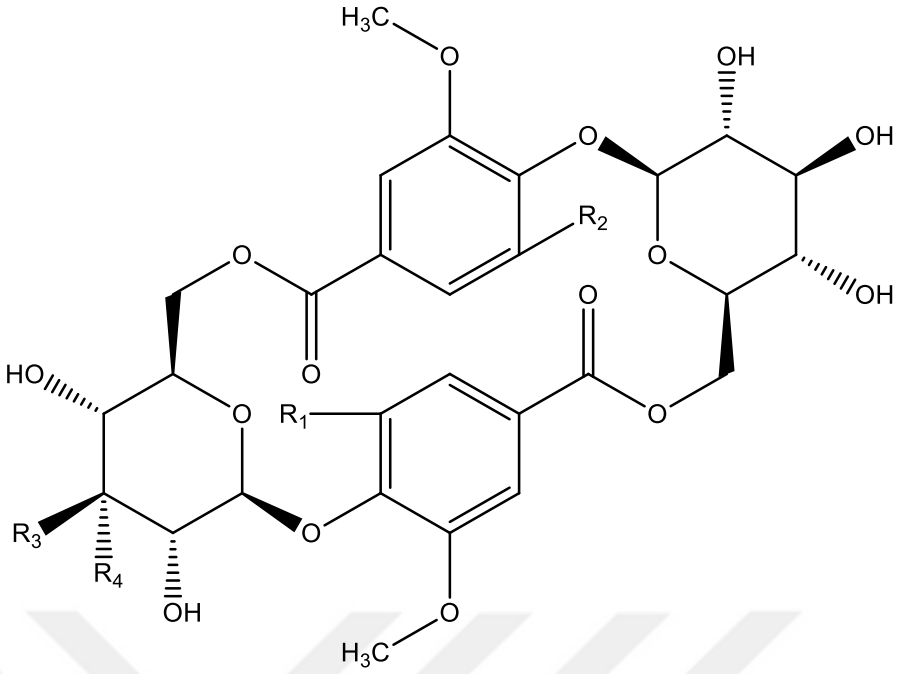


Table 24: Macrocyclic compounds isolated from *Clematis* species

Compound	R_1	R_2	R_3	R_4	Species	Ref.
Clemahexapetoside A	OCH_3	H	H	OH	*hexapetala*	(79)
Clemahexapetoside B	H	H	H	OH	*hexapetala*	(79)
Clemochinenoside A	OCH_3	OCH_3	OH	H	*hexapetala* *mandshurica*	(79, 80) (80)
Clemochinenoside B	OCH_3	H	OH	H	*armandii* *hexapetala* *mandshurica*	(78) (80) (80)

2.1.4.8. Steroid Compounds

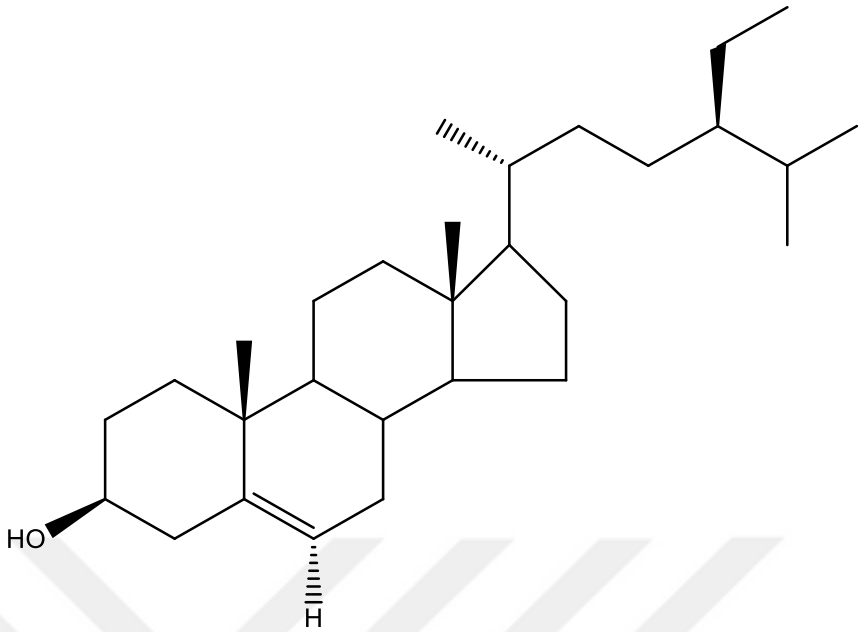


Table 25: Steroids isolated from *Clematis* species

Compound	Species	Ref.
Stigmasterol	*apiifolia*	(26)

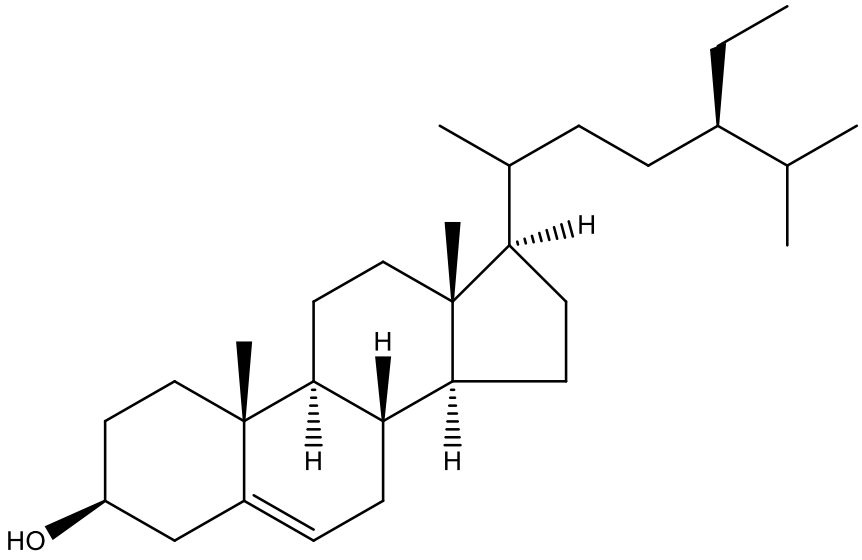


Table 26: Steroids isolated from *Clematis* species

Compound	Species	Ref.
β-sitosterol	*apiifolia*	(26)
	intricata	(26)
	ligusticifolia	(81)

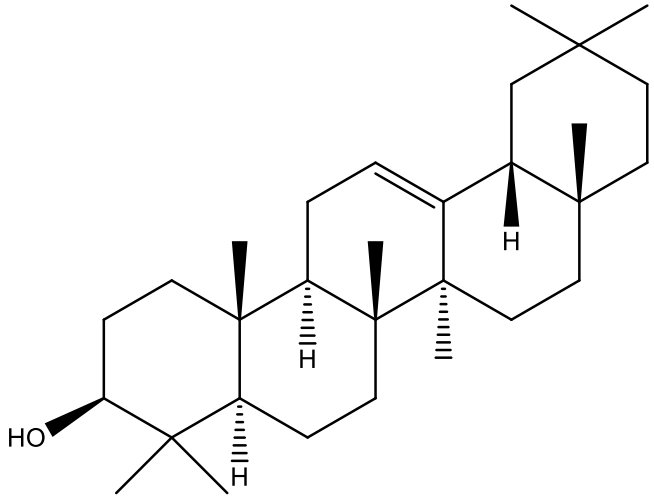


Table 27: Steroids isolated from *Clematis* species

Compound	Species	Ref.
β-amyrin	*montana*	(26)
	purpurea	(26)

2.1.4.9. Anemonin and Derivate Compounds

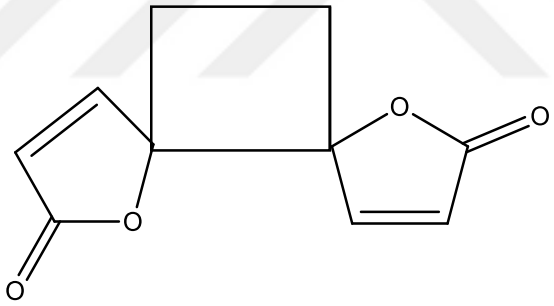


Table 28: Anemonin isolated from *Clematis* species

Compound	Species	Ref.
Anemonin	*crassifolia*	(40)
	chinensis	(81)
	hexapetala	(82)

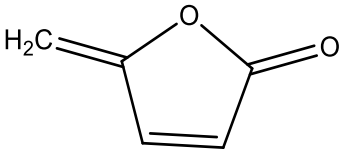


Table 29: Anemonin isolated from *Clematis* species

Compound	Species	Ref.
Protoanemonin	*apiifolia*	(42)
	aristata	(83)
	fawcettii	(83)
	gentianoides	(83)
	glycinoides	(83)
	microphylla	(83)
	pubescens	(83)

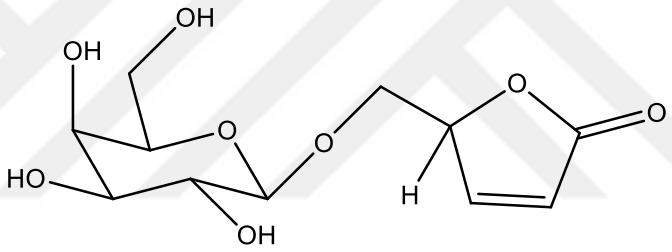


Table 30: Anemonin isolated from *Clematis* species

Compound	Species	Ref.
Ranunculin	*flammula*	(26)

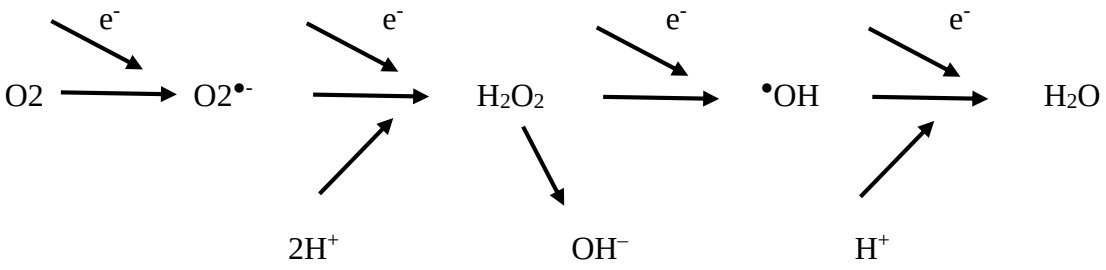
2.2. Oxidative Stress

Antioxidants have begun to draw more attention since the reactive oxygen species responsible for the formation of aging and degenerative diseases are associated with the stimulation of biological components. It has been proven that aging, cancer, atherosclerosis and other serious diseases are directly proportional to the oxidative damage to which DNA, low-density lipoprotein (LDL) and cell membrane are exposed (84).

The aim of current studies is to delay or stop this oxidative process. Antioxidants are both produced by cells in the body and can be taken through foods and supplements. The main natural antioxidants presenting in food and protecting the human body from harmful free radicals are mainly vitamins (vitamins A, C and E), flavonoids, carotenoids and polyphenols. Studies have also shown that phenolic compounds have significant antioxidant activities (85, 86).

2.2.1. Molecular Oxygen and Reactive Oxygen Species

Atoms containing unsubstituted (unpaired) electrons are defined as group of atom or molecular free radicals. Molecular oxygen (O_2) tends to form highly reactive oxygen species (ROS). A radical oxygen enters slow reaction with non-radical substances whereas, it easily reacts with other free radicals (87).



Reactive oxygen species are radicals such as peroxyl radicals ($ROO^{\bullet}$), hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{\bullet-}$), lipid peroxides (LOOH) and peroxynitrite ($^{-}OONO$) which rarely occurring during normal oxygen metabolism. These radicals cause cancer and cardiovascular diseases harming proteins and DNA (6).

Table 31: Reactive species (6, 88)

	Radicals		Non-radicals	
Reactive oxygen species	Superoxide	$O_2^{\bullet-}$	Hypobromous acid	HOBr
	Hydroxyl	$OH^{\bullet-}$	Hypochlorous acid	HOCl
	Peroxyl	$ROO^{\bullet}$	Hydrogen peroxide	H_2O_2
	Alkoxyl	$RO^{\bullet}$	Singlet oxygen	$^1O_2$
	Hydroperoxyl	$HO_2^{\bullet-}$	Ozone	O_3
Reactive nitrogen species	Nitrogenoxide	$NO^{\bullet-}$	Nitrous acid	HNO_2
	Nitrogendioxide	$NO2^{\bullet-}$	Diazottrioxide	N_2O_3
			Peroxynitrite	$ONOO^-$
			Alkylperoxynitrite	LOONO

2.2.1.1. Oxygen Derived Free Radicals

Superoxide Anion Radical

The superoxide radical ($O_2^{\bullet-}$) is the result of the reduction of molecular oxygen (O_2) by taking an electron (89). It is a weak oxidant but a powerful reducer with a millisecond half-life. $O_2^{\bullet-}$ is an important factor in oxygen toxicity, and the superoxide dismutase (SOD) enzyme protects the organism against it. As a weak oxidant, it is unlikely that $O_2^{\bullet-}$ may cause significant cell damage itself. However, $O_2^{\bullet-}$ can also trigger a series of reactions that can lead to an important oxidative stress. One of these is the Haber-Weiss reaction. The Haber-Weiss reaction causes the formation of OH, which is a strong reagent, interacting with $O_2^{\bullet-}$ and H_2O_2 in the presence of iron (90).

$$O_2^{\bullet-} + H_2O_2 \longrightarrow O_2 + OH^{\bullet} + OH^-$$

Hydroxyl Radical

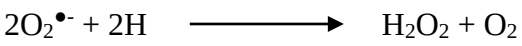
The hydroxyl radical ($OH^{\bullet}$), which reacts rapidly with many molecules such as sugars, amino acids, phospholipids, DNA and organic acids, is a highly reactive oxidant radical. Hydroxyl radicals occur in different ways, such as exposing to high-energy

ionizing radiation of water, reacting with the transistone metals of hydrogen peroxide (Fenton Reaction), degradation of the peroxynitrite radical, homolytic fission of the O-O bond in H_2O_2 formed by UV rays (91, 92).

2.2.1.2. Oxygen Derived Non-Free Radicals

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is produced from superoxide by the superoxide dismutase (SOD) enzyme.



$$2O_2^{\bullet-} + 2H \longrightarrow H_2O_2 + O_2$$

Hydrogen peroxide is not a free radical since it is not an unpaired electron, but it is considered free radical because of its potential to generate free oxygen radicals. Because it forms the hydroxyl radical ($OH^{\bullet}$), which is the most reactive and harmful free oxygen radical, in the presence of Fe^{+2} or other transition metals by the Fenton reaction and in the presence of the superoxide radical ($O_2^{\bullet-}$) by the Haber-Weiss reaction. Moreover, it is converted to hypochlorous acid (HOCl), a highly potent oxidant, through myeloperoxidase in leukocytes (93).

2.2.1.3. Reactive Nitrogen Species (RNS)

Severe studies conducted in recent years have led to a rapid change in the general rules of the biochemistry of free radicals. These studies have revealed that there are also non-reactive and non-short-lived free radicals. One of the most interesting examples of this is nitric oxide, which is not very reactive and has important physiological functions. Reactive nitrogen species, such as reactive oxygen species, also consist by the formation of more stable species by free radicals in the biological environment (94).

Nitrogen Oxide

Nitric oxide (NO) released from macrophages is a molecule synthesized from L-arginine via nitric oxide synthase (iNOS). When come together free oxygen radicals, it turns into peroxynitrite radical. NO is an essential mediator against pathogens, such as bacteria, fungi, virus and parasite in the host immune system. Besides, NO plays a substantial role in the arrangement of biological functions, for instance, neurotoxicity,

vasodilatation and neurotransmission. Nevertheless, redundant manufacture of the NO causes tissue damage and also various diseases (95, 96).

Peroxynitrite

Peroxynitrite is a radical occurred by the formation of nitric oxide and superoxide. It is not stable under physiological conditions. Peroxynitrite forms the thiyl radicals by reacting with thiols and causes thiol oxidation. It causes damage, reacting with all major biomolecules (protein, lipid, DNA), as well (94).

2.2.2. Damage of Free Radicals in Tissue and Cell

2.2.2.1. Lipid Peroxidation

Biomembranes and intracellular organelles are sensitive to oxidative attacks due to unsaturated fatty acids in membrane phospholipids. Reactive oxygen radicals can cause lipoprotein oxidation, especially LDL (97). The autoxidation processes of polyunsaturated lipids involve a radical chain reaction, which can begin on account of many causes. In addition, the lipoxygenase enzyme may start oxidation, as well (12). When membrane damage occurs, the essential metabolites in the cell go out of the cell. Moreover, Ca (II), an important agent for protein synthesis, rapidly increases in the cell and causes cell death (98).

2.2.2.2. Protein Oxidation

Amino acids generally tend to be more oxidized than lipids because of their carboxyl and amino groups. These molecules contain reduced carbon atoms which will undergo oxidative transformation in the side chains. Proteins with amino acids such as tryptophan, tyrosine, phenylalanine, histidine, methionine, cysteine containing unsaturated bonds and sulfur are easily affected by free radicals. After protein damage, reduction of enzyme activity, cytolysis, and even cell death may occur. In many studies, diseases such as Parkinson’s, Alzheimer’s, diabetes, renal tumor formation, and rheumatoid arthritis are associated with an increase in protein carbonyl groups (99, 100).

2.2.2.3. Oxidative Damage of DNA

The main target of oxidative damage in the DNA chain is purine, pyrimidine bases and sugar structure. The base damage disrupts the integrity of the strands, while the sugar damage causes breaks in the strands. $OH^{\bullet}$ radical, a strong reactive oxygen species, damages DNA by reacting to double bonds of DNA base. High amounts of reactive oxygen species within the cell activate transcription factors and related genes, and accelerate cancer formation by increasing DNA damage (101).

2.2.2.4. Oxidative Damage in Carbohydrates

Hydrogen atoms are initially removed from C-H bonds and α-hydroxyalkyl radicals are formed in the radical oxidation of carbohydrates. These radicals react with C-OH bonds in acid and base catalyzed reactions, resulting in the breakdown of glycosidic bonds and the formation of peroxyl radicals with O_2 (102).

2.2.3. Antioxidants and Defense Mechanisms

Antioxidants significantly inhibit or prevent oxidation of the substrate at low concentrations in the body or in food when compared to the oxidizable substrate. The antioxidants interest of the health authorities has increased in recent years. It is presently known that antioxidants help to prevent degenerative diseases associated with reactive nitrogen species and reactive oxygen species and reactive chlorine species in the body (12).

Antioxidants show their effects primarily in the following ways:

1. Cleaning of reactive oxygen species via enzymatic reactions or direct

2. Inhibition of reactive oxygen species by suppression

3. Attachment of metal ions and thus inhibition of radical formation reactions

4. Repair or cleaning of target molecules after damage (12).

2.2.3.1. Enzymatic Antioxidant Defense Mechanisms

Superoxide dismutase (SOD) is an antioxidant enzyme that catalyzes the alteration to O_2 and H_2O_2 of superoxide free radical ($O_2^{\bullet -}$) (103).

Glutathione, a tripeptide carrying the thiol group, acts as a substrate for many enzymes, such as transferases, peroxidases, which prevent or reduce the destructive

effects of free radicals. It protects biological membranes against lipid peroxidation. Glutathione peroxidase (GSH-Px) is the enzyme responsible for the degradation of hydroperoxides (104).

Superoxide anion radical is converted to H_2O_2 by superoxide dismutase. However, it is necessary to remove it from the medium owing to the fact that the formed H_2O_2 is converted to OH radical. The catalase enzyme (Cat) removes the formed H_2O_2 from the medium (89).

2.2.3.2. Non-Enzymatic Endogenous Antioxidant Defense Mechanisms

Melatonin is a very strong lipophilic antioxidant that removes the hydroxyl free radical ($OH^{\bullet}$), the most harmful free radical, from the middle.

Glutathione reacts with free radicals and peroxides to protect the cells against oxidative damage.

Bilirubin inhibits lipid peroxidation by scavenging superoxide and hydroxyl radicals.

Uric acid binds the iron and copper ions and suppresses the prooxidation of vitamin C. However, it does not inhibit lipid peroxidation (104).

2.2.3.3. Non-Enzymatic Exogenous Antioxidant Defense Mechanisms

Although vitamin E shows great singlet oxygen and OH radical-scavenging activity, *in vivo* antioxidant effect is also linked to the peroxyl and alkoxyl radicals-scavenging activity. It is indicated to react rapidly with peroxyl radicals and to have high activity. Chain breaking power is the basis of antioxidant activity (9, 13).

Vitamin C is mostly found in the aqueous parts of many animal tissues such as spinal cord, lung, eye. It is more than 1% in plants and some fruits. Many organisms can synthesize this compound by itself. However, a few species including human must take with the diet. Ascorbic acid is a potent reducing agent. It has the thermodynamic to can react with many oxidative free radicals. It quickly reacts even with weak oxidative agents such as $O_2^{\bullet}$ and $OOH^{\bullet}$ (9, 13).

Plants and foods have wide phenolic compound derivatives such as simple phenols, phenyl propanoides, benzoic acid derivatives, flavonoids, stylbens, tannins, lignans and lignins. The first detailed kinetic study of antioxidant activity of phenolic

compounds was performed by Boland and Ten-Have in 1947. Phenolic antioxidants interfere with lipid oxidation by easily donating electrons to lipid radicals. In addition, they are perfect hydrogen donors. The resulting radical intermediates are comparatively stable since they have resonance delocalization and their molecular oxygen cannot attack phenolic antioxidants the reason is that there are no suitable parts to connect (11-13).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Plant Material

The aerial parts of *Clematis viticella* L. were collected from Bursa, İnegöl (Şehitler Village) on 15 July, 2017. The plant material was authenticated by Prof. Dr. Erdem Yeşilada and a voucher specimen was deposited in the herbarium of Faculty of Pharmacy, Yeditepe University, İstanbul, Turkey. (Herbarium No: YEF17013)

3.1.2. Chemicals & Solvents

(-)-Epicatechin	Sigma Aldrich; E175316
2, 2-Diphenyl picryl hydrazil (DPPH)	Sigma Aldrich; 056K1147
2, 4, 6-Tris (2-pyridyl)-s-triazine (TPTZ)	Fluka; BCBB4473
Acetic acid	Riedel de Haen; 30990
Aluminum chloride	Merck; 8.01081.1000
Ammonium acetate	Carlo Erba; 313507
Ammonium molybdate tetrahydrate	Riedel de Haen; 30590
Ascorbic acid	Sigma Aldrich; 065K0003
Butylated hydroxytoluene	Sigma Aldrich; MKBD8339
Caffeic acid	Acros Organics; 114930250
Copper sulphate	Carlo Erba; 364757
Escin	Sigma Aldrich; E1378106
Ethanol	Sigma Aldrich; 46139
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	Sigma Aldrich; BCBC1356

Ferric chloride hexahydrate	Riedel de Haen; 41250
Ferrous chloride tetrahydrate	JT Baker; 0703801013
Ferrous sulphate heptahydrate	JT Baker; 0632701017
Ferrozine	Sigma Aldrich; MKBD0707
Folin-Ciocalteu Reagent (FCR)	Sigma Aldrich; BCBD5119
Gallic acid	Fluka; 1126284
Hydrochloric acid (37%)	Sigma Aldrich; SZBA2250
Methanol	Sigma Aldrich; SZE9365S
N, N-dimethyl-p-phenylenediamine (DMPD)	Sigma Aldrich; D4139106
Neocuproine	Sigma Aldrich; 120M1890V
Quercetin dehydrate	Sigma Aldrich; 116K1836
Sodium acetate trihydrate	Riedel de Haen; 33450
Sodium carbonate	Riedel de Haen; 2217A
Sodium hydroxide	Riedel de Haen; 60030
Sodium molybdate dihydrate	Riedel de Haen; 10102406
Sodium nitrite	Fluka; 4565841
Sodium phosphate monobasic	Riedel de Haen; 62840
Sulfuric acid (98%)	Riedel de Haen; 62260
Trolox	SigmaAldrich; BCBF4547V
Vanillin	Fluka; 1435805

3.1.3. Equipments

Balance	Ohaus Explorer
Beaker (50, 100, 250 mL)	
Centrifuge	Sigma 3-16 PK

Eppendorf tubes (1.5 mL)	
Erlenmayer flask (50, 100, 250 mL)	
Graduated cylinder (25, 50, 100 mL)	
Hairdryer	Arçelik
Lyophilizator	Christ Alpha 2-4 LD
Micropipette (100-1000 microlt)	Isolab
Micropipette (500-5000 microlt)	Rainin
Micropipette (5-50 microlt, 20-200 microlt)	Transferpette
Microplate reader	Thermo Multiskan Ascent
Milli Q water device	Millipore
Oven	Binder
pH meter	Mettler-Toledo MP220
Polypropylene tubes (16 x 100 mm)	
Refrigerator	Arçelik
Rotatory evaporator	Buchi, Heidolph
Spectrophotometer	Spekol 1300
Ultrasonic bath	Sonorex RK156BH
Volumetric flasks (5, 10, 25, 50, 100, 200, 500, 1000 mL)	
Vortex	Heidolph Reax
Waterbath	GFL

3.2. Methods

3.2.1. Chemical Studies

3.2.1.1. Extraction

The dried and powdered aerial parts of *Clematis viticella* (156 g) were macerated with 1000 mL of 80% aqueous MeOH in the dark for 3 days at room temperature. The resulting total extracts were filtered and methanol was completely evaporated in a rotary evaporator under reduced pressure. The remaining parts were frozen and then lyophilized (the yield was 22.10 g, 14.17%).

3.2.1.2. Simulation Process of *in vitro* Human Digestion

The simulation process of *in vitro* human digestion consisting of two consecutive steps was modified by Celep et al. from a previously reported study (14, 105). The simulated stomach solution was composed of pepsin enzyme and NaCl dissolved in distilled water, pH of the solution was adjusted to 2 with HCl. An aliquot of sample solution was taken and the volume was completed to 20 mL with the simulated stomach solution. The mixture was incubated at 37 °C in a shaking water bath for 2 hours. At the end of this step, the mixture was instantly cooled down in an ice bath and then an aliquot was taken as a representative of "post-gastric" sample (PG) and kept at −20 °C. A segment of cellulose dialysis tubing (molecular weight cut off 12 kDa) containing sufficient $NaHCO_3$ was placed inside the beaker. The mixture was placed at 37 °C in a shaking water bath for 15 minutes. After that, a mixture of 4 mg/mL pancreatin and 25 mg/mL bile salts were added to the medium and the solution was incubated under the same conditions for an additional 2 hours. In the later process, the same procedures were carried out without a cellulose tube. At the end of the incubation process, the solution left outside the dialysis tubing was taken as the OUT sample representing material that remained in the gastrointestinal tract (colon-available) and the solution that managed to diffuse into the dialysis tubing was taken as the IN sample (serum-available). The solution obtained from the later process is referred as IN+OUT since it contains both compartments. The pH of the solutions was adjusted to 2 to deactivate all enzymatic process and to improve storage conditions. Besides, all of the samples were centrifuged for 20 minutes at 15.000 rpm in order to precipitate bile acids. They were kept at -20°C for further analysis no longer than one week.

3.2.2. *In vitro* Antioxidant Activity Studies

3.2.2.1. Determination of Total Phenolic Content

The total phenolic content of the samples was defined according to the method of Singleton and Rossi (106). In this experiment, it is based on the formation of a blue molybdenum-tungsten complex in presence of phenolic using Folin-Ciocalteu Reagent (FCR). This complex can be defined spectrophotometrically at 765 nm.

The Chemicals and Reagents

- Na_2CO_3 20% in H_2O
- Folin-Ciocalteu Reagent (FCR)
- Gallic acid

The Experimental procedure

All samples were diluted to a concentration of 1 mg/mL. 75 µL of Na_2CO_3 and 100 µL of Folin-Ciocalteu Reagent were added to 20 µL of each sample. Then the composite was let to incubate in the dark at room temperature for 30 minutes. The absorbance was measured spectrophotometrically at 690 nm using 96-well microplate reader. Gallic acid was dissolved in water and used as the standard substance. Calibration curve was designed in the concentrations of 50, 100, 300, 500 µg/mL. All determinations were performed in triplicate. The total phenolic content of the samples was expressed as mg gallic acid equivalents (GAE) in 1 g dry extract.

3.2.2.2. Determination of Total Phenolic Acid Content

The total phenolic acid content of the samples was detected spectrophotometrically with respect to the method declared by Mihailović et al. in 2016 (107). The essence of this method is based on complex that results from the interaction between phenolic acids and sodium nitrite-sodium molybdate. The absorbance of this complex was measured at 490 nm.

The Chemicals and Reagents

- HCl 0.1 M
- NaOH 1 M
- Arnow reagent 10% w/v Na_2MoO_4 and 10% w/v $NaNO_2$
- Caffeic acid

The Experimental procedure

All samples were diluted to a concentration of 1 mg/mL. 1 mL of samples were mixed with 1 mL of Arnow reagent, 1 mL of 0.1 M HCl and 1 mL of 1 M NaOH. Later, the eventual volume was adjusted to 10 mL with distilled water. The absorbance of samples was measured immediately at 490 nm. Caffeic acid was dissolved in methanol and used as the standard substance. Calibration curve was schemed in the concentrations of 50, 62.5, 100, 125, 250 and 500 µg/mL. All definitions were performed in triplicate. The total phenolic acid content of the samples was stated as caffeic acid equivalents (CAE) in 1 g dry extract.

3.2.2.3. Determination of Total Flavonoid Content

The total flavonoid content of the samples was calculated according to the aluminum chloride colorimetric method developed by Woisky and Salatino (108). This method is based on the formation of decided acid complexes by $AlCl_3$ with the C-4 keto group and either C-3 or C-5 hydroxyl groups of flavones or flavonols. Moreover, $AlCl_3$ creates unstable acid complexes with ortho-dihydroxyl groups in the A or B ring of flavonoids.

The Chemicals and Reagents

- Ethanol 75%
- Aluminum chloride 10% in H_2O
- Potassium acetate 1 M
- Quercetin

The Experimental procedure

All samples were diluted to a concentration of 1 mg/mL. 50 µL of samples were mixed with 150 µL of 75% ethanol, 10 µL of 10% aluminum chloride solution, 10 µL of 1M potassium acetate. The mixture was incubated in the dark at room temperature for 30 minutes. The absorbance was read spectrophotometrically at 405 nm. Quercetin was dissolved in methanol and used as the standard substance. Calibration curve was designed in the concentrations of 15.625, 31.25, 62.5, 125, 250 and 500 µg/mL. All determinations were performed in triplicate. The total flavonoid content of the samples was expressed as quercetin equivalents (QE) in 1 g dry extract.

3.2.2.4. Determination of Total Proanthocyanidin Content

The total proanthocyanidin content of the samples was explored with regard to the vanilin-HCl method emphasized by Ariffin et al (109). Vanillin is protonated in an acidic medium and gives a weak electrophilic carbocation that reacts with the flavonoid ring at the C-6 or C-8 position. This intermediate compound which is dehydrated gives a red colored compound.

The Chemicals and Reagents

- Vanilin 1% in MeOH
- HCl 9 M
- (-)-Epicatechin

The Experimental procedure

All samples were diluted to a concentration of 1 mg/mL. 2.5 mL of 1% vanillin and 2.5 mL of 9 M HCl were added to 1 mL of sample in a capped glass tube. The mixture was allowed to incubate in the dark for 20 minutes at 30 °C. The absorbance was read spectrophotometrically at 492 nm. (-)-Epicatechin was dissolved in methanol and used as the standard substance. Calibration curve was plotted in the concentrations of 3.125, 6.25, 12.5, 25, 50, 100, 200 and 400 µg/mL. All determinations were performed in triplicate. The total proanthocyanidin content of the samples was expressed as (-)-epicatechin equivalents (ECE) in 1 g dry extract.

3.2.2.5. Determination of Total Saponin Content

The total saponin content of the samples was performed according to the vanilin-sulfuric acid method developed by Hiai et al (110). Because the essential structure for the reaction in this experiment is the OH group at C-3, which may be in the free or glycoside form.

The Chemicals and Reagents

- Vanilin 8% in MeOH
- H_2SO_4 72% in H_2O
- Escin

The Experimental procedure

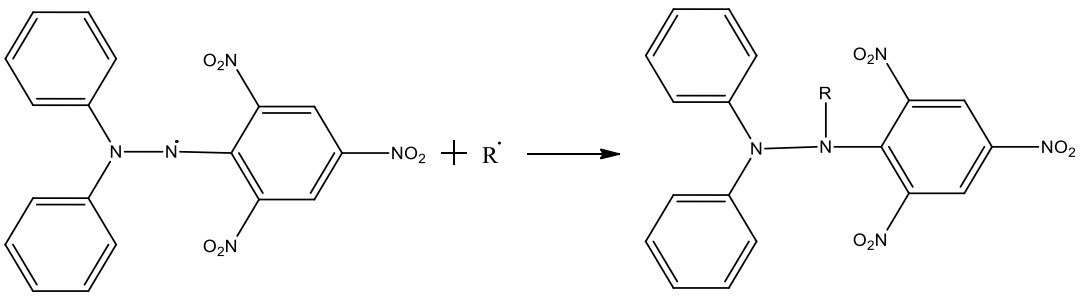
All samples were diluted to a concentration of 1 mg/mL. 0.5 mL of 8% vanillin and 5 mL of 72% H_2SO_4 were added to 0.5 mL of samples in a closed-cap polypropylene tube, respectively. The mixture was incubated at 70 °C in 10 min. The tubes are taken immediately to the ice bath and cooled down to room temperature. The absorbance was read spectrophotometrically at 560 nm. Escin was dissolved in H_2O and used as the standard substance. Calibration curve was plotted in the concentrations of 50, 62.5, 100, 125, 250, 500 and 1000 µg/mL. All determinations were performed in triplicate. The total saponin content of the samples was clarified as escin equivalents (EE) in 1 g dry extract.

3.2.2.6. Determination of DPPH Radical-Scavenging Activity

It is a method based on measuring the scavenging effects of DPPH radical, a stable organic nitrogen radical of antioxidants. The scavenging activity of the samples against DPPH radical was expressed by using the method described by Akter et al (111, 112). This radical is reduced hydrazine when it interacts with hydrogen donors. Purple colored DPPH radical gives maximum absorption at 517 nm. The absorbance decreases and the color changes from purple to yellow by adding antioxidant to the DPPH solution. This method is a simple and useful for evaluating the scavenging ability of antioxidants. However, some compounds (carotenoids) which show overlapping spectrum with DPPH at 517 nm makes difficult to interpret. Also, most of them enter the slow reaction with DPPH because of the steric hindrance. Thus, the method does not give an accurate assessment about the antioxidant capacity of the antioxidants entering the reaction with DPPH. Moreover, it is difficult to get reproducible results due to the fact that the color of DPPH is sensitive to light, air oxygen, humidity and pH.

The reaction mechanism is as below.

$DPPH^{\bullet}$ + Antioxidant-H $\rightarrow$ DPPH-H + $A^{\bullet}$



The Chemicals and Reagents

- DPPH 0.1 mM in MeOH
- BHT

The Experimental procedure

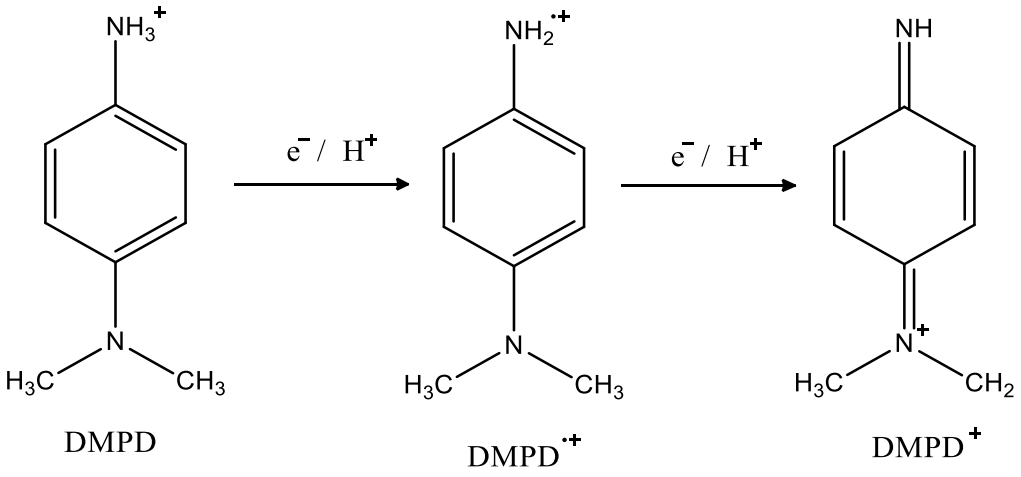
All samples were diluted to a concentration of 1 mg/mL. 25 µL of samples were separately added to 200 µL 0.1 mM DPPH solution prepared in methanol just before use. The mixture was incubated at room temperature in the dark for 50 minutes. The absorbance was calculated at 540 nm. MeOH was used in the control group and butylated hydroxy toluene (BHT) was used as the reference material. DPPH radical-scavenging activity of the control group, the reference material and the samples were calculated as follows:

$$\text{DPPH radical-scavenging activity} = \frac{(ABS_{Control} - ABS_{Sample})}{ABS_{Control}} X\ 100$$

"$ABS_{Control}$" is the absorbance value of the control group; "ABS_{Sample}" is the absorbance of the samples. The activity was stated as EC_{50} values corresponding to the concentration which shows 50% activity.

3.2.2.7. Determination of DMPD Radical-Scavenging Activity

The scavenging activity of the samples against DMPD radical was expressed by using the method described by Fogliano et al. in 1999 (113). In this method, the DMPD (N, N'-dimethyl-p-phenylenediamine) radical cation is formed by using $FeCl_3$ solution. The pink and stable DMPD radical takes an H atom therefrom entering the chemical reaction with the antioxidant substances and the antioxidants diminish the radical effect of DMPD. As a result, the pink color of the DMPD radical is disappeared by reaction with antioxidants. The absorbance value of the color is measured 492 nm.



DMPD DMPD$^{\bullet+}$ DMPD^{+}

The Chemicals and Reagents

- DMPD Reagent

  1. DMPD 100 mM

  2. Acetate Buffer 100 mM, pH 5.25

  3. $FeCl_3.6H_2O$ 50 mM

  The DMPD reagent was freshly prepared by mixing 1, 2 and 3 in the ratio of 5:500:1.

- Trolox

The Experimental procedure

All samples were diluted to a concentration of 1 mg/mL. 15 µL of samples were separately added to 210 µL of the DMPD solution prepared just before use. The mixture was incubated at room temperature in the dark for 50 minutes. The absorbance was measured at 492 nm. Trolox was used as the reference substance, and a calibration curve was plotted in the concentrations of 100, 125, 200, 250, 500 and 1000 µg/mL. The results were given as mg trolox equivalent (TE) per g material.

3.2.2.8. Determination of Ferric Reducing Antioxidant Power (FRAP)

FRAP activity was performed according to a method described by Benzie and Strain (114). This assay, a redox-linked colorimetric method, uses antioxidants as reductants. Ferric tripyridyl triazine complex (Fe^{3+} TPTZ) is reduced to ferrous form (Fe^{2+} TPTZ) at low pH. The reduced form has an intensive blue color and the change in absorption at 593 nm is measured.

The Chemicals and Reagents

- FRAP Reagent:

  1. Acetate buffer 300 mM, pH 3.6

  2. TPTZ 10 mM in 40 mM HCl

  3. $FeCl_3.6H_2O$ 20 mM

  The working FRAP reagent was freshly prepared by mixing 1, 2 and 3 in the ratio of 10:1:1.

- $FeSO_4.7H_2O$
- BHT (Butylated hydroxy toluene)

The Experimental procedure

All samples were diluted to a concentration of 1 mg/mL. 10 µL of samples and 30 µL of distilled water were mixed with 260 µL of working FRAP reagent in a microplate. The mixture was incubated at 37°C for 30 minutes. Later, the absorbance was recorded at 593 nm using a 96-well microplate reader. Water was used for the control group instead of sample. Calibration curve was plotted in the concentrations of 125, 250, 500 and 1000 µM of $FeSO_4.7H_2O$. Butylated hydroxy toluene was used as the reference substance. The results were expressed as µM $FeSO_4.7H_2O$ per g material.

3.2.2.9. Determination of Cupric Reduced Antioxidant Capacity (CUPRAC)

CUPRAC activity was recorded according to the method found by Apak et al. with some modifications made by Celep et al (112, 115). This experiment is based on the formation of a colored complex by reducing Cu (II) -neocuproine complex to Cu (I) -neocuproine in the presence of antioxidant and absorbance is measured at 450 nm since maximum absorption gives in this absorbance.

$$n\ Cu(Nc)_2^{2+} + Ar(OH)_n \longrightarrow Cu(Nc)_2^{+} + Ar(O=)_n + nH^{+}$$

The Chemicals and Reagents

- $CuSO_4$ 1 mM
- Neocuproine 7.5 mM in MeOH
- Ammonium acetate buffer 1000 mM, pH 7.0
- Ascorbic acid

The Experimental procedure

85 µL of each of 10 mM $CuSO_4$, 7.5 mM neocuproine, and 1 M ammonium acetate buffer (pH 7.0) solutions were mixed in a microplate. Later, 51 µL of distilled water and 43 µL of samples were added respectively. The mixture was incubated at room temperature for 1 hour. After the incubation period, the absorbance was read at 450 nm using a 96-well microplate reader. Ascorbic acid was used as the standard substance, and a calibration curve was plotted in the concentrations of 12.5, 25, 50, 100, 200 and 400 µg/mL. The results were given as mg ascorbic acid equivalent (AAE) per g material.

3.2.2.10. Determination of Total Antioxidant Capacity (TOAC)

The total antioxidant capacities of the samples were measured with regard to the phosphomolybdenum method found by Prieto et al. with small modifications made by Celep et al (112, 116). The assay is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at acidic pH.

The Chemicals and Reagents

- The Reagent Solution:
  - Sodium phosphate monobasic 28 mM
  - Ammonium molybdate 4 mM
  - Sulfuric acid 600 mM
- Ascorbic acid

The Experimental procedure

All samples were diluted to a concentration of 1 mg/mL. 300 µL of the reagent solution was mixed with 30 µL of warrantably diluted samples. The microplate containing the mixture was incubated at 95 ºC for 90 minutes in a water bath. After the

incubation period, the samples were cooled to room temperature, and the absorbance was read at 690 nm using a 96-well microplate reader. Ascorbic acid was used as the standard substance and a calibration curve was plotted in the concentrations of 12.5, 25, 50, 100, 200, 400 and 500 μg/mL. The results were given as mg ascorbic acid equivalent (AAE) per g material.

3.2.2.11. Statistics

The experiments were performed in triplicate. The results were assessed as mean ± standard deviation. Statistical comparisons were made using one-way analysis of variance (ANOVA) followed by Students–Newman– Keuls post hoc test for multiple comparisons. In addition, Pearson correlation coefficients were calculated. Statistically significant difference was detected as $p < 0.05$.

4. RESULTS

4.1. Results of *In vitro* Activity Studies

4.1.1. Total Phenolic Content of *Clematis viticella* Extract

The total phenolic content of 80% methanolic extract of *Clematis viticella* aerial parts is given in Table 32, Figure 3 according to the simulation process of *in vitro* human digestion. Although *Clematis viticella* had the highest total phenolic content before digestion simulation, this content was slightly decreased after the gastric phase of digestion. Furthermore, it was significantly decreased after the intestinal digestion.

Table 32: The total phenolic content of *Clematis viticella* aerial parts

	ND[C]	PG	IN	IN+OUT
Total Phenolic Content[A]	113.89 ± 1.13[B,a]	93.57 ± 5.50[b]	62.92 ± 1.97[c]	68.19 ± 3.66[c]

[A] Total phenolic content was expressed as mg gallic acid equivalents (GAE) in 1 g dry extract.

[B] Results were expressed as the mean of triplicates ± standard deviation (S.D.)

[a-c] Values with different letters within a row were significantly different ($p < 0.05$)

[C] The abbreviations for samples are ND: non-digested, PG: postgastric, IN: serum available, OUT: colon available

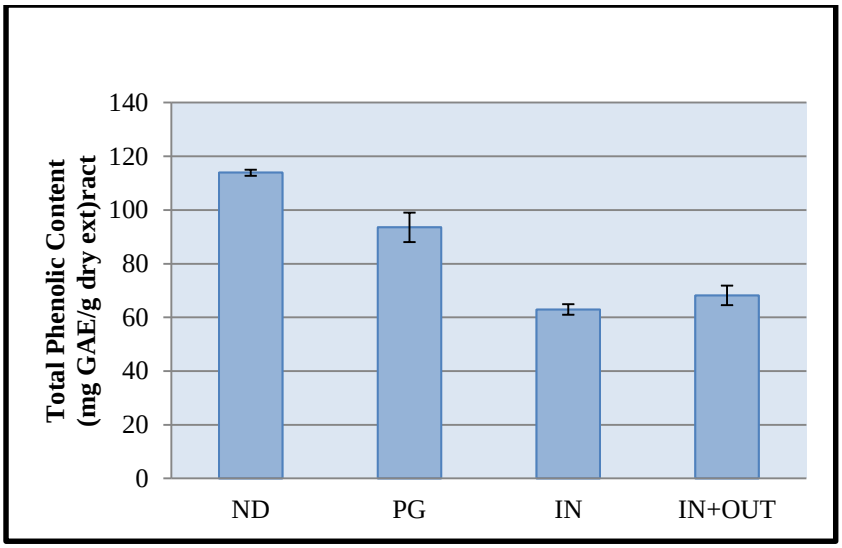


Figure 3: The total phenolic content of *Clematis viticella* aerial parts. The results are expressed as mg gallic acid equivalent in 1 g dry extract.

4.1.2. Total Phenolic Acid Content of *Clematis viticella* Extract

The total phenolic acid content of 80% methanolic extract of *Clematis viticella* aerial parts is given in Table 33, Figure 4 according to the simulation process of *in vitro* human digestion. It was also observed that majority of the phenolic acid content of the *Clematis viticella* extract (75.2%) passed through gastric phase of digestion. IN+OUT fraction seemed to possess the lowest value.

Table 33: The total phenolic acid content of *Clematis viticella* aerial parts

	ND[C]	PG	IN	IN+OUT
Total Phenolic Acid Content[A]	$55 \pm 0.91^{B,a}$	41.36 ± 0^{b}	18.03 ± 3.67^{c}	15.61 ± 1.05^{c}

[A] Total phenolic acid content was expressed as mg caffeic acid equivalents (CAE) in 1 g dry extract.

[B] Results were expressed as the mean of triplicates ± standard deviation (S.D.)

[a-c] Values with different letters within a row were significantly different ($p < 0.05$)

[C] The abbreviations for samples are ND: non-digested, PG: postgastric, IN: serum available, OUT: colon available

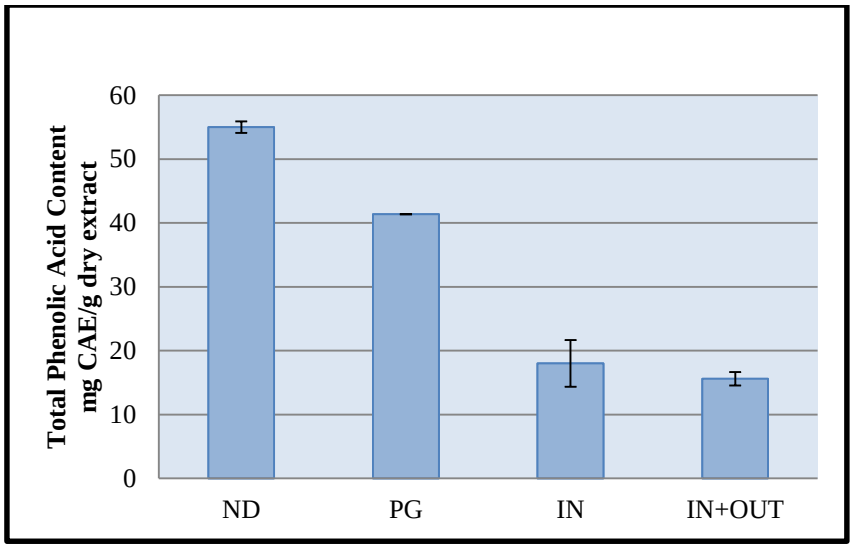


Figure 4: The total phenolic acid content of *Clematis viticella* aerial parts. The results are expressed as mg caffeic acid equivalent in 1 g dry extract.

4.1.3. Total Flavonoid Content of *Clematis viticella* Extract

The total flavonoid content of 80% methanolic extract of *Clematis viticella* aerial parts is given in Table 34, Figure 5 according to the simulation process of *in vitro* human digestion. It was observed that the total flavonoid content of *Clematis viticella* increased after the gastric phase of digestion. However, this increase was assessed as not significant.

Table 34: The total flavonoid content of *Clematis viticella* aerial parts

	ND[C]	PG	IN	IN+OUT
Total Flavonoid Content[A]	21.16 ± 2.46[B,a]	24.29 ± 1.45[a]	6.98 ± 0.57[b]	9.37 ± 0.27[b]

[A] Total flavonoid content was expressed as mg quercetin equivalents (QE) in 1 g dry extract.

[B] Results were expressed as the mean of triplicates ± standard deviation (S.D.)

[a-b] Values with different letters within a row were significantly different ($p < 0.05$)

[C] The abbreviations for samples are ND: non-digested, PG: postgastric, IN: serum available, OUT: colon available

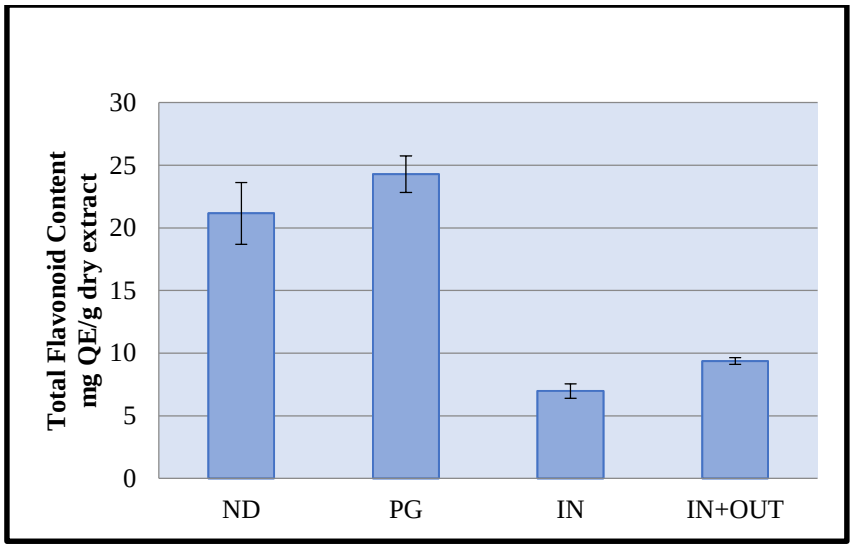


Figure 5: The total flavonoid content of *Clematis viticella* aerial parts. The results are expressed as mg quercetin equivalent in 1 g dry extract.

4.1.4. Total Proanthocyanidin Content of *Clematis viticella* Extract

The total proanthocyanidin content of 80% methanolic extract of *Clematis viticella* aerial parts is researched according to the simulation process of *in vitro* human digestion by using the vanilin-HCl method emphasized by Ariffin et al. [109] However, the content of total proanthocyanidins in both extract and digestive products has not been determined. (-)-Epicatechin was used as a reference substance.

4.1.5. Total Saponin Content of *Clematis viticella* Extract

The total saponin content of 80% methanolic extract of *Clematis viticella* aerial parts is given in Table 35, Figure 6 according to the simulation process of *in vitro* human digestion. *Clematis viticella* was found to have high saponin content prior to *in vitro* gastrointestinal digestion. However, significant decrease in PG and IN fractions was observed after digestion simulation.

Table 35: The total saponin content of *Clematis viticella* aerial parts

	ND[C]	PG	IN	IN+OUT
Total saponin Content[A]	$131.97 \pm 0.58^{B,a}$	73.63 ± 0.58^{b}	30.3 ± 1^{c}	42.97 ± 0.58^{d}

[A] Total saponin content was expressed as mg escin equivalents (EE) in 1 g dry extract.

[B] Results were expressed as the mean of triplicates ± standard deviation (S.D.)

[a-d] Values with different letters within a row were significantly different ($p < 0.05$)

[C] The abbreviations for samples are ND: non-digested, PG: postgastric, IN: serum available, OUT: colon available

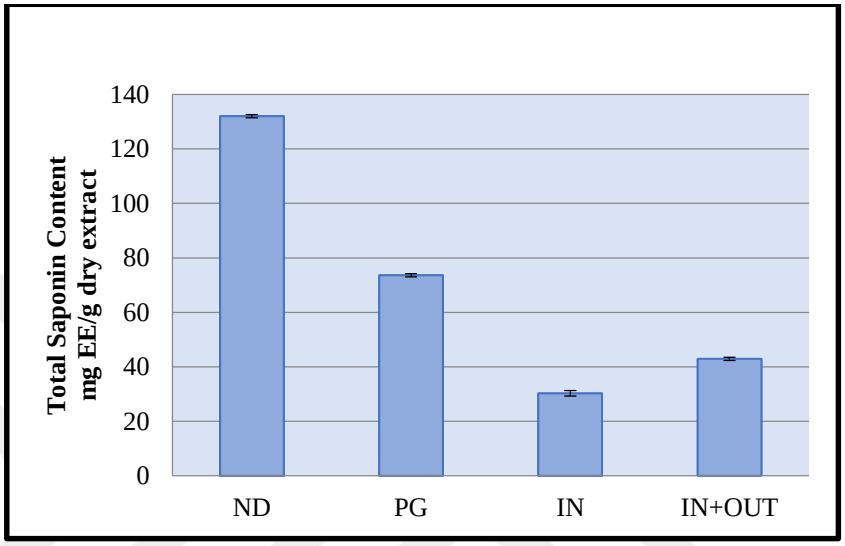


Figure 6: The total saponin content of *Clematis viticella* aerial parts. The results are expressed as mg escin equivalent in 1 g dry extract.

4.1.6. DPPH (2,2-Diphenyl-1-picrylhydrazyl) Radical-Scavenging Activity

DPPH radical-scavenging activitiy of 80% methanolic extract of *Clematis viticella* aerial parts are given in Table 36, Figure 7 according to the simulation process of *in vitro* human digestion. The results are expressed as "half maximal effective concentration" (EC_{50}). Although the non-digested fraction of *Clematis viticella* showed high DPPH radical-scavenging activity, the BHT used as reference substance showed the highest activity. However, the metal reducing activity of the extract significantly decreased both after the gastric phase of digestion and after the intestinal digestion. In addition, the free radical scavenging activity of the bioaccessible fraction (IN+OUT sample) was significantly higher than bioavailable fraction (IN sample).

Table 36: DPPH radical-scavenging activity of *Clematis viticella* aerial parts

	ND[C]	PG	IN	IN+OUT	BHT[*]
DPPH radical-scavenging activity[A]	395.64± 22.64[B,a]	488.85± 42.88[b]	922.56± 22.68[c]	718.45± 12.31[d]	77.84± 0.5[e]

[A] DPPH radical scavenging activity was expressed as EC_{50} in µg/mL.

[B] Results were expressed as the mean of triplicates ± standard deviation (S.D.)

[a-e] Values with different letters within a row were significantly different ($p < 0.05$)

[C] The abbreviations for samples are ND: non-digested, PG: postgastric, IN: serum available, OUT: colon available

[*] Butylated hydroxytoluene

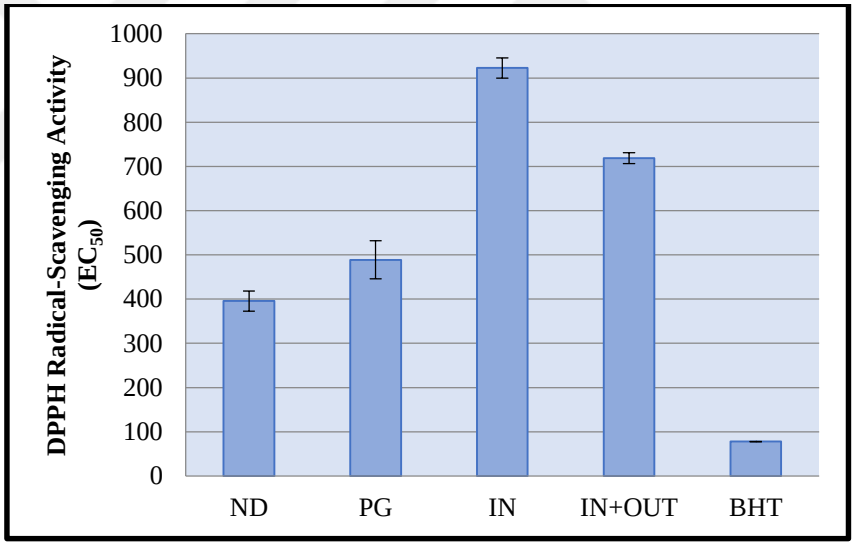


Figure 7: DPPH radical-scavenging activity of *Clematis viticella* aerial parts. The results are expressed as EC_{50} in µg/mL.

4.1.7. DMPD (N, N-dimethyl-p-phenylendiamine) Radical-Scavenging Activity

DMPD radical-scavenging activity of 80% methanolic extract of *Clematis viticella* aerial parts in Table 37, Figure 8 according to simulation process of *in vitro* human digestion. The results were stated as mg trolox equivalents (TE) per g dry extract. The DMPD radical-scavenging activity of *Clematis viticella* also showed similar results to DPPH radical-scavenging activity.

Table 37: DMPD radical-scavenging activity of *Clematis viticella* aerial parts

	ND[C]	PG	IN	IN+OUT
DMPD radical-scavenging activity[A]	60.55 ± 1.19[B,a]	54.80 ± 3.28[b]	33.58 ± 1.5[c]	35.37 ± 0.69[c]

[A] DMPD radical-scavenging activity was expressed as mg trolox equivalents (TE) in 1 g dry extract.

[B] Results were expressed as the mean of triplicates ± standard deviation (S.D.)

[a-c] Values with different letters within a row were significantly different ($p < 0.05$)

[C] The abbreviations for samples are ND: non-digested, PG: postgastric, IN: serum available, OUT: colon available

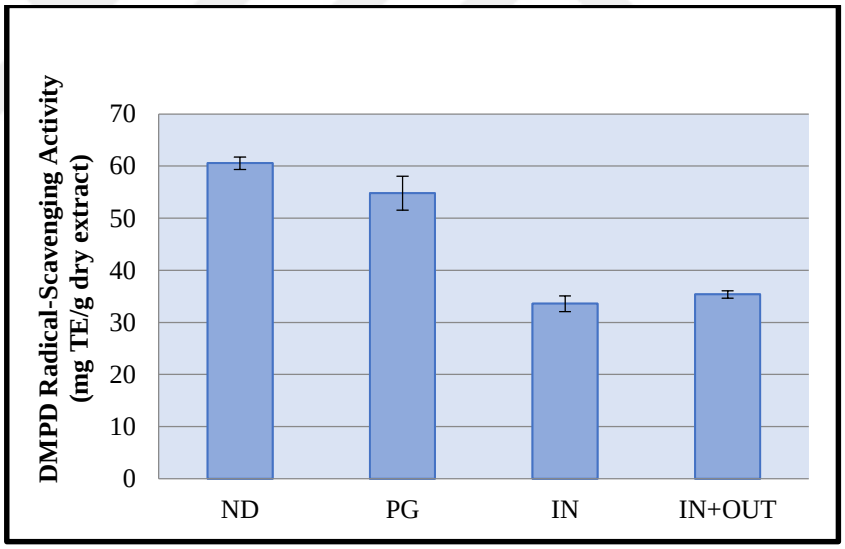


Figure 8: DMPD radical-scavenging activity of *Clematis viticella* aerial parts. The results are expressed as mg trolox equivalents in 1 g dry extract.

4.1.8. Ferric Reducing Antioxidant Power Assay (FRAP)

Ferric reducing antioxidant power assay of 80% methanolic extract of *Clematis viticella* aerial parts is giveen in Table 38, Figure 9 according to simulation process of *in vitro* human digestion and expressed as µM $FeSO_4$ equivalents per g dry extract. *Clematis viticella* extract and digestive products had perceptible ferric reducing antioxidant power in comparison with the reference compound, BHT. A major part of

Clematis viticella extract (73.47%) passed through gastric phase of digestion. A little part of it (38.57%) passed serum phase.

Table 38: FRAP of *Clematis viticella* aerial parts

	ND[C]	PG	IN	IN+OUT	BHT[*]
Ferric reducing antioxidant power[A]	974.05 ± 7.11[B,a]	715.68 ± 41.68[b]	375.71 ± 50.6[c]	369.18 ± 3.77[c]	2353.18±54.54[d]

[A] FRAP activity was expressed as µM FeSO4 equivalents in 1 g dry extract.

[B] Results were expressed as the mean of triplicates ± standard deviation (S.D.)

[a-d] Values with different letters within a row were significantly different ($p < 0.05$)

[C] The abbreviations for samples are ND: non-digested, PG: postgastric, IN: serum available, OUT: colon available

[*] Butylated hydroxytoluene

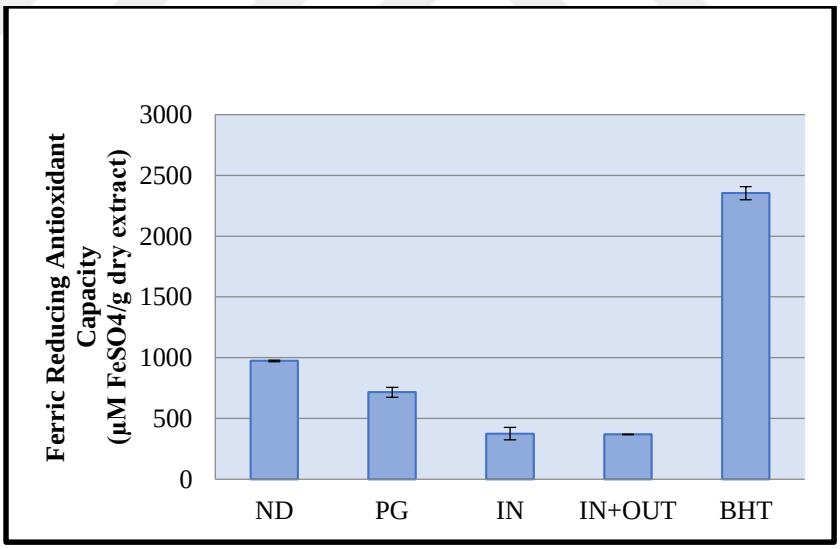


Figure 9: FRAP of *Clematis viticella* aerial parts. The results are expressed as µM $FeSO_4$ equivalents in 1 g dry extract or reference substance.

4.1.9. Cupric Reducing Antioxidant Capacity (CUPRAC)

Cupric reducing antioxidant capacity of 80% methanolic extract of *Clematis viticella* aerial parts is given in Table 39, Figure 10 according to simulation process of *in vitro* human digestion. The results were expressed as mg ascorbic acid equivalents (AAE) per g dry extract. Results of both non-digested and digested samples in the cupric reducing capacity assay was similarity shown in the ferric reducing power.

Table 39: CUPRAC of *Clematis viticella* aerial parts

	ND[C]	PG	IN	IN+OUT
Cupric reducing antioxidant capacity[A]	240.01 ± 3.02[B,a]	192.76 ± 3.45[b]	98.78 ± 0.87[c]	97.5 ± 0.73[c]

[A] CUPRAC activity was expressed as mg ascorbic acid equivalents (AAE) in 1 g dry extract.

[B] Results were expressed as the mean of triplicates ± standard deviation (S.D.)

[a-c] Values with different letters within a row were significantly different ($p < 0.05$)

[C] The abbreviations for samples are ND: non-digested, PG: postgastric, IN: serum available, OUT: colon available

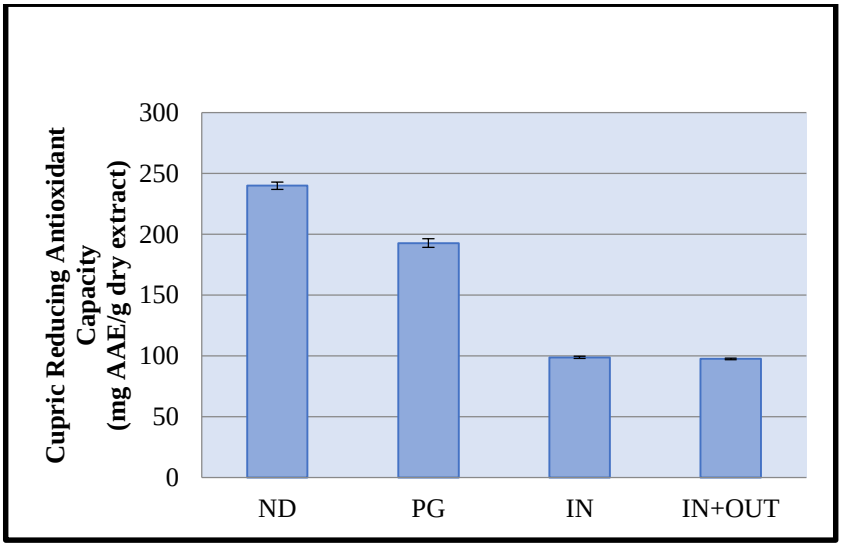


Figure 10: CUPRAC of *Clematis viticella* aerial parts. The results are expressed as mg ascorbic acid in 1 g dry extract.

4.1.10. Total Antioxidant Capacity (TOAC)

Total antioxidant capacities of 80% methanolic extract of *Clematis viticella* aerial parts is given in Table 40, Figure 11 according to simulation process of *in vitro* human digestion. The results were expressed as mg ascorbic acid equivalents (AAE) per g dry extract. Although *Clematis viticella* had the highest total antioxidant capacity before digestion simulation, this content was slightly decreased after the gastric phase of digestion. Moreover, it was significantly decreased after the intestinal digestion.

Table 40: TOAC of 80% methanolic extract of *Clematis viticella* aerial parts.

	ND[C]	PG	IN	IN+OUT
Total antioxidant capacity[A]	$59.75 \pm 0.3^{B,a}$	45.89 ± 0.53^{b}	28.26 ± 0.37^{c}	27.65 ± 0.3^{c}

[A] Total antioxidant activity was expressed as mg ascorbic acid equivalents (AAE) in 1 g dry extract.

[B] Results were expressed as the mean of triplicates ± standard deviation (S.D.)

[a-c] Values with different letters within a row were significantly different ($p < 0.05$)

[C] The abbreviations for samples are ND: non-digested, PG: postgastric, IN: serum available, OUT: colon available

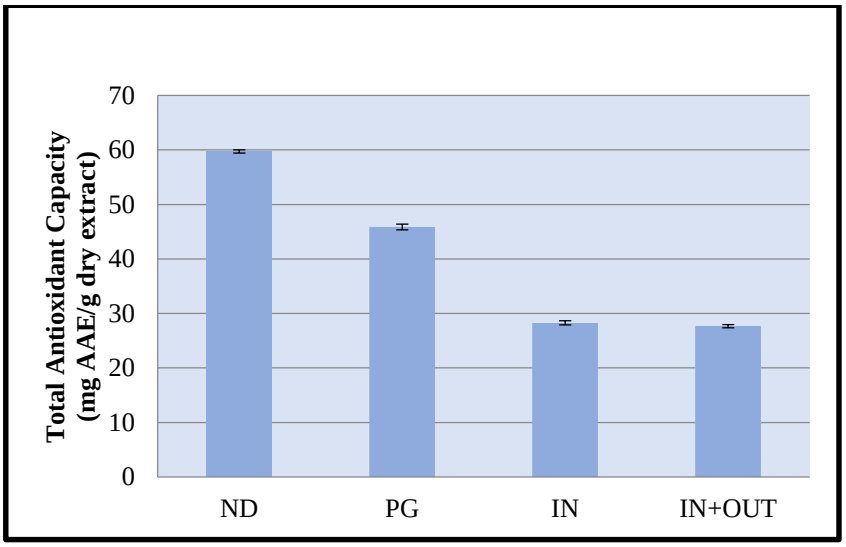


Figure 11: TOAC of 80% methanolic extract of *Clematis viticella* aerial parts. The results are expressed as mg ascorbic acid in 1 g dry extract.

4.2. Bioavailability and Bioaccessibility Results of *In vitro* Activity Studies

4.2.1 Bioavailability Results of *In vitro* Activity Studies

Bioavailability is the degree and the rate at which compounds can reach the effect site within the body. The bioavailability indexes of 80% methanolic extract of *Clematis viticella* aerial parts were determined as the ratio of the activity in non-digested (ND) samples to the activity in IN samples, representing the serum and colon-available contents (117).

Table 41: Bioavailability results of *Clematis viticella* aerial parts

Name of the analysis	Bioavailability index (BAvI) (IN/ND) X 100
Total Phenolic Content	55.25%
Total Phenolic Acid Content	32.78%
Total Flavonoid Content	32.99%
Total Saponin Content	22.96%

4.2.2 Bioaccessibility Results of *In vitro* Activity Studies

Bioaccessibility is an important initial step for compounds to display their biological activities. The bioaccessibility indexes of 80% methanolic extract of *Clematis viticella* aerial parts were determined as the ratio of the activity in non-digested (ND) samples to the activity in IN+OUT samples, representing the serum and colon-available contents (118).

Table 42: Bioaccessibility results of *Clematis viticella* aerial parts

Name of the analysis	Bioaccessibility index (BAcI) [(IN+OUT)/ND] X 100
Total Phenolic Content	59.87%
Total Phenolic Acid Content	28.38%
Total Flavonoid Content	44.28%
Total Saponin Content	32.56%

5. DISCUSSION

Every living organism, with the exception of anaerobic microorganisms, requires molecular oxygen as an electron acceptor for energy production. Since oxygen itself is a powerful oxidant, secondary oxidation reactions in normal physiological metabolism are inevitable. Oxygen-derived radicals which are called as reactive oxygen species (ROS) constitute the most important class of radical species in living systems (9). Because the radical oxygen enters slow reaction with non-radical substances whereas, it easily reacts with other free radicals (87).

Free radicals are formed by the removal of an electron from a non-radical atom or molecule, or by the addition of an electron to a non-radical atom or molecule (119). Since these molecules are very unstable, they interfere with other molecules and damage them in order to become stable (120). When attacking a molecule, they steal their electrons, oxidize, and also cause this new molecule to turn into a free radical. The reactions that start in this way give rise to living cell to be damaged (121).

There are radical or non-radical reactive species in the human body. The most important of these are called as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (6). There are various defense systems against reactive oxygen and nitrogen species. These systems are generally grouped under the heading ‘‘Antioxidant System’’. Oxidants are constantly produced in the organism while the antioxidant system prevents these oxidants and their adverse effects. This is a constant balance. If this balance is broken in favor of the oxidants, it is called ‘‘Oxidative Stress’’ (7, 8). Oxidative stress is a major problem for living organisms and can lead to the formation of reactive oxygen species which can cause cellular damage. This is the case for many diseases such as cancer, diabetes and cardiovascular diseases. Although the human body has a number of antioxidant mechanisms and enzymes, it should be supplemented by consumption of antioxidant-rich foods. This situation underlines our assumption twice that the value of plants in our country has unique importance (9).

The production of endogenous enzymes, which are natural antioxidants of the body, will have decreased over the time. For this reason, defense mechanisms are weakening and the free radical balance of the body is deteriorating. It is very important to take natural antioxidant nutrients to regain the balance. Antioxidant substances (natural antioxidants), found in foods taken from natural sources, prevent or delay the

occurrence of early-onset aging and diseases that are the main causes of premature death in the community, such as cancer and heart diseases, by reducing the effects of free radicals (122). The main natural antioxidants presenting in food and protecting the human body from harmful free radicals are mainly vitamins (vitamins A, C and E), flavonoids, carotenoids and polyphenols. Studies have also shown that phenolic compounds have significant antioxidant activities (85, 86).

The first detailed kinetic study of antioxidant activity of phenolic compounds was performed by Boland and Ten-Have in 1947 (11). Then correlation between antioxidant activity and phenolic compounds was supported by unlimited work using different methods. However, with the advance of science, it has been noted that the *in vitro* antioxidant capacity of phytochemicals does not reflect the real health benefits. Thus, simulation process of *in vitro* human digestion is expressed as an important initial step in the analysis of the activity profile of phytochemicals. Exactly, the biological properties of phytochemicals found in the plants are influenced by the metabolism, bioaccessebility and bioavailability of these molecules in the body. To put it in another way, molecules are needed to be bioaccessible so as to exhibit their bioactivity after biotranformation. The bioavailability of phytochemicals is attached to diversified factors which include chemical state of compound, the molecular weight, differences in enzymatic digestion, absorption, plant matrix, polarity, interactions with macromolecules, etc. GI digestion is the most crucial factor among them. Bioactive ingredients, especially of natural origin, can expose chemical and structural changes that might cause variations in the biological activity during the digestion process. Whereby, tracing the changes occurring throughout digestion, for instance, enzymatic activity, altered pH and mechanical action is significant in the evaluation of bioactivity and bioavailability (123-125).

It is clear that the *in vitro* simulation method cannot contain all stages of the human digestive system the reason is that it does not completely forge the active transportation processes. Nevertheless, when *in vitro* digestion method is compared with *in vivo* and clinical investigations, it is simpler, cheaper and more reproducible. Moreover, the method makes possible the scanning of a broad range of samples and experimental conditions. It also permits the estimation of phytochemical modifications interrelated to the GI digestion (14, 105, 123).

Clematis species (Ranunculaceae), including particularly in the treatment of rheumatic diseases in Turkey, are used to eliminate the symptoms of many diseases. Among these species, *Clematis viticella* L. aerial parts have been widely used traditionally in rheumatic complaints in Kocaeli and its vicinity, but the number of studies conducted is limited (15). The chemical composition of ethanolic extract of *Clematis viticella* aerial parts was investigated first time by Kırmızıbekmez et al. in 2018. At the end of this study, flavonoid glycosides, phenolic acids and one phenylethanol derivative were isolated. Later, compounds which are isolated from *C. viticella* were assessed for anti-inflammatory activities on inhibitory effects on the release of neopterin, NO and TNF-α markers. Apart from this study, there is not biological activity and phytochemical studies on *Clematis viticella* are available (76). In the light of these studies, we planned to appraise the phytochemical profile and the antioxidant capacity of *Clematis viticella* aerial parts growing in Turkey, before and after simulation model of *in vitro* human digestion.

Firstly, total phenol, phenolic acid, flavonoid, proanthocyanidin and saponin contents of *Clematis viticella* were evaluated before and after simulated human digestion. However, the content of total proanthocyanidin in both extract and digestive products has not been determined. Although total phenol, phenolic acid, flavonoid and saponin contents were found the most in ND fractions, they were found the least in IN and IN+OUT fractions and no significant difference was observed between IN and IN+OUT fractions, except total saponin content. Due to the fact that total saponin content was strikingly reduced in bioavailable IN fraction (126). This sharp decrease in total contents can be related to pH changes in the digestion medium, particularly the alkaline pH of the intestines. The other cause of the change is the impact of GI digestive enzymes because they ease the release of phytochemicals from the sample matrix. Further different studies have shown that phenolic compounds are not stable and they are exposed to hydrolysis after GI digestion (105, 127). Several authors have also reported that the total phenol content of different plant extracts is reduced after simulated human digestion (128, 129). Conversely, there is also a study showing that total phenol content increases after intestinal phase (118). These variations can result from the diversities in the digestion simulation process or directly from the studied sample.

Antioxidant capacities of both digested and non-digested samples were demonstrated using different methods. Experiments with different mechanisms are needed to fully evaluate antioxidant activity. The antioxidant activity of different *Clematis* species (*C. armandii*, *C. chinensis*, *C. flammula*, *C. rehderiana* and *C. trichotoma*) was investigated using various methods. Different extracts of these plants exerted significant *in vitro* antioxidant activity. However, in none of these studies the GI digestion process was taken into account and they were only performed *in vitro* (48-51).

Both free radical scavenging activities and metal reducing activities of the samples are investigated in the present work. Moreover, total antioxidant capacities of digested and non-digested samples were calculated. The DPPH radical-scavenging activity of the sample slightly decreased after the gastric phase of the digestion. Nonetheless, that of sample significantly was reduced after intestinal digestion and bioavailable IN fraction showed the least activity. The results of DMPD radical-scavenging activity were almost identical to those of DPPH radical-scavenging activity. It was also found the least in IN fraction. There was a 44.54% loss of activity in the IN fraction of extract compared to its non-digested counterpart. Similarly, the FRAP and CUPRAC activities of the samples were also reduced following the simulated digestion process. However, metal reducing activities of the samples were found the least in IN+OUT fractions. The results also exhibited a reduction in the total antioxidant capacity (TOAC) of the samples after simulation process of *in vitro* human digestion. The reduction of antioxidant activity after *in vitro* digestion simulation was shown by different studies (14, 127, 130).

When all the results are considered, the decrease in the antioxidant activity may be derived from the lower concentration of phenolic compounds after the simulation of GI digestion compared to nondigested samples. pH alterations might induce the changes in the antioxidant activity, as well (127, 130). Moreover, the interaction of antioxidant phenolics with other compounds of the samples such as minerals, vitamins, volatile compounds might result in changes in their bioactivity and bioavailability, as well (129, 131).

Clematis viticella should be considered as a good source of bioaccessible antioxidants and related phytochemical substances in phytotherapy which is a form of

complementary therapy applied through the use of drugs and preparations made from drug to assist in treatment to protect against diseases to heal and alleviate diseases.

Taking into consideration one can reach the result that the simulation process of *in vitro* human digestion significantly affected the antioxidant capacity of *Clematis viticella*. Moreover, this study showed potential oral bioavailability and bioaccessibility of total phenol, phenolic acid, flavonoid, and saponin contents found in the aerial parts of *Clematis viticella*. The results showed that both phenolic content and antioxidant activity decreased after simulated human digestion. Furthermore, it should be considered that non-phenolic compounds conduce to antioxidant capacity. Consequently, these results indicate that it resulted from the complicated interaction of compounds in the plant matrix in an *in vitro* simulated human digestion.

The data obtained from this study has provided a scientific explanation for the ethnopharmacological use of *Clematis viticella* by both the activity and the chemical analyses. In addition, the antioxidant activity profile and phytochemical content of *Clematis viticella* are investigated for the first time in this study by subjecting it to the *in vitro* simulation model of the human digestive tract.

This thesis will be a pioneer for future researches which will be held in order to investigate the antioxidant activity mechanism or the identification of effective components of *Clematis viticella*.

6. CONCLUSION

This study was designed to investigate both antioxidant activity potentials and total phytochemical profiles of the 80% methanolic extract and the gastrointestinal digestion products of *Clematis viticella* aerial parts by subjecting it to the *in vitro* simulation model of the human digestive tract. By this way, it was aimed to show the relationship between antioxidant activity and phenolic profile.

The results obtained from this study exhibited that *Clematis viticella* aerial parts showed antioxidant activity potential. The *in vitro* tests displayed that samples had free radical-scavenging activity (DPPH radical-scavenging activity and DMPD radical-scavenging activity) and the capacity to reduce metal ions that participate in the free radical generation (Ferric Reducing Antioxidant Power, Cupric Reducing Antioxidant Capacity and Total Antioxidant Capacity). Non-digested samples showed the highest antioxidant activity in all tests. Besides, the lowest antioxidant activity was observed after intestinal digestion.

At the same time, this study showed potential oral bioavailability and bioaccessibility of total phenol, phenolic acid, flavanoid, and saponin content found in the aerial parts of *Clematis viticella*. The phenolic compound content, such as antioxidant activity, also decreased after simulated human digestion.

The changes in the antioxidant activity and phenolic profile may be affected by pH alterations. In addition, the interaction of antioxidant phytochemicals with other compounds of the samples such as minerals, vitamins, volatile compounds can also result in changes in their bioavailability and bioactivity. Furthermore, it should be considered that non-phenolic compounds conduce to antioxidant capacity. Consequently, these results indicate that it resulted from the complicated interaction of compounds in the plant matrix in an *in vitro* simulated human digestion.

According to the results obtained, it is shown that *Clematis viticella* has high antioxidant activity because it contains high phenolic compounds and saponins. It is recommended that *Clematis* species which is known to be used for alternative treatment among the population should be encouraged to medical use by working on advanced phytochemical studies.

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