T.C.
YEDITEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF PHARMACOGNOSY

COMPARATIVE CHEMICAL AND BIOACTIVITY STUDIES ON AN ENDEMIC SPECIES *CRATAEGUS TURCICUS* DÖNMEZ AND SOME *CRATAEGUS* SPECIES GROWING IN TÜRKİYE

DOCTOR OF PHILOSOPHY THESIS

Tansu TURNALAR ÜLGER, Pharm.

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Assoc. Prof. Dr. Etil Güzelmeriç

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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 ..... and numbered .....

Prof. Dr. Bayram YILMAZ

Director of Institute of Health Science

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 has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

06.11.2023

Tansu Turnalar Ülger

DEDICATION

This thesis is dedicated to my beloved family with deepest love and gratitude.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

12-HHT: 12-hydroxyheptadecatrienoic Acid

12-LOX: 12-Lipooxygenase

AA: Arachidonic Acid

ABTS: 2,2’azinobis(3-ethylbenzothiazoline-6-sulfonic acid

Ac: Acetyl

ADC2: Automatic Developing Chamber 2

Al(III): Aluminum Ion

ALT: Alanine Aminotransferase

ANOVA: Analysis of Variance

AST: Aspartate Aminotransferase

BHA: Butylated Hydroxylanisole

C.M.: *Crataegus monogyna*

C.O.: *Crataegus orientalis*

C.P.: *Cratageus pentagyna*

C.T.: *Crataegus turcicus*

C3GE: Cyanidin-3-*O*-glucoside Equivalent

CCE: Cyanidin Chloride Equivalent

CE: Catechin Equivalent

COX-1: Cyclooxygenase-1

Ctrl+LPS: Control Group Only Stimulated With LPS

Cu: Copper

Cu(I)-Nc: Bis(neocuprione) copper (I) cation

Cuprac: Cupric Reducing Antioxidant Capacity

$CuSO_4$: Copper(II) sulfate

COX-2: Cyclooxygenase-2

DAD: Diode Array Detector

DDPH: α,α-diphenyl-β-picrylhydrazyl

DMEM: Dulbecco’s Modified Eagle's Medium

EFAs: Essential Fatty Acids

EGCG-E: Epigallocatechin Gallate Equivalent

F-C: Folin-Ciocalteu

Fe^{3+}: Ferric Ion

Fe^{3+}-TPTZ: ferric 2,4,6-tripyridyl-s-triazine

FRAP: Ferric Reducing Antioxidant Power Assay

GAE: Gallic Acid Equivalent

Gal: Galactose

Gall: Galloyl

Glc: Glucose

Glcro: Glucronide

GRAS: Generally Recognised As Safe

H_2SO_4: Sulfuric Acid

$H_3PMo_{12}O_{40}$: Phosphomolybdic Acid ($H_3PMo_{12}O_{40}$)

$H_3PW_{12}O_{40}$: Phosphotungstic Acid ($H_3PW_{12}O_{40}$)

HCl: Hydrochloric Acid

HDL-C: High-Density Lipoprotein Cholesterol

HFD: High-Fat Diet

HPLC: High-Performance Liquid Chromatography

HPTLC: High-Performance Thin-Layer Chromatography

IC_{50}: The Half Maximal Inhibitory Concentration

ICH: International Conference on Harmonisation

IL-6: Interleukin-6

IND: Indomethacin

iNOS: Inducible Nitric Oxide Synthase

IUPAC: International Union of Pure and Applied Chemistry

LDH: Lactate Dehydrogenase

LDL-C: Low-Density Lipoprotein Cholesterol

L-NAME: N^{ω} nitro-L-arginine methyl ester

LOD: Limit of Detection

LOQ: Limit of Quantification

LPO: Lipid Peroxidation

LPS: Lipopolysaccharides (from *Escherichia coli*)

MeOH: Methanol

Mo: Molybdate (Mo)

MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole)

NAFLD: Non-Alcoholic Fatty Liver Disease

NANC: Neurons

$NaNO_2$: Sodium nitrite

NaOH: Sodium Hydroxide

NO: Nitric Oxide

NP: Natural Product

NYHA: New York Hearth Association

PBS: Phosphate-Buffered Saline

PEG 400: Polyethylene Glycol 400

Pen: Pentose

PGD_2: Prostaglandin D_2

PGE_2: Prostaglandin E_2

$PGF_{2\alpha}$: Prostaglandin F_2 Alpha

PGI_2: Prostaglandin I_2

PLAs: Phospholipases

QE: Quercetin Equivalent

R_F: Retention Factor

Rha: Rhamnose

ROS: Reactive Oxygen Species

RSD: Relative Standard Deviation

RT-PCR: Reverse Transcription Polymerase Chain Reaction

SD/S: Standard Deviation per Second

SD: Sprague-Dawley

TAC: Total Anthocyanin Content

TC: Total Cholesterol

TCM: Traditional Chinese Medicine

TE: Trolox Equivalent

$TEAC_{FRAP}$: Trolox Equivalent Antioxidant Capacity of FRAP

TFC: Total Flavonoid Content

TG: Triglycerides

THF: Tetrahydrofuran

TLC: Thin-Layer Chromatography

TNF- α: Tumour Necrosis Factor-alpha

TPAC: Total Proanthocyanidin Content

TPC: Total Phenolic Content

TPTZ: 2,4,6-Tris(2-pyridyl)-s-triazine

t_R: Retention Time

Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

TXA2: Thromboxane A_2

TXB2: Thromboxane B_2

WHO: World Health Organization

ABSTRACT

Ülger Turnalar T, (2023), Comparative Chemical and Bioactivity Studies on an Endemic Species *Crataegus turcicus* Dönmez and Some *Crataegus* Species Growing In Türkiye. Yeditepe University, Institute of Health Sciences, Department of Pharmacognosy, Ph.D. Thesis, İstanbul.

Crataegus turcicus Dönmez is a tree endemic species to Türkiye. This study aimed to assess its flower-bearing branches, leaves, and fruits in comparison with other well-known *Crataegus* species (*C. monogyna*, *C. pentagyna*, and *C. orientalis*) in terms of chemical composition and bioactivities. Firstly, the content of total phenolics (TPC), flavonoids (TFC), proanthocyanidin (TPAC), and anthocyanidin (TAC) were evaluated in different organs of *Crataegus* species. The highest TPAC was found in the hydroalcoholic extract of *C. turcicus* flower-bearing branches. Moreover, its all extracts had comparatively higher amounts of TPC, TFC, and TAC compared to other *Crataegus* species. The chemical screening by high-performance thin-layer chromatography (HPTLC) showed that *C. turcicus* plant parts were rich in chlorogenic acid, neochlorogenic acid, quercetin, vitexin derivatives, epicatechin, procyanidin, and cyanidin glycosides and their quantities were evaluated by high-performance liquid chromatography (HPLC). Based on *in-vitro* antioxidant activity tests, the flower-bearing branches of *C. turcicus* showed the highest antioxidant activity by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) test among the assessed antioxidant assays. Additionally, hydroalcoholic extracts of *C. turcicus* at 1 mg/mL concentration significantly reduced LPS-induced nitric oxide, tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) production in RAW 264.7 cells more potently than indomethacin (positive control). In addition to its remarkable anti-inflammatory activity, *C. turcicus* extracts exhibited analgesic activity by reducing prostaglandin E_2 (PGE_2) levels.

Keywords: *Crataegus* L.; *Crataegus turcicus*; phenolic profiling; antioxidant activity; anti-inflammatory activity; analgesic activity

ÖZET

Ülger Turnalar T, (2023), Türkiye'de Yetişen Bazı *Crataegus* Türlerinin, Endemik Tür Olan *Crataegus turcicus* ile Karşılaştırmalı Kimyasal ve Biyolojik Aktivite Çalışmaları. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmakognozi ABD, Doktora Tezi, İstanbul.

Crataegus turcicus Dönmez Türkiye'ye endemik bir ağaç türüdür. Bu çalışmada *Crataegus turcicus* Dönmez bitkisinin çiçekli dalları, yaprakları ve meyvelerinin kimyasal bileşim ve biyoaktivite çalışmaları açısından diğer iyi bilinen *Crataegus* türleri (*C. monogyna, C. pentagyna* ve *C. orientalis*) ile karşılaştırılması amaçlanmıştır. İlk olarak toplam fenolik (TFMM), flavonoit (TFM), proantosiyanidin (TPM), ve antosiyanidin (TAM) madde miktarları saptandı. En yüksek TPM değeri *C.turcicus'*un çiçekli dallarının hidroalkolik ekstresinde bulundu. Ayrıca, tüm bitki organlarının, diğer *Crataegus* türleri ile karşılaştırıldığında, TFMM, TPM ve TAM açısından daha yüksek miktarlarda olduğu gözlendi. Yüksek Performaslı İnce Tabaka Kromatografisi (YPİTK) sonuçları, *C. turcicus*'un farklı bitki kısımlarının klorojenik asit, neoklorojenik asit, kersetin, vitexin türevleri, epikateşin, prosiyanidin ve siyanidin glikozitleri bakımından zengin olduğunu ortaya koydu ve kantitatif analiz Yüksek Performanslı Sıvı Kromatografisi (YPSK) ile gerçekleştirildi. Bir dizi *in vitro* antioksidan aktivite sonucuna göre, *C. turcicus'*un çiçekli dalları, 2,2,-difenil-1-pikrilhidrazil (DPPH) testinde en yüksek antioksidan aktiviteyi gösterdi. Ayrıca, *C. turcicus'*un 1 mg/mL konsantrasyonundaki hidroalkolik ekstreleri, indometazinden (pozitif kontrol) daha etkili bir şekilde RAW 264.7 hücrelerinde LPS ile indüklenen nitrik oksit, tümör nekroz faktör-alfa (TNF-α) ve interlökin-6 (IL-6) üretimini azalttı. *C. turcicus*, anti-enflamatuvar etkinliğinin yanı sıra, prostaglandin E_2 (PGE_2) seviyesini azaltarak analjezik aktivite de gösterdi.

Anahtar kelimeler: *Crataegus* L.; *Crataegus turcicus*; fenolik profili; antioksidan aktivite; antienflamatuvar aktivite; analjezik aktivite

1. INTRODUCTION AND PURPOSE

Crataegus species are small thorny trees or shrubs with 3-7 lobed, 1.5-5 cm long, smooth, broad-ovate or obovate leaves which have toothed margins. The genus *Crataegus* has white to pink and pink to red colored flowers in clusters, and red, black or yellow drupe fruits with meaty white flesh. Each fruit has calyx remnants at the top (1, 2). Also, some *Crataegus* species can grow up to 12 m long (2). *Crataegus* species are mostly distributed in Europe, Asia, North Africa, and North America (2, 3). This genus includes around 280 species (4). In Türkiye, 24 naturally grown species of the genus *Crataegus* are indicated (5). These species are called "alıç, yemişen, geyik dikeni, alış, alaş, aluş, elaç, halıç, haluç, kızlar yemişi, kuş yemişi, yemişen, yemişken, and yemişen" among the local people (6). The *Crataegus* plant is known as hawthorn, considering its names in other languages, the plant got its name from its thorny and bushy physical characteristics (2). When the phytochemical profile of the all parts of *Crataegus* species is examined, phenolic groups such as flavonoids (hyperoside, rutin, isoquercitrin, vitexin, vitexin-2"-*O*-rhamnoside, orientin, isoorientin), oligomeric procyanidins (procyanidins B2 and B5, epicatechin) and some phenolic acids (chlorogenic acid, neochlorogenic acid, protocatechuic acid, caffeic acid) are found as major compounds (7-9). Oligomeric procyanidins are predominant in fruits of the *Crataegus* species, while flavonols, flavonol glycosides and C-glycosyl flavones are dominant in the leaves and flowers of the *Crataegus* species (9).

In medieval times, fruits of *Crataegus* species were used to heal digestive disorders and roots of the plant were applied on small injuries. In Europe, traditional usage of *Crataegus* species (fruits, leaves and flowers) is on heart diseases due to their spasmolytic, cardiovascular-enhancing, hypotensive, and antiatherosclerotic effects. Therefore, European scientists started their first experiments to reveal the pharmacological activities of *Crataegus* species on heart diseases (10). After that the use of *Crataegus* species against heart diseases became popular in Europe. Traditional usage of *Crataegus* species in México is different, *Crataegus* species have been utilized to heal respiratory ailments including cough, flu, bronchitis and asthma (3). In Traditional Chinese Medicine (TCM) berries of Chinese hawthorn have been used for some diseases such as disturbed digestion, diarrhea, feeling of fullness, in appetite problems of children, arteriosclerosis, hypercholesterolemia, improve circulation, remove blood stasis, hyperlipidemia, and hypertension (3, 10). In Türkiye, fruits of hawthorn have been used

traditionally for diuretic and antidiarrheal purposes (11). Present studies on different parts of *Crataegus* species clarified that the plant has numerous biological activities and effects such as hypotensive (12), hypolipidemic (13), antioxidant (14), antimicrobial (15), gastroprotective (16), anti-inflammatory (16) activities and cardioprotective (17), anti-anxiety (18), anti-depression (18), cardiotonic (19), anti-atherogenic (20, 21), hypoglycemic (22), and diuretic (23) effects.

Overproduction of free radicals and reactive metabolites, also known as reactive oxygen species (ROS) or oxidants, in organisms results in oxidative stress. Oxidative stress affects the whole organism, this effect leads to damage of important cellular molecules (24, 25). To prevent the toxic effect of oxidative stress, organism's defense mechanism steps in. When organism's protective system is insufficient, antioxidants play a crucial role in neutralizing toxic effect of ROS by converting them into nonradical metabolites (26). The presence of ROS can contribute to the development of various illnesses, one of which is chronic inflammation (24). Inflammation is a naturally occurring and biological feedback of the organism to pathogens and it is relevant to many illnesses for instance bacterial and viral infections, contact with allergens, exposure to radiation and harmful substances, autoimmune and chronic diseases, excessive weight, the consumption of alcoholic beverages, smoking, and a diet rich in calories (25). Bioactive natural substances, particularly phenolic compounds, can potentially function as a preventive treatment owing to their antioxidant and anti-inflammatory properties. One of the chemically and pharmacologically well defined food supplements of *Crataegus* spp. (Hawthorn) could play a significant role in averting oxidative stress, given its diverse array of phenolic components (27, 28). However, there are limited studies on *Crataegus turcicus* Dönmez which is endemic to Türkiye. It was first identified in the Ardanuç (Artvin, Türkiye) district by Dönmez and Dönmez (29).

Within the scope of this study, hydroalcoholic extracts of flower-bearing branches, leaves and fruits of the *C. turcicus* with other *Crataegus* species in this experiment (*C. pentagyna*, *C. monogyna* which are registered in European Pharmacopoeia as an officinal plant (30) and *C. orientalis*) were comparatively evaluated in terms of chemical composition, antioxidant, anti-inflammatory, and analgesic activities. Firstly, total phenolic content (TPC), total flavonoid content (TFC), total proanthocyanidin content (TPAC), and total anthocyanin content (TAC) were assessed. Then, high-performance thin-layer chromatographic (HPTLC) method was used to

identify chlorogenic acid, neochlorogenic acid, quercetin, vitexin, vitexin-2"-*O*-rhamnoside, epicatechin, procyanidin, and cyanidin-3-*O*-glycoside and their quantities except for cyanidin-3-*O*-glycoside were evaluated by high-performance liquid chromatography (HPLC). Furthermore, antioxidant, anti-inflammatory, and analgesic activities of the extract were assessed in *in-vitro* test. To evaluate antioxidant activity, several *in-vitro* assays were performed including DPPH radical scavenging, ABTS radical cation decolorization, FRAP, and CUPRAC assays. Additionally, inhibition of lipopolysaccharide (LPS)-induced nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) production were determined to evaluate anti-inflammatory activity. Besides, analgesic activity was evaluated by prostaglandin E_2 (PGE_2) inhibition in LPS-activated RAW264.7 cells.

2. LITERATURE REVIEW

2.1. Botanical Information

2.1.1. Rosaceae Family

Rosaceae family consists of plants that can be either woody or herbaceous and in some cases, they may have thorns. The leaves of these plants are typically arranged alternately, often with stipules, and frequently have a serrated edge. Leaves can be simple or compound. Inflorescence often happens on spur shoots and plants bear them in trees and shrubs. Flowers are hermaphrodite or unisexual. They are actinomorphic, perigynous and epigynous. Sepals are 4 or 5 and mostly free. Epicalyx can be present or absent. Petals are 4 or 5, free, or absent. Stamens are one or numerous. Ovary is superior to inferior and consists of one or many carpels. Fruits have dry or fleshy receptacle. Seeds are usually without endosperm. The dichotomous key provided in 'The Flora of Turkey and the East Aegean Islands' to identify genera of Rosaceae family is given below (31).

1. Leaves simple, sometimes ± deeply lobed or dissected
 2. Annual or perennial herbs; flowers apetalous; epicalyx present
  3. Stamens l (-2); small annual with dense leaf-opposed cymes…….....***22. Aphanes***
  3. Stamens 4(-10); perennial, cymes corymbose or panicled……..***21. Alchemilla***
 2. Shrubs or trees; flowers with petals; epicalyx absent
  4. Ovary superior; fruit a drupe with a single stone (sometimes dehiscent), or composed of several follicles
   5. Carpels 5; fruit composed of several follicles; stipules absent…....……..***1. Spiraea***
   5. Carpel 1; fruit a drupe; stipules present, sometimes deciduous *(Primus* group)
    6. Flowers solitary, fasciculate, umbellate or in few-flowered (up to 12-flowered) lax racemes
     7. Fruit dehiscent; pericarp becoming dry (Almonds)…….***9. Amygdalus***
     7. Fruit indehiscent; pericarp fleshy and juicy
      8. Drupes glabrous, or if sometimes puberulent or a little pubescent then small, c. 1 cm diam.
       9. Drupes pruinose; leaves convolute in bud (Plums, etc.)……...***5. Prunus***
       9. Drupes without a bloom; leaves conduplicate in bud (Cherries).....***6. Cerasus***
      8. Drupes velutinous or tomentose, or exceptionally glabrous then large, over 2 cm

diam.

10. Stones smooth; leaves convolute in bud, broadly ovate, at most 1½ x as long as broad(Apricot)…….....……...……***7. Armeniaca***

10. Stones distinctly pitted; leaves conduplicate in bud, elliptic-lanceolate to oblong-lanceolate, c. 3 x as long as broad (Peach)…….…...***8.Persica***

6. Flowers 15 or more, in elongate but compact racemes

11. Leaves deciduous; racemes with leaves at base……..***3. Padus***

11. Leaves evergreen; racemes with bracts but no leaves at base….***4. Laurocerasus***

4. Ovary ± inferior; fruit a pome (see family description)

12. Flowers solitary

13. Flowers less than 1 cm diam.; fruit red or black……***25. Cotoneaster***

13. Flowers more than 1 cm diam.; fruit brown, greenish or yellow

14. Branches unarmed; fruit more than 5 cm long, with leathery carpel walls (Quince)……..….***30. Cydonia***

14. Branches usually thorny; fruit less than 5 cm long, with bony carpel walls (Medlar)…….…….***24. Mespilus***

12. Flowers in 2- to many-flowered inflorescences

15. At least some leaves lobed (occasionally almost all leaves with lobes replaced by deep teeth, then leaves cuneate)

16. Flowers in compound corymbs……..***28. Sorbus***

16. Flowers in simple corymbs or umbels

17. Often spiny; walls of carpels becoming woody in fruit……***27. Crataegus***

17. Unarmed; walls of carpels becoming cartilaginous in fruit

18. Leaves trilobed; sepals persistent in fruit…… ***33. Eriolobus***

18. Leaves with more than 3 lobes; sepals deciduous in fruit

32. x Malosorbus

15. Leaves not lobed

19. Leaf margin entire

20. Petals 5 mm or less, rounded; carpel walls becoming woody in fruit

25. Cotoneaster

20. Petals 10-15 mm, elongate: carpel walls becoming cartilaginous in fruit

35. Amelanchier

19. Leaf margin serrate or dentate

21. Inflorescence a compound corymb or panicle

22. Leaves up to 25 cm long; inflorescence a panicle; fruit yellow, pyriform or ellipsoidal, 20-30(-100) mm (Loquat)……. ***29. Eriobotrya***

22. Leaves up to 11 cm long; inflorescence a compound corymb; fruit red. globose, 9-16 mm diam.

23. Shrub, usually spiny; leaves evergreen; carpels completely connate. ***26. Pyracantha***

23. Unarmed tree; leaves deciduous; carpels partly free……...***28. Sorbus***

21. Inflorescence a simple corymb, umbel or raceme

24. Inflorescence a raceme; ovary and fruit incompletely 4-10-locular with 1 ovule per loculus…….***35. Amelanchier***

24. Inflorescence an umbel or corymb; ovary and fruit 2-5-locular with 2 ovules per loculus

25. Fruit globose to pyriform with numerous grit cells; styles free (Pears) ***34. Pyrus***

25. Fruit globose and umbilicate without or with only a few grit cells; styles connate at base (Apples)……. ***31. Malus***

1. Leaves compound, with separate leaflets

26. Small tree, unarmed; ovary inferior; fruit a pome…….***28. Sorbus***

26. Shrubs, sometimes armed, or herbs; ovary superior; fruit not a pome

27. Armed shrubs

28. Inflorescence a dense, head-like spike; petals absent; armature of persistent spiny branch-tips……..……..***19. Sarcopoterium***

28. Inflorescence lax, paniculate or corymbose; petals present; armature of prickles scattered along branches

29. Fruit of several fleshy druplets borne on **a** convex receptacle ***11. Rubus***

29. Fruit a deep, fleshy, urceolate hypanthium containing several longstyled achenes……..…….***23. Rosa***

27. Herbs or unarmed shrubs

30. All leaves ternate or palmate

31. Epicalyx absent; fruit of several fleshy druplets……..***11. Rubus***

31. Epicalyx present; fruit of dry achenes (in *Fragaria* borne on a fleshy receptacle)

32. Petals absent…….***21. Alchemilla***

32. Petals present

33. Petals 1-2 mm; stamens 5(-10)**……*14. Sibbaldia***

33. Petals more than 2 mm; stamens usually more than 10

34. Leaves ternate; flowers white; receptacle much enlarged in fruit and fleshy…….……*13. Fragaria*

34. Leaves palmate or ternate; flowers white, yellow or reddish; receptacle neither enlarged nor fleshy in fruit……*12. Potentilla*

30. At least basal leaves 1-2-pinnate

35. Flower and fruit surrounded by a 10-fid involucre *18. Aremonia*

35. Involucre absent

36. Epicalyx present

37. Styles long, at least part of styles persistent in fruit

38. Distal part of styles deciduous; corolla shorter than calyx; fruit of 50-250 achenes……*15. Geum*

38. Distal part of style persistent; corolla longer than calyx; fruit of 5-15 achenes……..……..……..*16. Orthurus*

37. Styles short, in fruit deciduous in their entirety…*12. Potentilla*

36. Epicalyx absent

39. Petals absent; inflorescence a dense, head-like spike *20. Sanguisorba*

39. Petals present; inflorescence a lax raceme, paniculate cyme, panicle or elongate spike

40. Inflorescence a lax spike or raceme; receptacle with a crown of hooked spines……...……..……*17. Agrimonia*

40. Inflorescence a panicle or paniculate cyme; receptacle spineless

41. Stipules present; leaves 1-pinnate; fruit a group of achenes *10. Filipendula*

41. Stipules absent; leaves 2-pinnate; fruit of 3(-5) cartilaginous follicles……..……..….*2. Aruncus*

2.1.2. *Crataegus* genus

Crataegus is a genus comprising of deciduous trees or shrubs that commonly bear thorns. Leaves are alternate, simple and exhibit various shapes, ranging from lobed to subpinnate, with smooth or serrate edges. The inflorescence is corymbose and emerges from spur shoots. Flowers are typically composed of 5 petals and lack an epicalyx. Hypanthium is connected to the carpels. Petals are typically white or pink-colored and longer than the persistent sepals. Stamens are range from 5 to 25. Carpels are range from 1 to 5 and typically united on the inner margin, at least near the base. Fruits are drupaceous and usually with a mealy flesh. Fruits come in a range of colors, including yellow, red, dark purple or black. Hybrids are common within this genus. It is usually necessary to have fertile material, preferably encompassing both flower and fruit, for reliable identification. The dichotomous key provided in 'The Flora of Turkey and the East Aegean Islands' to identify the genus *Crataegus* is given below (31). However, *Crataegus turcicus* is not available in the 'The Flora of Turkey and the East Aegean Islands' since it has not been updated yet.

1. Styles 3-5; fruit with 3-5 pyrenes
 2. Inflorescence lax; peduncles and pedicels long
  3. Leaves broadly cuneate or truncate at base; lobes spreading horizontally ***1. pentagyna***
  3. Leaves cuneate or narrowly cuneate at base; lobes diverging at an acute angle ***2. davisii***
 2. Inflorescence dense; peduncles and pedicels short
  4. Leaves glandular-serrate
   5. Leaves l.5-2.5(-5) cm, sparsely villous, green……..***3. tanacetifolia***
   5. Leaves 3-5 cm, densely grey-villous…… ***4. x bornmuellcri***
  4. Leaves eglandular
   6. Fruit reddish-orange, pyrenes (4-)5; calyx reflexed in fruit……...…...***5. orientalis***
   6. Fruit red; pyrenes (2-)3-4; calyx ± spreading in fruit…….. ***6. szovitsii***
1. Styles 1-3; fruit with 1-3 pyrenes
   7. Styles 2-3; fruit yellow or orange, with 2-3 pyrenes
    8. Branches unarmed; fruit 15-25 mm diam……***7. pontica***
    8. Branches thorny; fruit 12-18 mm diam……***8. aronica***

7. Styles 1-2; fruit red to purple (or colour unknown), with 1-2 pyrenes

9. Leaves with at least lower divisions almost or quite divided to the midrib

10. Leaves persistently pilose above, lobes at most shallowly serrate; flowers 8-15 mm diam……..….***16. monogyna subsp. azarella***

10. Leaves glabrous above when mature, lobes deeply serrate; flowers c. 17 mm diam…….. ***14. stevenii***

9. Leaf lobes not divided to the midrib

11. Styles 2; fruit with 2 pyrenes

12. Inflorescence glabrous; leaves glabrous or with a few hairs on the under¬ side of the midrib

13. Leaves 3-lobed, base narrowly cuneate; fruit ovoid-ellipsoid, 6-8 mm diam……...….***9. sinaica***

13. Leaves usually 5-7-lobed, base broadly cuneate; fruit globose, 12-18 mm diam……..***12. atrosanguinea***

12. Inflorescence and leaves (especially beneath) pubescent

14. Upper leaves on the flower shoots narrowly cuneate; fruit ovoidglobose ***10. meyeri***

14. Upper leaves on flower shoots broadly cuneate; fruit ellipsoidal ***11. dikmensis***

11. Styles 1; fruit with 1 pyrene

15. Leaves 1-3 cm; fruit with erect sepals…….***17. microphylla***

15. Leaves usually more than 3 cm; fruit with reflexed sepals

16. Leaves green beneath, outer margin of at least the lower lobes serrate for c. 1/2 their length; fruit dark purple……...***13. curvisepala***

16. Leaves green or glaucous beneath, if green then lobes shallow or re¬ placed by teeth, lobes serrate at apex only; fruit red, brownish-red or colour unknown

17. Leaves on fertile shoots usually 5-Iobed, upper lobes usually with narrow incisions……..***16. monogyna***

17. Leaves on fertile shoots 3-lobed or with lobes replaced by teeth

18. Leaves green beneath, with 3 shallow lobes or lobes replaced by teeth ***9. sinaica***

18. Leaves glaucous beneath, with 3 deeply incised lobes ***15. pscudoheterophylla***

2.1.3. *Crataegus turcicus* Dönmez

C. turcicus is a small tree or shrub and can grow up to 6 meters. The plant is thornless or rarely thorny, if exists length of thorns range from 15 to 30 (or even up to 50) mm. The twigs of this plant can be sparsely to moderately villous. The buds are around 1.5 to 2 mm in length and 1 to 2.3 mm in diameter. The leaves of *C. turcicus* are green and sparsely villous on the upper surface and slightly greyish-green and villous on the lower surface. The margin of the leaves is irregularly serrate, and the base is widely cuneate to truncate. Lobes of the leaves are acute, although can be rarely obtuse. Petiole is 5-15 to 20-33mm. Stipules are 5-10 to 2-5 mm in length, D-shaped to semilunar, coarsely serrate to lacerate and have 5 to 15 teeth, although some are entirely serrate. Hypanthium is 3-4 X 3-4 mm. Sepals are 2-3.5 X 1-2 mm, triangular in shape, shortly cuspidate and with an entire margin. Petals are 4-6 X 4-5 mm. The fruit is slightly oblong, exhibiting dark red, sparsely villous all over. The flesh of the fruit is yellowish and at maturity, sepals become reflexed (29).

Flowering of *C. turcicus* is from June to July. Fruits ripen around September to October. The distribution of *C. turcicus* is known from only Artvin (Ardanuç) district and its altitude is 1800-1900 m (29).

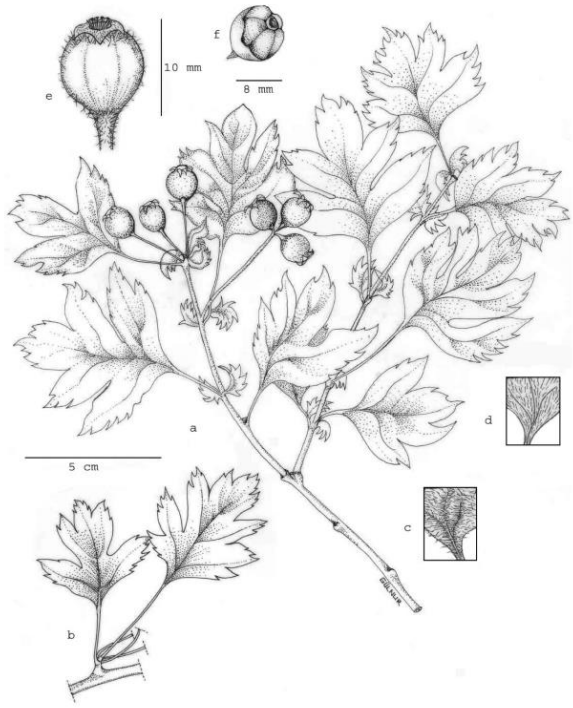


Figure 1. *Crataegus* turcicus Dönmez (29).

2.2. Traditional Medicinal Uses of *Crataegus* Species

The use of *Crataegus* species dates back to ancient times, especially as folk medicine (32). Some authors suggest that the first mention of the plant can be found in the *Tang ben Chao* pharmacopeia of 659 A.D. in Chinese medicine, while ancient Greek writers, such as Dioscorides, referred to Oxyakantha in the first century A.D., although it is still questionable whether he was referring to hawthorn. In 1753, *C. oxycantha* L. was the name given to hawthorn by Linnaeus, then Jacquin separated the species into *C. oxycantha* and *C. monogyna* (10). In the late 1800s first experiments on the clinical use of *Crataegus* sp. for heart diseases started in Europe (10). Then the use of *Crataegus* species on heart diseases became popular in Europe after that plant began to be utilized for the same purpose in North America (3).

Some *Crataegus* species which are used as folk medicine around the world include *C. pinnatifida* (China), *C. pubesens* (Mexico), *C. cuneata* (Japan), *C. laevigata* and *C. monogyna* (Europe), *C. oxycantha* and *C. aronica* (Middle East), *Cratague phaenopyrum* (America) and *Cratageus ambigua* (Russia) (10). In chinese the fruit of *C. pinnatifida* is called shanzha and in mexico the fruit of *C. mexicana* is called tejocotes (32). *Crataegus* sp. Has been classified as "traditional herbal medicinal product" by the Committee for Herbal Medicinal Products of the European Medicines Agency, based on its traditional use and because of it is generally recognized as safe (GRAS) (33).

In Arabian traditional medicine, a decoction made from the leaves and unripe fruits of *C. aronia* is utilized as a remedy for cardiovascular illnesses, cancer, diabetes and erectile dysfunction (34).

In Mexico, *Crataegus* extracts are used to treat the initial phases of diabetes (35). Furthermore, in Mexico, *Crataegus* species have been employed for the management of respiratory ailments such as cough, flu, bronchitis and asthma (3).

In China, fresh or dried fruit of *C. pinnatifida* is used for lower plasma lipids. In addition in China, fruits of *C. pinnatifida* Bunge var. Typica Schneider and *C. pinnatifida* Bunge are used to improve circulation, remove blood stasis, treat indigestion, diarrhea, abdominal pain, hyperlipidemia and hypertension (32).

In Europe, the fruit, leaves and flowers of *Crataegus* sp. have been traditionally used as agent with various medicinal properties such as spasmolytic, cardiovascular enhancing, anti-hypertensive, and antiatherosclerotic effects. (36). After the use of

Crataegus species on heart diseases became popular in Europe, plant began to be utilized for the same purpose in North America (3).

In Türkiye to treat a diverse range of ailments including asthma, flu, cold, rheumatic pain, stomachache, nephritis, hemorrhoids and cardiac diseases, different parts of *Crataegus* spp. as flower, sprout, root, and leaf have been utilized in traditional medicine. Furthermore, flower and fruit of the plant are utilized to treat diseases such as cardiovascular problems, hypertension, and diabetes. In certain local regions of Türkiye, vinegar of *Crataegus* spp. is produced and flowers and fruits of the plant are ingested as a tea (32, 37).

2.3. Biological Activity Studies on *Crataegus* species

2.3.1. Studies on Cardioprotective Activity

A randomized, placebo-controlled, double-blind clinical study was carried out on patients with congestive heart failure New York Heart Association (NYHA) class II by using *Crataegus* Extract WS® 1442. Patients were treated for 12 weeks with 240 mg/d WS® 1442 or matching placebos. In terms of exercise tolerance, WS® 1442 group showed significant improvement and the drug was well tolerated. As a result, this study showed that *Crataegus* Extract WS® 1442 could be an effective alternative treatment for congestive heart failure NYHA class II (38).

Veveris et al. (39) studied on cardioprotective effects of *Crataegus* Extract WS® 1442 by using Wistar rats with myocardial injury. WS® 1442 or placebo was given orally to Wistar rats, which were divided into different groups randomly, for 7 days. According to the result of the experiment, administering *Crataegus* Extract WS® 1442 orally to rats provided cardioprotective effects and prevented myocardial dysfunction and infarction caused by a prolonged occlusion of the left anterior descending coronary artery.

In comparison to cardioactive drugs such as ouabain, epinephrine, milrinone and propranolol, the antiarrhythmic impact of *C. oxycantha* extract was examined and it was determined that the extract had a remarkable impact in comparison to standard cardiac medications. The extract displayed antiarrhythmic activity by stimulating rhytm in inactive cardiac muscle cells. Moreover, the extract resulted in a negative chronotropic effect without blocking β-adrenergic receptors (40).

In another study, aqueous extract of *Crataegus* leaves was given to male, Wistar rats suffering from myocardial injury, for 15 days. At the end of the treatment blood

samples were collected from rats and biochemical parameters such as serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), oxidative stress levels of NO and lipid peroxidation (LPO) were measured. As a result, aqueous extract of *Crataegus* leaves demonstrated protective effect on heart against ischemia-reperfusion induced myocardial injuries (41).

Bernatoniene et al. (42) studied the effect of fruits of *C. monogyna* Jacq. on the respiration of isolated rat heart mitochondria. Male Wistar rats were utilized for this study and they were killed in accordance with the guidelines by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Purposes (License No: 0006). After that, heart mitochondria were isolated. The %70 hydroalcoholic extract of *C. monogyna* Jacq. fruits were contained 139 ± 8 mg/100 mL of total phenolic compounds,1.26 ± 0.1 mg/mL quercetin, 29.32 ± 0.5 mg/mL hyperoside, 2.64 ± 0.2 mg/mL rutin, 0.49 ± 0.01 mg/mL quercitrin, 44.92 ± 0.9 mg/mL epicatechin, 0.71 ± 0.06 mg/mL procyanidin B2 and 3.46 ± 0.03 mg/mL chlorogenic acid. After the assessment of mitochondrial respiration, membrane potential and H_2O_2 generation, their outcomes showed that the fruit extract of *C. monogyna* Jacq had a role in cardioprotective activity.

In a randomized, double-blind, and placebo-controlled study, *Crataegus* Extract WS® 1442 was used 1800 mg or 900 mg of doses to patients with chronic congestive heart failure (NYHA class III). According to their results, patients who were administered 1800 mg of WS® 1442 showed a statistically remarkable increase in their maximum workload tolerance during cycling exercise, compared to both those who received a placebo and those who were given 900 mg of WS® 1442, after 16 weeks of treatment. In addition, WS® 1442 significantly reduced heart failure symptoms as reported by the patients, compared to the placebo and the 1800 mg dose of WS® 1442 was rated highest in both efficacy and tolerability (43).

Jayalakshmi et al. (44) investigated influence of fruit extract of *C. oxycantha* on rat heart which had experimentally induced myocardial infarction. The study involved male albino Wistar rats that were divided into five groups with six rats in each group. The control group was labeled as Group I, while Group II received *C. oxycantha* extract for 30 days. Group III received isoproterenol via subcutaneous injection for 2 days and group IV received *C. oxycantha* extract orally for 30 days, followed by isoproterenol injection on the 31st and 32nd days. Lastly, group V was given captopril orally as a positive control for 30 days, followed by isoproterenol injection on the 31st and 32nd days. According to their results, the pretreatment of *C. oxycantha* extract helped to preserve the antioxidant

status of the mitochondria, prevented damage to mitochondrial lipids caused by isoproterenol and prevented a decrease in Kreb's cycle enzymes in the hearts of rats. Their results suggest that *C. oxycantha* extract is comparable to captopril as a cardioprotectant.

2.3.2. Studies on Anti-Hypertensive Activity

Koçyıldız et al. (45) studied the effects of *C. tanacetifolia* leaves extract on the blood pressure and structure of the coronary artery. Experiments performed with albino male Wistar rats. Induction of hypertension in rats provided with L-NAME (N^{ω} nitro-L-arginine methyl ester). Male albino Wistar rats were divided into four groups with six rats in each group. Group I as a control group received saline, Group II was hypertensive group and Group III received 100 mg/kg *C. tanacetifolia* leaves extract after induction of hypertension via L-NAME. Lastly, group IV, labeled as hypertensive group, received flavonoid compound called hyperoside. Hyperoside was produced from an extract of *C. tanacetifolia* leaves and administered at a dose of 6 mg/kg/day. According to their results, *C. tanacetifolia* leaves extract prevented the onset of high blood pressure even in the presence of NO inhibition.

Garjani et al. (46) investigated the effects of hydroalcoholic (%70), chloroform and ethylacetate extracts obtained from the *C. meyeri* flowering tops on male Wistar rats under open-chest anesthesia with myocardial ischemia. According to their findings, the hydroalcoholic extract demonstrates a notable decline in mean arterial blood pressure (hypotensive activity) even when administered at a low dosage. Specifically, the administration of 5 mg/kg resulted in a considerable reduction in mean arterial blood pressure.

Walker et al. (47) a conducted randomized controlled trial to examine the impact of *C. laevigata* extract on hypertension in patients with type 2 diabetes who were already taking prescribed medication. During the 16-week trial period, either 1200 mg of *C. laevigata* extract (n=39) or a placebo (n=40) was administered to a total of 79 patients with type 2 diabetes. Their findings revealed that the group treated with *C. laevigata* extract experienced a greater reduction in diastolic blood pressure compared to the placebo group, but no significant difference was observed between the two groups in terms of the reduction of systolic blood pressure from baseline. Thus, their study suggested that *C. laevigata* extract has a hypotensive effect.

Naderi et al. (48) demonstrated anti-hypertensive effect of leaves and flowers of the *C. curvisepala*. The experiment was a double-blind, placebo-controlled clinical trial

and conducted with 92 men and women with mild hypertension. The data was analyzed through Student's t-test for statistical evaluation. Patients received 20 drops of hydroalcoholic extracts of leaves and flowers (at a ratio of 2:1) from *C. curvisepala* or placebo three times a day for 4 months. Their results showed that after 3 months, the plant extract led to a significant decrease in both systolic and diastolic blood pressure. In conclusion, their findings indicated that hydroalcoholic extracts of leaves and flowers from *C. curvisepala* extract exhibits a time-dependent antihypertensive effect.

In a randomized, double-blind, placebo-controlled study conducted with 60 patients suffering from stage I hypertension, hawthorn extract capsule 450 mg (Willmar Schwabe Pharmaceuticals, Karlsruhe, Germany) or placebo was given twice a day for 12 weeks. Patient's blood pressure was measured while in seated position, both at the beginning of the treatment and at the end of the 4^{th}, $8^{th,}$ and 12^{th} weeks of the treatment period. Their finding demonstrated that the hawthorn extract group experienced a significant reduction in both systolic and diastolic blood pressure after 12 weeks, while placebo group showed only a non-significant decrease. These results indicated that the hawthorn extract capsule possesses a significant antihypertensive ability (49).

2.3.3. Studies on Hypolipidemic Activity

Kausar et al. (50) conducted a study on hyperlipidemic albino rats to investigate the impact of the ethanolic extract of dried berries of *C. monogyna* alone and in combination with Simvastatin on their serum lipid profile. The study lasted for a period of 8 weeks. A total of 100 albino rats were randomly assigned to 5 groups, with each group consisting of 20 animals. The groups were labeled as A (control), B (experimental control), C (*C. monogyna* extract), D (simvastatin) and E (combination of *C. monogyna* extract and simvastatin). The study's results showed that ethanolic extract of *C. monogyna* berries was as effective as Simvastatin in reducing cholesterol levels. However, when compared to the combination of *Crataegus* extract and Simvastatin, *Crataegus* alone was found to be more effective. Both drugs have similar efficacy in reducing triglycerides and lowering LDL levels. As a conclusion, the study indicated that *Crataegus* has a great potential as a natural agent for reducing lipids, without any adverse effects.

Chen et al. (51) investigated the effects of *C. pinnatifida* drink (Shan Zha) on hyperlipidemic patients. Total of 30 volunteers, who were clinically diagnosed with hyperlipidemia and consisted of 25 females and 5 males, participated in their study.

During 1-month experimental period, the patients continued their usual daily life, activities and diet but refrained from taking any medications. They consumed *crataegus* drink twice a day (250 mL per serving) for one month while visiting the laboratory. Blood samples were collected from their veins while fasting in the early morning at the start and end of the experimental period. Their results indicated a significant reduction in serum cholesterol, triglycerides, and LDL levels among hyperlipidemic patients after consuming the *C. pinnatifida* drink.

Another study examined the impact of consuming a beverage made from the fruit of *C. pinnatifida* on atherosclerotic mice with high blood lipid levels. The study involved 24 atherosclerotic mice that were divided into 4 groups: Group A, which was the negative control group, Group B, which was the positive control group, Group C, which received high-cholesterol diet and 6mg/kg/day of Simvastatin, and Group D, which received high-cholesterol diet and *Crataegus* fruit drink. The study spanned a duration of 8 weeks. The study finding indicated that in comparison to the positive control group (Group B), both simvastatin group (Group C) and the *Crataegus* fruit compound group (Group D) exhibited a significant reduction in the ratio of low-density lipoprotein cholesterol (LDL-C) to serum cholesterol (TC). Additionally, Group D showed a greater reduction in triglyceride (TG) levels than Group C (52).

Shao et al. (53) studied on aqueous and ethanolic extracts of *C. pinnatifida* fruits, in terms of their hypolipidemic activity on rats. After the adaptation period, all rats were randomly assigned to one of eight groups (n=8). The control group (Group 1) received intragastric administration of 10 mL/kg body weight of distilled water twice a day. The remaining groups (Groups 2-8) received intragastric administration of 10 mL/kg body weight of the high-fat emulsion once daily. After 6 hours Group 2 received intragastric administration of 10 mL/kg body weight of distilled water, acting as the model group. Groups 3-5 received intragastric administration of aqueous extract at low, medium and high doses, which were equivalent to approximately 0.6, 1.2 and 2.4 g/kg/day of crude drug, respectively. Similarly, Groups 6-8 received intragastric administration of ethanolic extract at low, medium and high doses, which were equivalent to approximately 0.6, 1.2 and 2.4 g/kg/day of crude drug, respectively. According to their results, both *Crataegus* extracts administered to hyperlipidemic rats showed a dose-dependent reduction in levels of serum total cholesterol (TC), triglycerides (TG) and low-density lipoprotein cholesterol (LDL-C), as well as improvement in levels of high-density lipoprotein

cholesterol (HDL-C). Notably, the ethanolic extract at a low dose exhibited a greater reduction in TC and LDL-C levels than the aqueous extract at a high dose.

2.3.4. Studies on Hypoglycemic Activity

Aierken et al. (54) investigated the hypoglycemic activity of *C. pinnatifida* fruit extract on Sprague-Dawley (SD) rats. A group of 54 male rats were randomly divided into 6 groups. These groups included the normal control group, type II diabetic model group, the type II diabetic group treated with *Crataegus* extract at high (200 mg/kg), middle (100 mg/kg) and low (50 mg/kg) concentrations and type II diabetic group treated with Orlistat. Their findings showed that administering *Crataegus* extract at high, middle and low doses resulted in a considerable drop in fasting blood glucose levels among type II diabetic rats, as well as an increase in serum insulin levels.

In another study, hydroalcoholic fruit extract of *C. monogyna* was given to male Wistar rats at doses of 100, 200 or 400 mg/kg for 3 weeks. In order to cause diabetes in the rats, a single intraperitoneal dosage of 60 mg/kg streptozotocin was given. Blood glucose levels were measured at the conclusion of the study. The findings indicated a noteworthy rise in mean blood glucose levels of rats treated with streptozotocin in comparison to the control group. Moreover, administering doses of 100, 200 and 400 mg/kg of the *C. monogyna* extract demonstrated a considerable decrease in mean blood glucose levels as opposed to the diabetic group (55).

Jouad et al. (56) studied the hypoglycemic activity of *C. oxycantha* leaves on male Wistar rats. Decoction of *C. oxycantha* leaves was given to rats at two different oral doses. Intravenous administration of Streptozotocin led to the development of diabetes in rats. Six rats from each of the normal and diabetic groups were distributed randomly among 4 groups. The control group was given distilled water, while the treated groups were administered an aqueous extract of *C. oxycantha* leaves at 150 or 300 mg/kg doses or standard reference drug (Metformin®) at a 500 mg/kg dose. To conclude, the hypoglycemic effect of an aqueous extract of *C. oxycantha* leaves was observed in rats with streptozotocin induced diabetes but not in normal rats. Their results suggest that the hypoglycemic activity of the leaves extract was not linked to insulin secretion since basal plasma insulin levels did not significantly differ between untreated and *C. oxycantha* treated groups of normal and diabetic rats given oral doses of 150 or 300 mg/kg of the aqueous *C. oxycantha* extract.

Ljubuncic et al. (57) investigated the hypoglycemic effect of leaves and unripe fruits of *C. aronia* syn. *azarolus*. Decoction of leaves and unripe fruits of the plant was given orally to diabetic and control rats daily, at 50 mg dried plant/mL or 350 mg dried plant/mL concentrations for 24 days. According to their results, higher concentration of decoction significantly reduced the blood glucose levels in the control rats. Additionally, both 50 mg dried plant per milliliter or 350 mg dried plant per milliliter concentrations of the decoction dramatically lowered the blood glucose levels in the diabetic rats.

2.3.5. Studies on Anti-inflammatory Activity

Tadic et al. (58) studied the potential anti-inflammatory activity of hydro-alcoholic extract obtained from a combination of *C. monogyna* and *C. oxycantha* berries (in a 1:1 ratio), using carrageenan-induced rat paw edema model. The researchers administered doses of 50, 100 and 200 mg/kg of the extracts orally. Indomethacin, which was dissolved in DMSO, was utilized as a reference at a dose of 4 mg/kg, which led to a 50% reduction in paw edema. The control animals were given 1 mL/kg of DMSO. After one hour of administering the extract or indomethacin, a carrageenan-saline solution (0.5% in 1 mL) was injected into the right hind paw's plantar surface, while a pure saline solution (0.9% NaCl, 0.1 mL) was injected into the left hind paw, serving as the non-inflamed paw control group. In a manner that depends on the dose, the hydro-alcoholic extract of *Crataegus* was found to decrease the carrageenan-induced paw edema in rats. At doses 50, 100 and 200 mg/kg, the edema reduction was 20.8%, 23% and 36.3%, respectively. The reduction they achieved by the highest dose was statistically significant and amounted to 72.4% of the effect produced by indomethacin, which was given at a dose that produced a 50% reduction in paw edema.

The ethanolic extract of *C. orientalis* leaves was analyzed by Bor et al. (59) to investigate any potential anti-inflammatory activity. To evaluate the anti-inflammatory activity, the carrageenan-induced hind paw edema model was employed. The test involved administering 40 μL (1%) of carrageenan into the right hind paw of each rat, 35 minutes after the IP injection of the test sample. The left hind paw was injected with 40 μL of saline solution as a control. The reference drug used was diclofenac at a dose of 10 mg/kg. According to their results, the ethanolic extract of *C. orientalis* leaves demonstrated significant anti-inflammatory activity and inhibited carrageenan-induced paw edema in rats in a dose-dependent manner. The extract exhibited inhibitory effects of edema at 270 minutes post-carrageenan injection, with values of 45.2%, 86.6% and

96.8% for doses of 50, 100 and 200 mg/kg, respectively. At 180 and 270 minutes, the 200 mg/kg dose of extract was significantly more effective than diclofenac (10mg/kg).

Kmail et al. (60) studied anti-inflammatory properties of *C. azarolus* L. extract by using *in vitro* mono and co-culture systems. In conclusion, the secretion levels of TNF-α, IL-6 and IL-10 were observed to be modulated by the *Crataegus* extract.

A different study examined the anti-inflammatory properties of *C. orientalis* leaves and berries. The ability to reduce inflammation was investigated through the inhibitory activity of cyclooxygenase-1 (COX-1) and 12-lipooxygenase (12-LOX) by using the intact cell system (platelets). To conclude, the extract from *C. orientalis* leaves demonstrated a dose-dependent reduction in the production of 12-HHT and TXB2 through COX-1 pathway, with IC50 values below the lowest concentration tested. Similarly, the extract from *C. orientalis* berries also displayed significant inhibitory activity towards the production of these metabolites, although not as strongly as leaves extract (61).

In another research, the aim was to investigate the anti-inflammatory properties of water fraction of *C. pinnatifida* Bunge var. *typical* Schneider on RAW 264.7 cells stimulated with lipopolysaccharide (LPS) and to understand the underlying mechanisms. In order to assess the effects of the water fraction of fruits, the level of NO production in LPS-treated RAW 264.7 cells was measured using an ELISA method, while cytotoxicity was measured using an MTT assay. Furthermore, the study analyzed the expression of iNOS, Cyclooxygenase-2 (COX-2), TNF-α, IL-6 and IL-1β mRNA using a reverse transcription polymerase chain reaction (RT-PCR). The finding of study demonstrated that the water fraction of *C. pinnatifida* Bunge var. *typical* Schneider fruit was safe to use and significantly reduced the production of NO in LPS-stimulated RAW 264.7 cells. The water fraction also suppressed the expression of COX-2, TNF-α, IL-6 and IL-1β (62).

Abdel-Rahman et al. studied anti-inflammatory properties of defatted methanolic extract of *C. sinaica* fruits through an *in vivo* experiment. A model of carrageenan-induced rat paw edema was employed to evaluate the anti-inflammatory effect of methanolic extract of *C. sinaica* fruits in comparison to indomethacin (20 mg/kg p.o.). The rats were divided into 5 groups, group 1 was administered distilled water daily and served as a control group. Groups 2-4 were orally treated with extract of *C. sinaica* at doses of 50, 100 and 200 mg/kg, respectively. Group 5 was given indomethacin at a dose of 20 mg/kg orally and was designated as the standard group. The paw volumes of the rats were measured at 1, 2, 3 and 4 hours after the carrageenan injection. The

administration of methanolic extract of *C. sinaica* fruits resulted in a significant reduction in edema rate when compared to the control group. The anti-inflammatory inhibition rates and potencies were observed to be dose-dependent, with the highest values achieved for all doses within the first hour following the carrageenan injection (63).

2.3.6. Studies on Anti-Noniceptive Activity

Abdel-Rahman et al. studied anti-noniceptive activity of defatted methanolic extract of *C. sinaica* fruits through an in vivo experiment. The analgesic effects of peripheral activity were evaluated through a writing test, which involved dividing a group of 25 animals randomly into 5 groups of 5 animals each. The first group was assigned as the negative control, while 2 to 4 were given varying doses of plant extract (50, 100 and 200 mg/kg, respectively). The fifth group was administered acetylsalicylic acid (150 mg/kg) and used as the standard group. To induce pain, all animals were given an intraperitoneal injection of acetic acid (0.7% aqueous solution) at a dosage of 10 mL/kg body weight, 30 minutes before treatment. Pain was measured by quantifying the number of abdominal writhes over a 20-minutes period. Results showed a significant decrease in the number of writhes in animals treated with methanolic extract of *C. sinaica* fruits at 100 and 200 mg/kg, as well as acetylsalicylic acid. To evaluate central analgesic activity, a hot-plate test was used, and rats were randomly assigned to 1 to 5 groups, each containing 5 animals. The first group was considered the negative control, while groups 2, 3 and 4 were given doses of 50, 100 and 200 mg/kg plant extract, respectively. Group 5 was administered acetylsalicylic acid as the standard group. According to their results, aminals treated with methanolic extract of *C. sinaica* fruits at all dose levels did not exhibit any significant differences compared to the acetylsalicylic acid group, starting from 1 hour after administration (63).

In a study conducted by Bor et al. (59), the antinoniceptive effects of ethanolic extract of *C. orientalis* leaves were investigated using the hot-plate, tail immersion and writhing tests. In the hot-plate, doses of 100 and 200 mg/kg were found to have significant antinoniceptive effects. Similarly, significant antinoniceptive effects were onserved in all doses in the tail-immersion test. A notable reduction in writhing responses was observed with increasing dosage during writhing test. Based on these experimental results, the ethanolic extract of *C. orientalis* was found to exhibit remarkable antinoniceptive activity.

2.3.7. Studies on Gastroprotective Activity

Tadic et al. (58) investigated the potential gastroprotective activity of hydro-alcoholic extract obtained from combination of *C. monogyna* and *C. oxycantha* berries in a 1:1 ratio. To evaluate the gastroportective activity of the extract, an experimental model involving the induction of acute gastric mucosa damage by absolute ethanol was employed. The investgated extract, which was dissolved in DMSO, was administered orally in doses ranging from 50-200 mg/kg, 60 minutes prior to the ethanol administration. Ranitidine was used as a reference grug, given orally in doses of 5-20 mg/kg. The control animals received the vehicle in a dose of 1 mL/kg, also 60 minutes before ethanol. In conclusion, their results showed that the ethanolic extract of *Crataegus* berries showed notable dose-dependent gastroprotective activity, similar to the reference drug ranitidine. Notably, the highest administered dose of the extract (200 mg/kg) displayed an even more pronounced protective effect compared to a dose of 20 mg/kg of ranitidine.

Moeini et al. (64) conducted a study to assess the effectiveness and safety of using *C. monogyna* Jacq. fruit to treat patients with gastroesophageal reflux disease. The study included 80 male and female patients who has been clinically diagnosed with the disease and they were allocated into two groups, namely the experimental group and the control group, randomly. Patients in the experimental group were given *C. monogyna* syrup, which contained 66.7% sugar and 10% dried extract of *C. monogyna* Jacq. fruit extract, while those in control group received a placebo syrup. Both groups were instructed to take 5 mL of syrup after each meal for 4 weeks. The severity of the gastroesophageal reflux disease symptoms was evaluated using validated questionnaire before and after the treatment in both groups. The results showed that after 4 weeks of treatment, the experimental group exhibited a significant improvement in the two main symptoms of the disease, namely heartburn and regurgitation, compared to the control group. the symptoms were alleviated by 93.5% and 94.2% respectively.

2.3.8. Studies on Hepatoprotective Activity

Mecheri et al (65) was investigated the hepatoprotective effects of the *n*-butanol extract from the leaves of *C. oxycantha* in acute liver damage caused by Doxorubicin. Female rats were given either *C. oxycantha* (100 mg/kg body weight) or vitamin E as a reference antioxidant (100 mg/kg body weight) orally for 10 days, with or without

hepatotoxicity induced by a single intraperitoneal injection of doxorubicin (15 mg/kg on the 8th day). On the 11th day, blood and liver samples were examined to assess biomarker levels and histopathological alterations. The study concluded that administration of the *n*-butanol extract derived from the leaves of *C. oxycantha* resulted in notable enhancements in liver enzyme activities and oxidative stress markers.

Saeedi et al. (66) conducted a study to investigate the protective and therapeutic effects of *C. oxycantha* on non-alcoholic fatty liver disease (NAFLD) induced by a high-fat diet in rat models. 45 Wistar rats were divided into 8 groups, including a control group, a high-fat diet (HFD) group, an HFD group with diet change, an HFD group with diet change and *C. oxycantha* extract (20 mg/kg), a co-treatment group of HFD and *C. oxycantha* extract 10, 20 and 40 mg/kg and a normal diet group with *C. oxycantha* extract 40 mg/kg. *C. oxycantha* extract was administered orally. According to their results, the diet change from high fat to low fat improved liver damage, but the consumption of *C. oxycantha* extract (10 and 20 mg/kg) significantly reduced the levels of all examined liver biomarkers. The hepatoprotective effect of *C. oxycantha* extract was most prominent in the animals treated with dose of 20 mg/kg extract.

2.3.9. Studies on Anxiolytic and Antidepressant Activity

Popovic-Milenkovic et al. (67) investigated the anxiolytic activity of *C. nigra* Wald. Et Kit. Fruits. The anxiety effect of the investigated extract was measured utilizing the elevated plus-maze test. The animals were divided into 6 groups of 8 animals, with 4 groups receiving different concentrations of the hydro-alcoholic extract of *C. nigra* (10, 100, 300 and 600 mg/kg). 2 groups served as controls, receiving either vehicle (normal saline) or diazepam (1 mg/kg), to control and measure the anxiety effect. The groups that underwent treatment were administered the extracts intraperitoneally and anxiety induced by arm height. In conclusion, the hydroalcoholic extract of *C. nigra* significantly increased the total time spent in the open arms compared to the control at doses 300 and 600 mg/kg, with the most pronounced effect observed at the dose of 300 mg/kg. Although the extract of *C. nigra* did not show a significantly greater effect than the diazepam control.

Another study investigated the anxiolytic and antidepressant effects of *C. pinnatifida* compared to the effects of escitalopram on stressed mice. According to their results, *C. pinnatifida* was effective in producing anxiolytic and antidepressant-like effect

comparable to escitalopram through the activation of 5-HT1A receptors and an increase in BDNF levels in the hippocampus and prefrontal cortex (68)

2.3.10. Studies on Antimicrobial Activity

Tadic et al. (58) investigated antimicrobial activity of ethanolic extract of *C. monogyna* and *C. oxycantha* (1:1) berries. In conclusion, the study demonstrated that the extract had a moderate bactericidal effect, particularly against gram-positive bacteria such as *M. flavus, B. subtilis* and *L. monocytogenes,* with percentages of 80%, 62.2% and 60.7%, respectively, when compared to the reference antibiotic streptomycin. However, the extract did not show any effect against *C albicans.*

Another study investigated the antimicrobial properties of alcohol, hydroalcohol and aqueous extracts of *C. oxycantha* L. berries. The findings showed that the ethanolic extract effectively inhibited the growth of most tested microorganisms, except for two bacterial species, *Bacillus subtilis* and *Straphlyocıccus aureus,* and one fungal species, *Aspergillus niger* (69).

In another study, agar diffusion tests were conducted to evaluate the antimicrobial activity of *C. oxycantha* extracts. The findings of the study showed that all tested extracts have antibacterial properties against *S. aureus* but have no inhibitory effect on *E. coli*. The antibacterial behavior of *C. oxycantha* extracts was found to be influenced by the extraction solvent. The *n*-butanol fruit extract was found to be the most active against *S. aureus,* followed by the *n*-butanol leaves extract and the ethyl acetate fruit extract. However, only fruit extracts obtained with both *n*-butanol and ethyl acetate solvents demonstrated bactericidal activity, which was not observed in leaves extract (70).

Djeddi et al. (71) examined the antimicrobial activity of a methanolic extract of *C. monogyna* against 9 pathogenic bacterial strains using the disk diffusion method. A positive control, cefazoline, was also included the study. Their results showed that the methanolic extract of *C. monogyna* exhibited strong antimicrobial activity, with the highest dilution of 1000 mg/L showing significant activity against *A. baumannii, K. pneumonia, E. intermedius* and *E. cloacae*. Additionally, even at the lowest dilution of 100 mg/L, the extract displayed high activity against *A. banumannii* and moderate effects against *E. coli1* and *E. coli2* were observed.

Benli et al. (72) conducted a research to investigate antimicrobial activity and mode of action of *C. tanacetifolia* plant extract, an endemic species. The study involved extracting the plant's leaves with methanol and subjecting the resulting extract to the drop

method to test its efficacy against 10 bacterial and 4 yeast strains. Their findings revealed that the plant extract demonstrated potent antibacterial effects against *Bacillus subtilis, Shigella, Staphylococcus aureus* and *Listeria monocytogenes*, among the tested microorganisms.

2.3.11. Studies on Antioxidant Activity

The research examined the antiradical properties of aqueous and ethanolic *C. monogyna* fruit extracts by utilizing spectrophotometry through the DPPH radical scavenging assay and ABTS radical cation decolorization assay. The results indicated that both extracts displayed antiradical activity, but the ethanolic extract demonstrated greater efficacy in scavenging free radicals when compared to the aqueous extract (73).

In another investigation, the antioxidant properties of alcohol, hyroalcoholic, and aqueous extracts of *C. oxycantha* were assessed, using the DPPH radical scavenging acitivity assay. The findings of the investigation revealed that the fruit extracts exhibited substantial antioxidant activity, with a DPPH radical transformation rate reaching as high as 89.9% in the methanol-water (50/50, *v/v*) extract (69).

Ebrahimzadeh et al. (74) investigated the antioxidant activity of aqueous and methanolic extracts of *C. pentagyna* fruits using various *in vitro* assay systems, including DPPH, NO radical scavenging activity, linoleic acid and iron ion chelating power. Their results indicated that the aqueous extract exhibited higher antioxidant activity in the DPPH radical scavenging assay. However, both extracts showed weak antioxidant activity in reducing power determination and Fe^{2+} chelating ability assays. Both extracts displayed high activity in the ferric thiocyanate (FTC) method. Finally, in the nitric oxide radical scavenging activity assay, the methanolic extract demonstrated higher antioxidant activity.

Özyürek et al. (74) conducted a study to evaluate the antioxidant capacities of various *Crataegus* species that grow in Türkiye. The investigation encompassed the analysis of 52 samples flowers and leaves, representing 17 taxa across 14 distinc *Crataegus* species, which were naturally occurring in Türkiye, for their antioxidant activity and capacity. Four different methods, including CUPRAC, FRAP, ABTS/Persulfate and Folin-FCR assays, were employed to ascertain the antioxidant capacities of the samples. Each plant's leaves and flowers were examined individually for the study. According to their study, *Crataegus x sinaica* Boiss. Nothosubsp. *C. sinaica* was found to be the most effective species among the flower extracts, while *C. pentagyna*

Waldst and Kit. Ex. Wild. were the most active among the extracts of leaves. Notably *C. monogyna* Jacq. samples showed significantly high antioxidant activity.

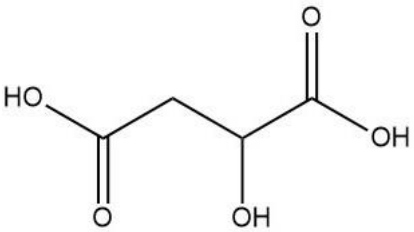
Issaadi et al. (75) investigated the antioxidant properties of flowers and fruits of *C. oxycantha* from Bejaia (Northeastern Algeria) using various analytical techniques, including scavenging assays such as FRAP and Fremy's salt by electronic paramagnetic resonance. The authors concluded that both the flowers and fruits exhibited robust antioxidant activity.

Another study examined the ability of aqueous and ethanolic extracts obtained from leaves, flowers, and fruits of *C. monogyna* to scavenge H_2O_2 radicals and their total antioxidant activity. The scavenging ability of extracts was compared with butylated hydroxylanisole (BHA) and α-tocopherol standards. The study found that the extracts could scavenge H_2O_2 radicals in a concentration-dependent manner and the ethanolic extract of leaves from *C. monogyna* had the highest scavenging activity value among the extracts at 30.13%. Moreover, the study used the ferric thiocyanate method in a linoleic acid system to determine the total antioxidant activity of extracts and standard compounds. In addition, their results revealed that *C. monogyna* extracts exhibited strong antioxidant activity, with 100 μg/mL of extracts inhibiting lipid peroxidation of linoleic acid emulsion by 84.80% to 93.52%, α-tocopherol showed only 40.49% inhibition of peroxidation. The antioxidant activity of the 100 μg extracts of *C. monogyna* and standards followed a decreasing order: water extract of *C. monogyna* leaves (93.52%) > water extract of *C. monogyna* flowers (93.06%) > ethanolic extract of *C. monogyna* fruits (92.09%) > ethanolic extract of *C. monogyna* leaves (90.92%) > ethanolic extract of *C. monogyna* (90.61%) flowers > water extract of *C. monogyna* (84.80%) fruits > α-tocopherol (40.49%) (76).

Djeddi et al. (71) conducted an *in vitro* investigation using the DPPH method to evaluate the antioxidant activity of the methanolic extract of *C. monogyna* Jacq. The results of their study indicated that the plant extract demonstrated a strong radical scavenging effect, with the highest activity observed at the concentration of 1000 mg/L (95.88%). The activity of the extract decreased with the decreasing concentration, with values of 94.86% and 94.07% observed at 750 mg/L and 500 mg/L, respectively. The lowest scavenging activity was observed at the lowest concentration tested, which was 100 mg/L (84.23%).

2.4. Phytochemical Studies on *Crataegus* Species

2.4.1. Organic acids from *Crataegus* species



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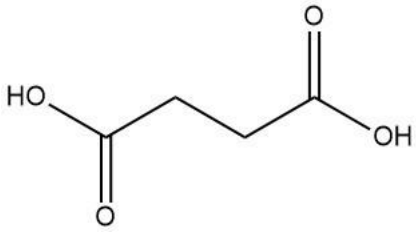

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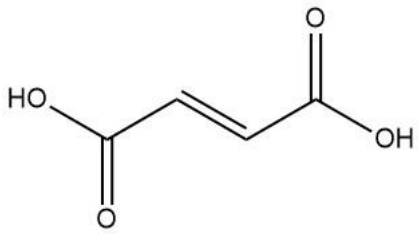
Table 1. Organic acids from *Crataegus* species.

Compounds	Species		Reference
Malic acid **(1)**	*C. aestivalis* *C. azarolus* *C. brettschneideri* *C. cuneata* *C. hupehensis* *C. kansuensis* *C. maximowiczii* *C. opaca* *C. pinnatifida*	*C. rufula* *C. sanguinea* *C. scrabrifolia* *C. monogyna* *C. atrosanguinea* *C. orientalis* *C. meyeri* (leaves and fruits)	(77), (78), (79), (80), (81)
Succinic acid **(2)**	*C. azarolus* *C. monogyna* *C. pinnatifida* *C. atrosanguinea* *C. orientalis* *C. meyeri* (leaves and fruits)		(81), (82), (83), (84)



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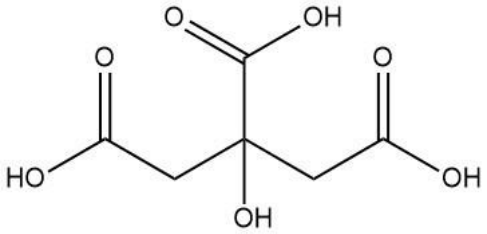

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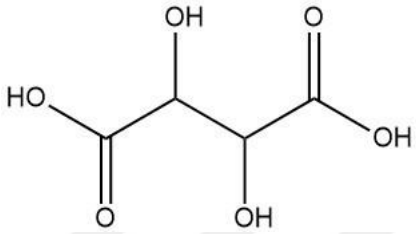

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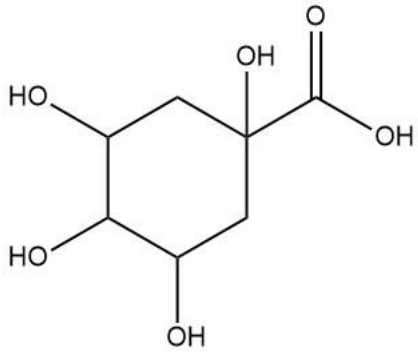
Table 2. Organic acids from *Crataegus* species.

Compounds	Species		Reference
Fumaric acid **(1)**	*C. monogyna* *C. atrosanguinea* *C. orientalis* *C. meyeri* (leaves and fruits)		(81)
Citric acid **(2)**	*C. aestivalis* *C. azarolus* *C. brettschneideri* *C. cuneata* *C. hupehensis* *C. kansuensis* *C. maximowiczii* *C. opaca*	*C. pinnatifida* *C. rufula* *C. sanguinea* *C. scrabrifolia* *C. monogyna* *C. atrosanguinea* *C. orientalis* *C. meyeri* (fruits)	(81)
Tartaric acid **(3)**	*C. cuneata* *C. maximowiczii* *C. pinnatifida* *C. sanguinea* *C. scrabrifolia* *C. monogyna* *C. atrosanguinea* *C. orientalis* *C. meyeri*(leaves and fruits)		(81)

2.4.2. Phenolic acids from *Crataegus* species



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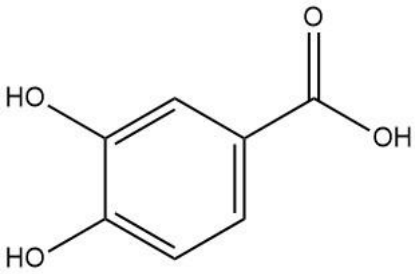

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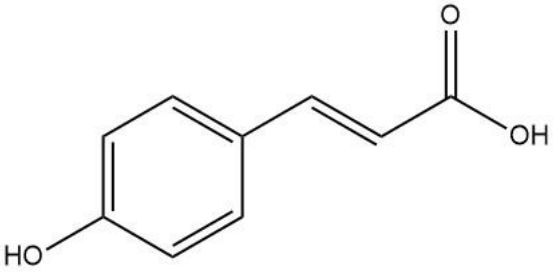

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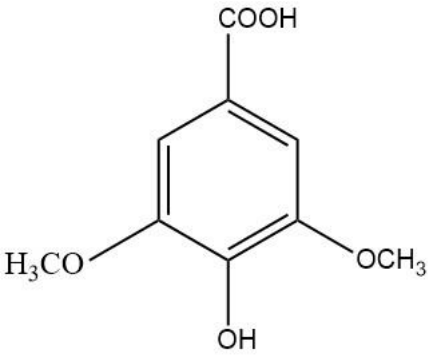
Table 3. Phenolic acids from *Crataegus* species.

Compounds	**Species**	**Reference**
Quinic acid **(1)**	*C. monogyna* (fruits)	(85)
Protocatechuic acid **(2)**	*C. pinnatifida* *C. monogyna* *C. atrosanguinea* *C. orientalis* *C. meyeri* (fruits)	(3), (81)
p-coumaric acid **(3)**	*C. monogyna* *C. atrosanguinea* *C. orientalis* *C. meyeri* (fruits)	(81)



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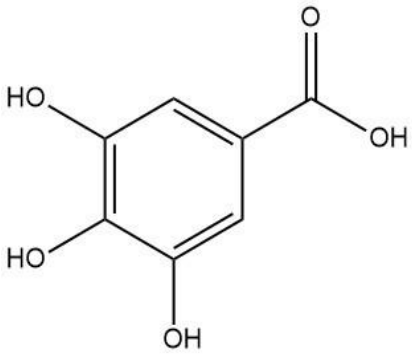

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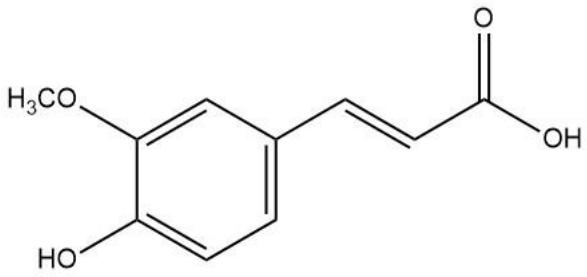
Table 4. Phenolic acids from *Crataegus* species.

Compounds	Species	Reference
Syringic acid **(1)**	*C. monogyna* *C. atrosanguinea* *C. orientalis* *C. meyeri* (fruits)	(81)
Gallic acid **(2)**	*C. cuneata* *C. monogyna* *C. atrosanguinea* *C. orientalis* *C. meyeri* (fruits)	(9), (81), (85)



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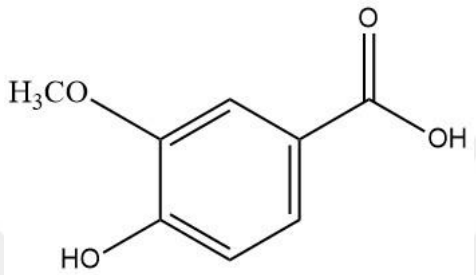

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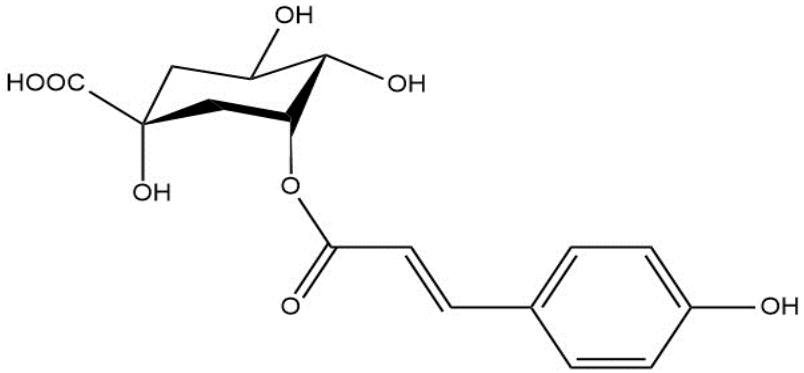
Table 5. Phenolic acids from *Crataegus* species.

Compounds	**Species**	**Reference**
Ferulic acid **(1)**	*C. monogyna* *C. atrosanguinea* *C. orientalis* *C. meyeri* (fruits)	(81)
Vanillic acid **(2)**	*C. monogyna* (fruits)	(86)



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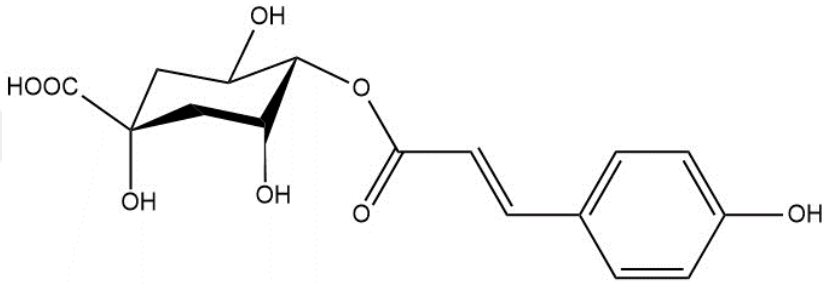

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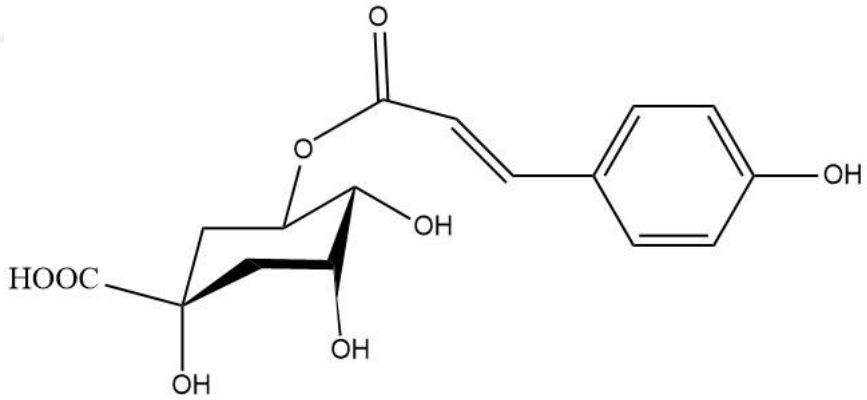

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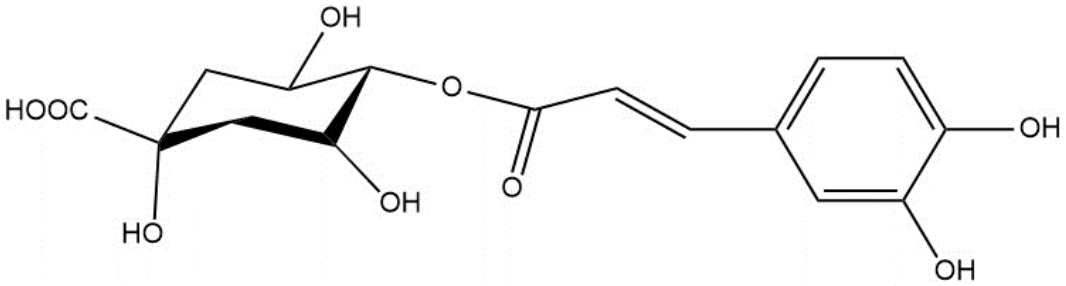
Table 6. Phenolic acids from *Crataegus* species.

Compounds	**Species**	**Reference**
3-*O*-*p*-coumaroylquinic acid **(1)**	*C. pinnatifida* (fruits)	(87)
4-*O*-*p*-coumaroylquinic acid **(2)**	*C. pinnatifida* (fruits)	(87)
5-*O*-*p*-coumaroylquinic acid **(3)**	*C. pinnatifida* (fruits)	(87)



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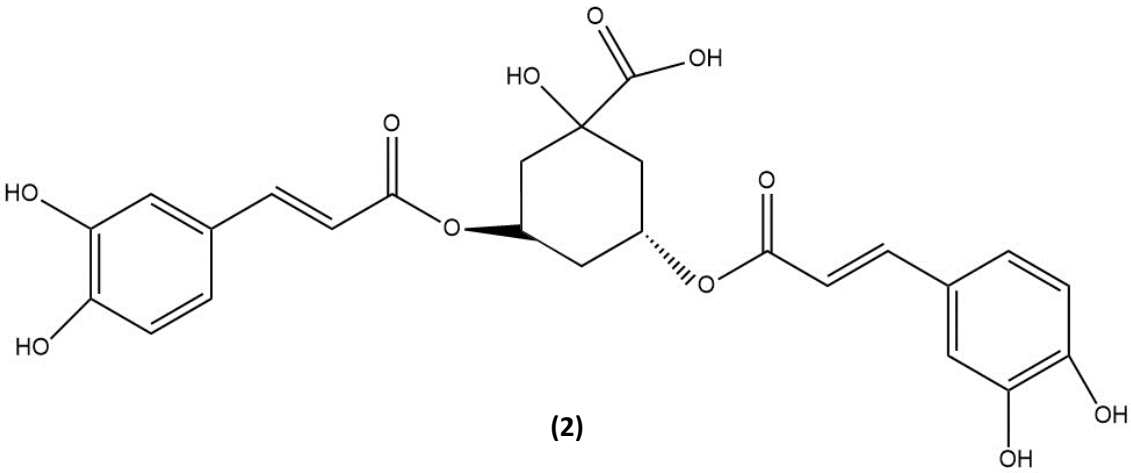

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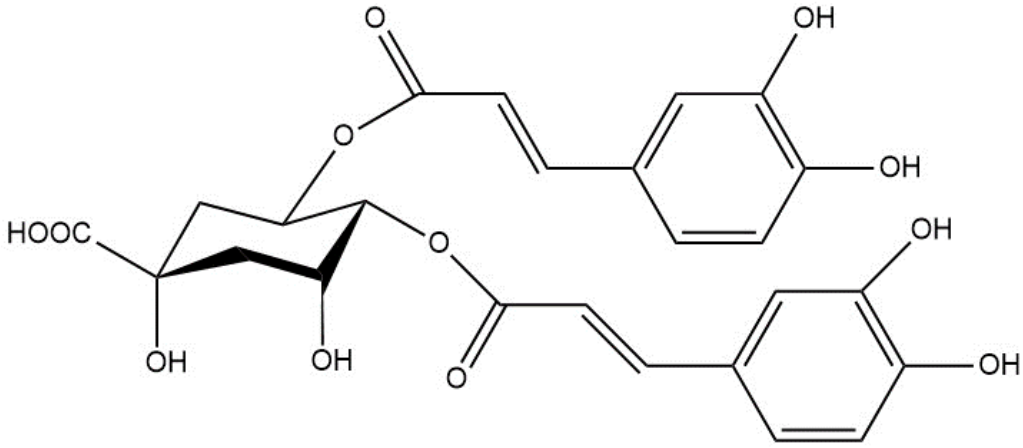

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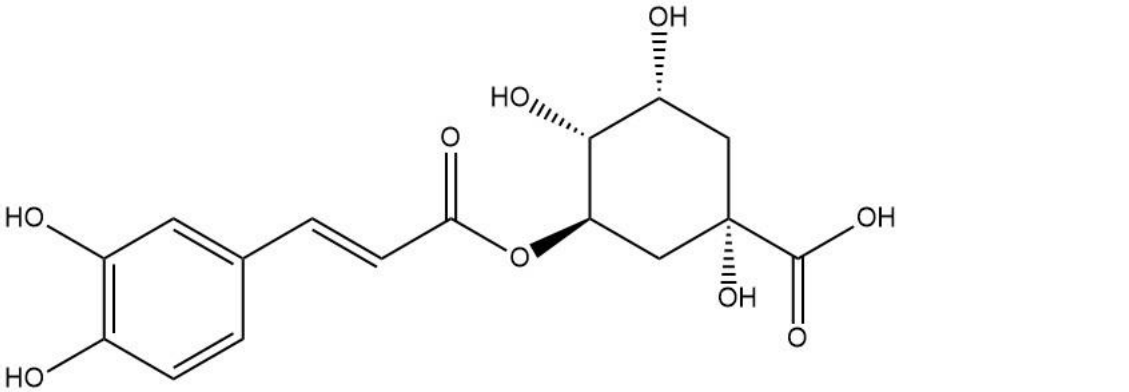
Table 7. Phenolic acids from *Crataegus* species.

Compounds	**Species**	**Reference**
4-*O*-caffeoylquinic acid **(1)**	*C. pinnatifida* (fruits)	(87)
3,5-di-*O*-caffeoylquinic acid **(2)**	*C. pinnatifida* (fruits)	(87)
4,5-di-*O*-caffeoylquinic acid **(3)**	*C. pinnatifida* (fruits)	(87)



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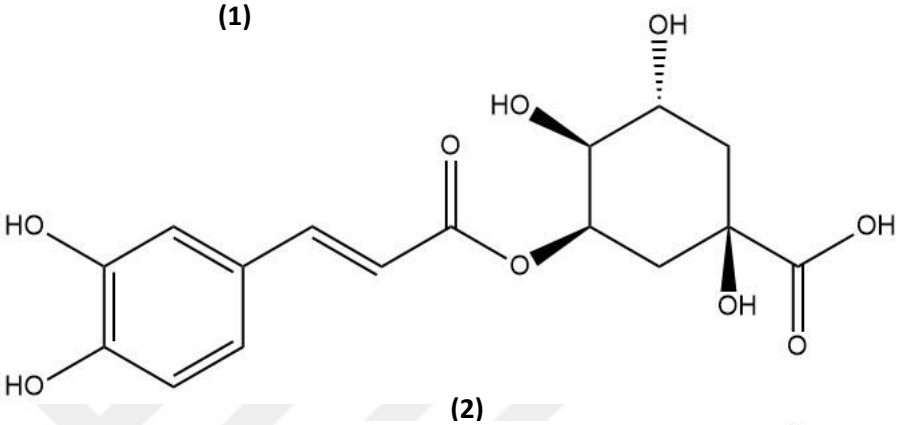

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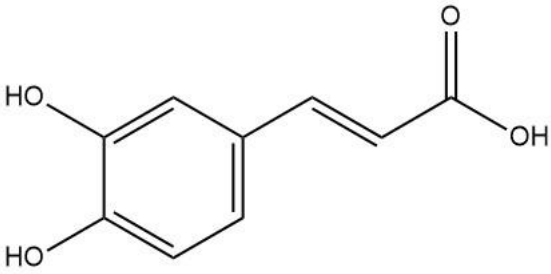

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Table 8. Phenolic acids from Crataegus species.

Compounds	**Species**			**Reference**
Chlorogenic acid **(1)**	*C. pinnatifida* *C. pentagyna* *C. pseudomelanocarpa* *C. monogyna* *C. meyeri* *C. songarica* *C. curvisepala* *C. pseudoheterophylla*	*C. sakrasnensis* *C. turkestonica* *C. szovitisii* *C. orientalis* *C. atrosanguinea* *C. persica* *C. brettschneideri* *C. rhipidophylla*	*C. scrabrifolia* *C. wilsonii* *C. aurantia* *C. grayana* *C. laevigata* *C. azarolus* (leaves and fruits)	(9), (81), (85), (87)
Neochlorogenic acid **(2)**	*C. pinnatifida* *C. grayana* (leaves and fruits)			(9)
Caffeic acid **(3)**	*C. pinnatifida* *C. laevigata* (leaves and fruits)			(9)

2.4.3. Flavonoids from *Crataegus* species

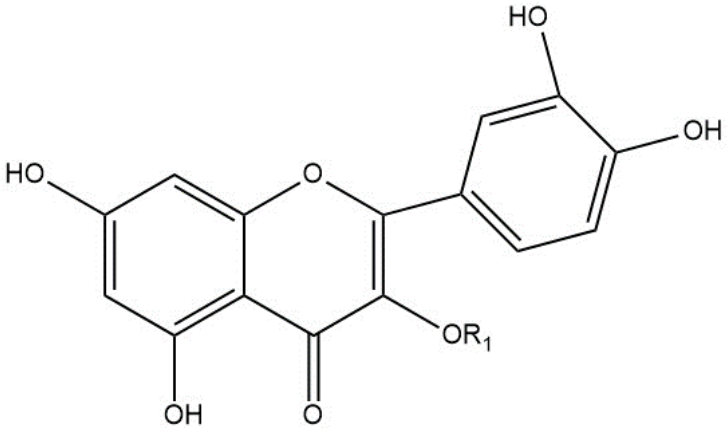


Table 9. Flavonoids from *Crataegus* species.

Compounds	R_1	Species	Reference
Hyperoside (Quercetin-3-*O*-galactoside)	Gal.	*C. rhipidophylla* *C. rhipidophylla* *C. pinnatifida* *C. brettschneideri* *C. scabrifolia* *C. cuneata* *C. hupehensisi* *C. sanguinea* *C. maximowiczii* *C. wilsonii* *C. aurantia* *C. kansuensis* *C. grayana* *C. monogyna* *C. laevigata* *C. azarolus* *C. rhipidophylla* *C. pentagyna* *C. songarica* *C. curvisepala* *C. pseudoheterophylla* *C. meyeri* *C. sakranensis* *C. turkestanica* *C. szovitisii* *C. orientalis* *C. persica* (leaves)	(9), (7)
Quercetin	H	*C. pinnatifida* *C. azarolus* *C. pentagyna* *C. pseudomelanocarpa* *C. meyeri* *C. monogyna* *C. songarica* *C. pseudoheterophylla* *C. turkestanica* *C. szovitisii* (leaves)	(9), (7)

Gal: Galactose.

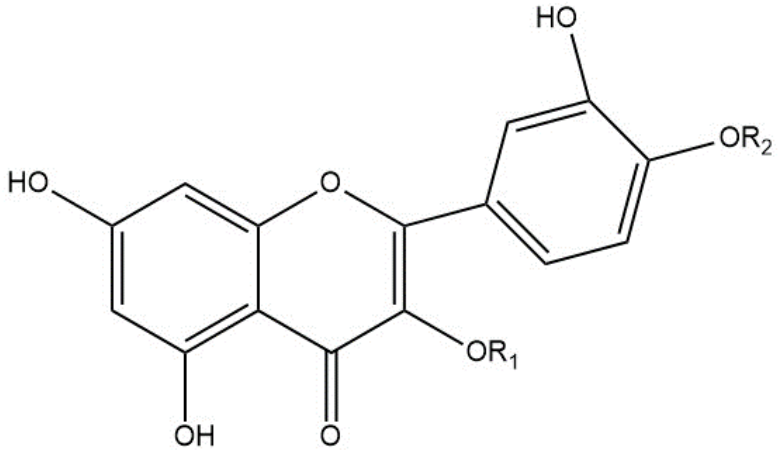


Table 10. Flavonoids from *Crataegus* species.

Compounds	R_1	R_2	Species	Reference
Rutin (Quercetin-3-*O*-*β*-D-rutinoside)	Rha. -Glc.	H	*C. pinnatifida* *C. scabrifolia* *C. cuneata* *C. hupehensisi* *C. sanguinea* *C. maximowiczii* *C. wilsonii* *C. aurantia* *C. laevigata* *C. azarolus* *C. rhipidophylla* *C. pentagyna* *C. pseudomelanocarpa* *C. songarica* *C. curvisepala* *C. pseudoheterophylla* *C. meyeri* *C. sakranensis* *C. turkestanica* *C. szovitisii* *C. orientalis* *C. atrosanguinea* *C. persica* (leaves)	(9), (7)
Quercetin-3-*O*-rhamnoside-galactoside	Rha. -Gal.	H	*C. pinnatifida* (leaves)	(9)
Spiraeoside (Quercetin-4'-glucose)	H	Glc.	*C. azarolus* (leaves)	(9)

Gal: Galactose, Glc: Glucose, Rha: Rhamnose.

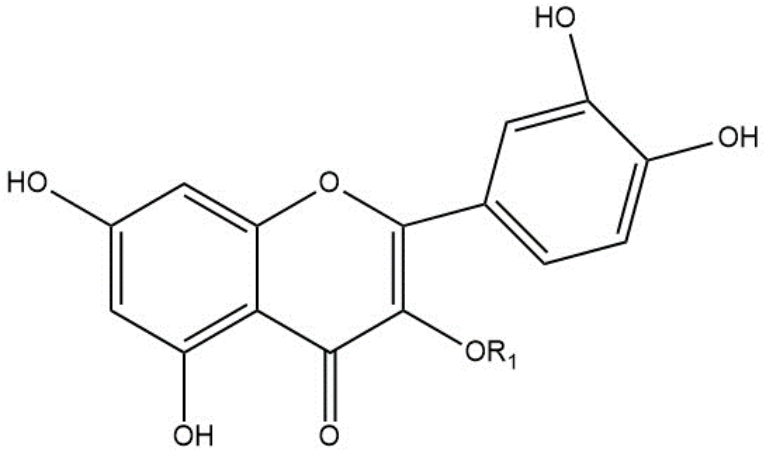


Table 11. Flavonoids from *Crataegus* species.

Compounds	**R_1**	**Species**	**Reference**
Quercetin 3-*O*-(6" galloyl) glucosider	Gall. -Glc	*C. monogyna* (leaves)	(85)
Isoquercitrin	Glc.	*C. pinnatifida* *C. brettschneideri* *C. scabrifolia* *C. cuneata* *C. monogyna* *C. laevigata* *C. azarolus* *C. rhipidophylla* *C. pentagyna* (leaves)	(9), (3)

Gall: Galloyl, Glc: Glucose.

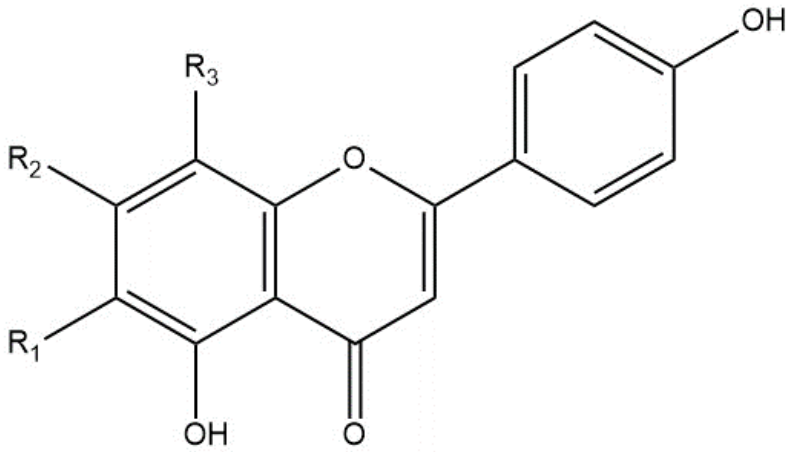


Table 12. Flavonoids from *Crataegus* species.

Compounds	R_1	R_2	R_3	Species		Reference
Vitexin	H	OH	Glc	*C. pinnatifida* *C. pseudomelanocarpa* *C. monogyna* *C. meyeri* *C. songarica* *C. curvisepala* *C. pseudoheterophylla* *C. sakrasnensis* *C. turkestonica* *C. szovitisii* *C. orientalis*	*C. atrosanguinea* *C. persica* *C. brettschneideri* *C. scrabrifolia* *C. wilsonii* *C. aurantia* *C. grayana* *C. rhipidophylla* *C. azarolus* (flowers and leaves)	(7)
Isovitexin	Glc	OH	H	*C. monogyna* *C. laevigata* *C. pentagyna* *C. rhipidophylla* (leaves)		(3)
Vitexin-2"-*O*-rhamnoside	H	OH	Glc-2"-O-Rha	*C. pinnatifida* *C. pseudomelanocarpa* *C. monogyna* *C. meyeri* *C. songarica* *C. curvisepala* *C. pseudoheterophylla* *C. sakrasnensis* *C. turkestonica* *C. szovitisii* *C. orientalis*	*C. atrosanguinea* *C. persica* *C. brettschneideri* *C. scrabrifolia* *C. wilsonii* *C. aurantia* *C. grayana* *C. rhipidophylla* *C. azarolus* (flowers and leaves)	(7)
Vitexin-4"-O-rhamnoside	H	OH	Glc-4"-O-Rha	*C. microphylla* (leaves)		(3)

Glc: Glucose, Rha: Rhamnose.

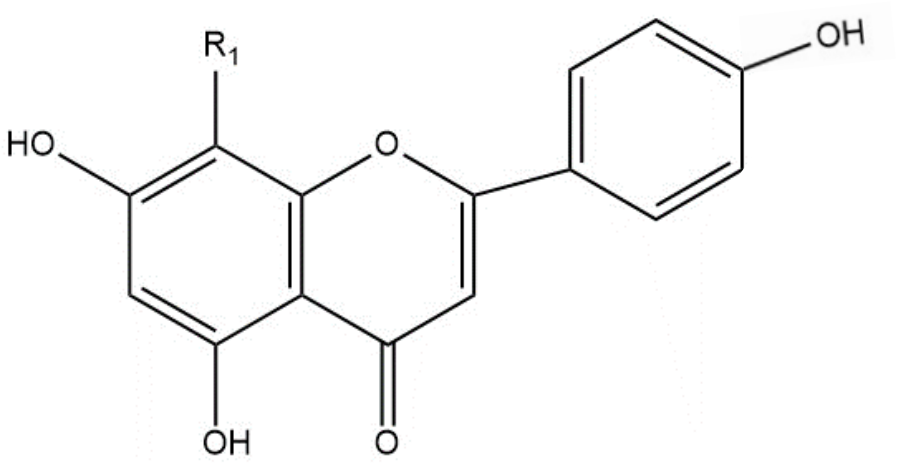


Table 13. Flavonoids from *Crataegus* species.

Compounds	R_1	Species	Reference
2"-*O*-Acetylvitexin	2-*O*-Ac-Glc	*C. pinnatifida* (leaves)	(9)
3"-*O*-Acetylvitexin	3-*O*-Ac-Glc	*C. pinnatifida* (leaves)	(9)
4"-*O*-Acetylvitexin	4-*O*-Ac-Glc	*C. pinnatifida* (leaves)	(9)
6"-*O*-Acetylvitexin	6-*O*-Ac-Glc	*C. pinnatifida* (leaves)	(9)
Vitexin-4"-*O*-glucoside	Glc-4"-*O*-Glc	*C. cuneata* *C. pinnatifida* *C. monogyna* (leaves)	(3)
Vitexin-2"-*O*-glucoside	Glc-2"-*O*-Glc	*C. pinnatifida* (leaves)	(3)
Vitexin-2"-*O*-(4"-*O*-Acetyl)-rhamnoside	Glc-2"-*O*-Rha	*C. monogyna* (leaves)	(9)

Ac: Acetyl, Glc: Glucose, Rha: Rhamnose.

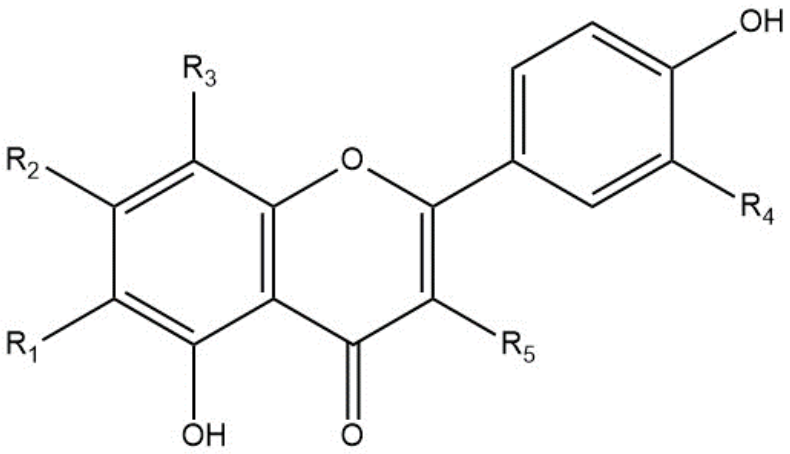
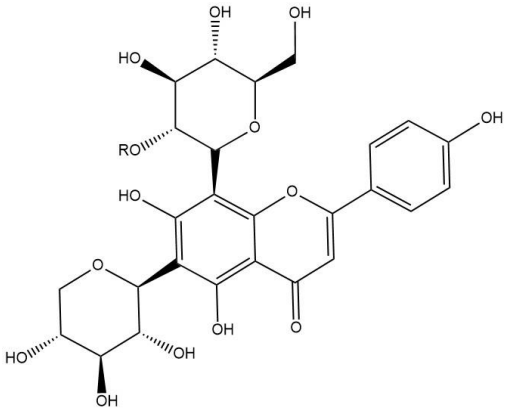


Table 14. Flavonoids from *Crataegus* species.

Compounds	R_1	R_2	R_3	R_4	R_5	Species	Reference
Orientin	H	OH	Glc	OH	H	*C. laevigata* *C. monogyna* *C. pentagyna* (flowers)	(3), (9)
Isoorientin	Glc	OH	H	OH	H	*C. laevigata* *C. monogyna* *C. pentagyna* (flowers)	(3), (9)
Orientin-2"-*O*-rhamnoside	H	OH	Glc-2"-*O*-Rha	OH	H	*C. pentagyna* (flowers)	(3), (9)
Isoorientin-2"*O*-rhamnoside	Glc-2"-*O*-Rha	OH	H	OH	H	*C. pentagyna* (flowers)	(9)
Luteolin	H	OH	H	OH	H	*C. pentagyna* (flowers)	(3)
Luteolin-7-*O*-glucoside	H	*O*-Glc	H	OH	H	*C. pentagyna* *C. turcicus* *C. monogyna* (fruits)	(88), (89)
Luteolin-7-*O*-*β*-glucuronide	H	*O*-Glcro	H	OH	H	*C. xmacrocarpa* *C. rhipidophylla* (leaves)	(9)
Kaempferol-3-*O*-neohesperidose	H	OH	H	H	O-Glc-2"-*O*-Rha	*C. monogyna* (pollen)	(9)
Sexangularetin-3-*O*-glucoside	H	OH	OCH_3	H	*O*-Glc	*C. pentagyna* (leaves) *C. monogyna* (pollen)	(9)
Sexangularetin-3-*O*-neohesperidoside	H	OH	OCH_3	H	*O*-Glc-2"-*O*-Rha	*monogyna* (pollen)	(9)

Glc: Glucose, Glcro: Glucronide, Rha: Rhamnose.



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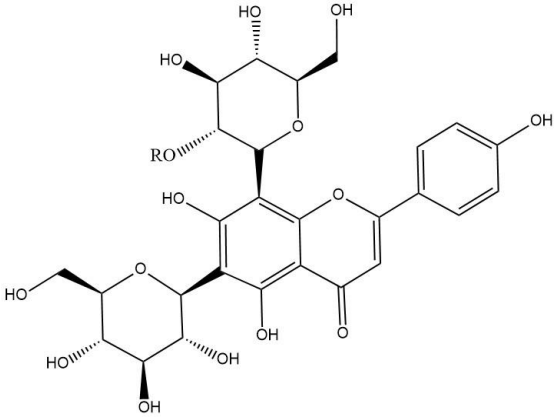

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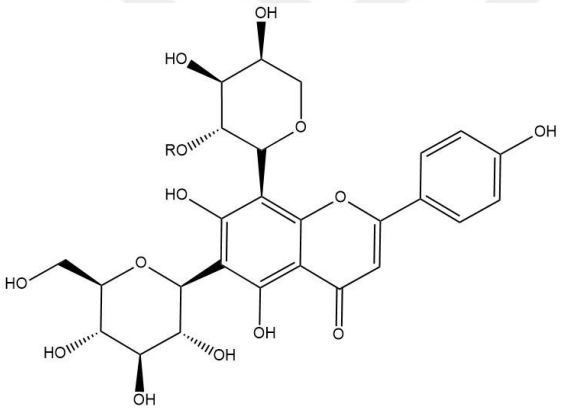

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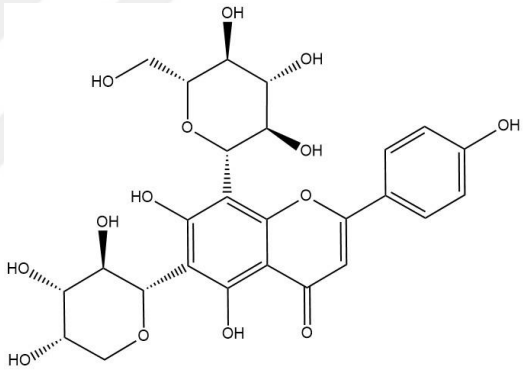

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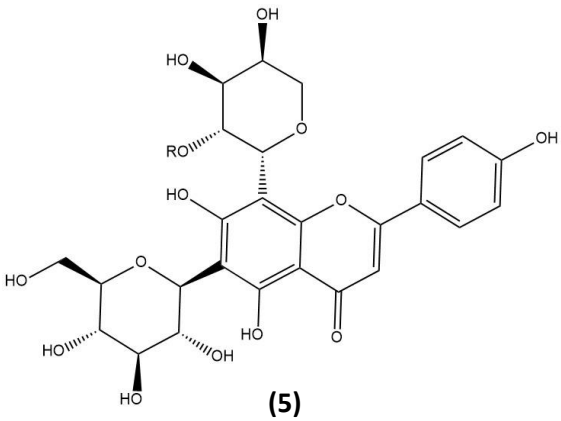

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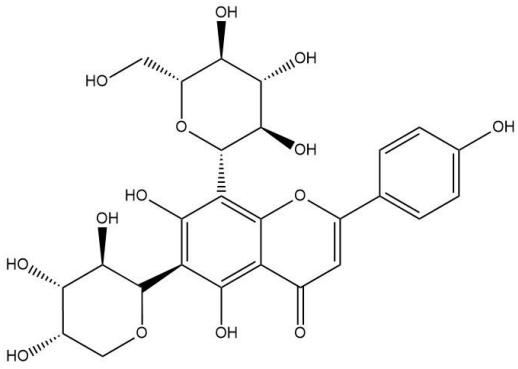
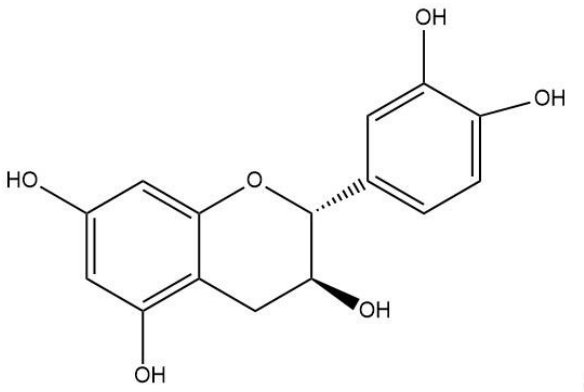


(6)

Table 15. Flavonoids from *Crataegus* species.

Compounds	**Species**	**Reference**
Vicenin-1 **(1)**	*C. monogyna* (leaves)	(90)
Vicenin-2 **(2)**	*C. monogyna* (leaves)	(90)
Schaftoside **(3)**	*C. monogyna* (leaves)	(90)
Isoschaftoside **(4)**	*C. monogyna* (leaves)	(90)
Neoschaftoside **(5)**	*C. monogyna* (leaves)	(90)
Neoiso-scaftoside **(6)**	*C. monogyna* (leaves)	(90)



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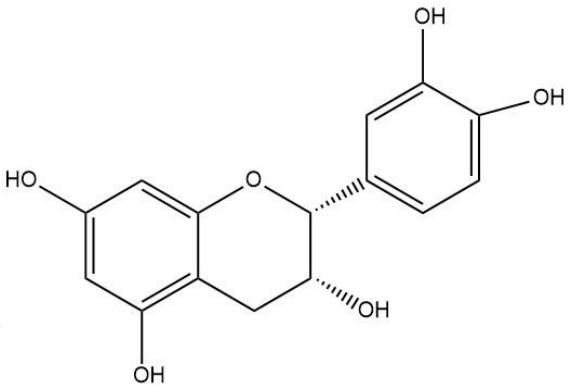

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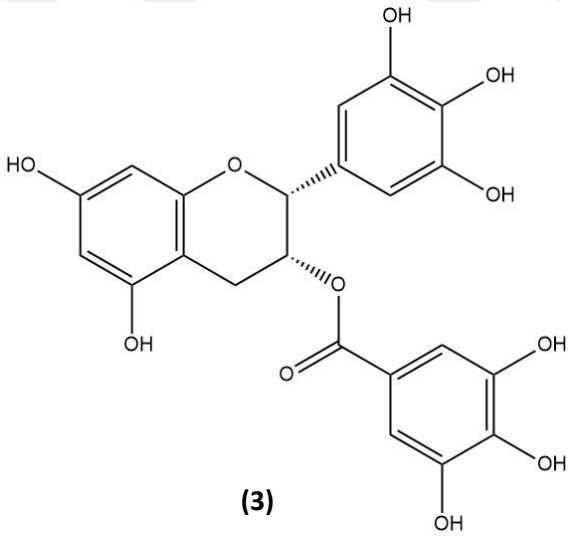
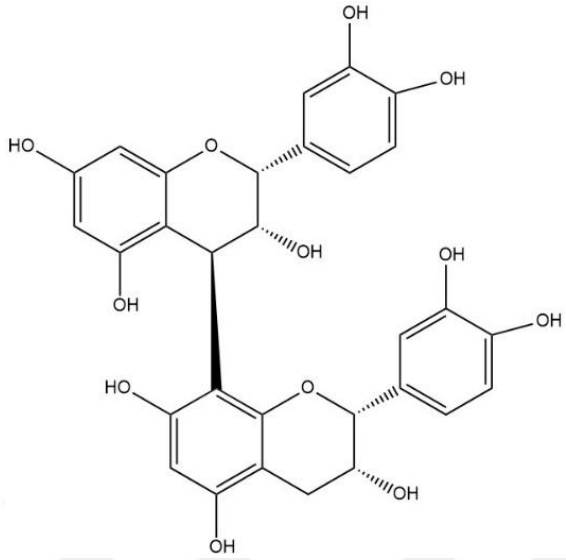


(3)

Table 16. Flavan-3-ols from *Crataegus* species.

Compounds	**Species**	**Reference**
Catechin **(1)**	*C. monogyna* *C. atrosanguinea* *C. orientalis* *C. meyeri* (fruits, leaves)	(81), (90)
Epicatechin **(2)**	*C. monogyna* *C. atrosanguinea* *C. orientalis* *C. meyeri* (fruits, leaves)	(81), (90)
Epigallocatechin gallate **(3)**	*C. monogyna* (leaves)	(85)

2.4.4. Proanthocyanidins from *Crataegus* species



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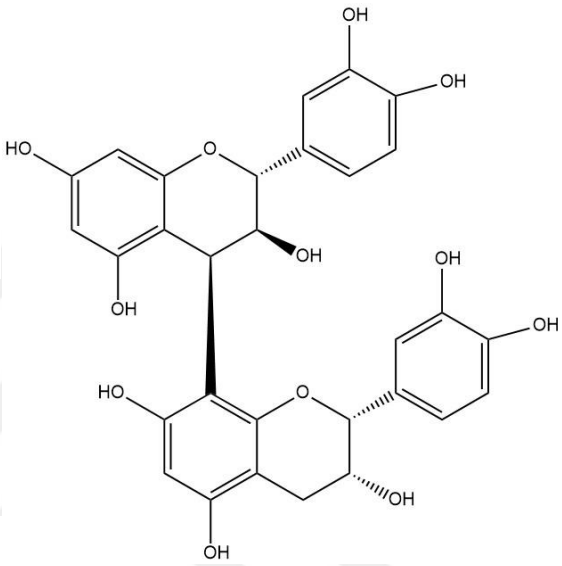

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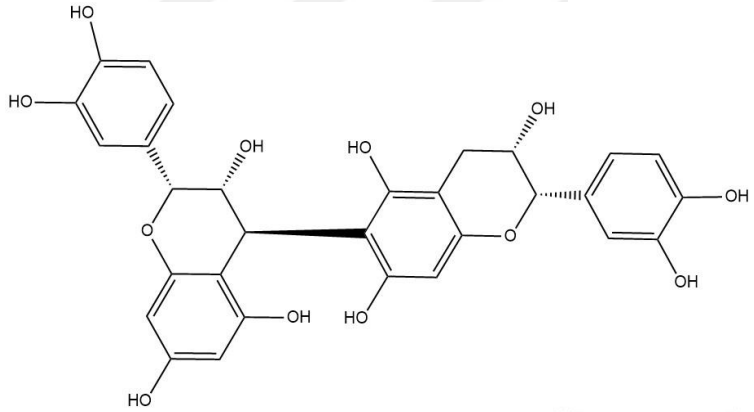

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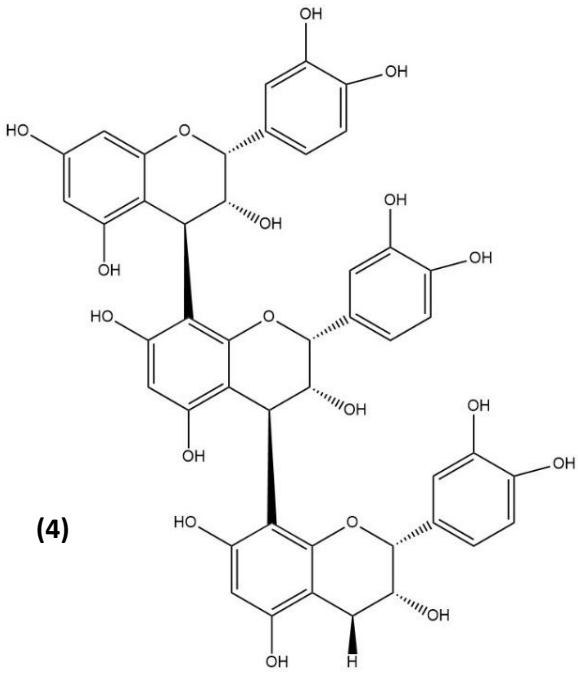

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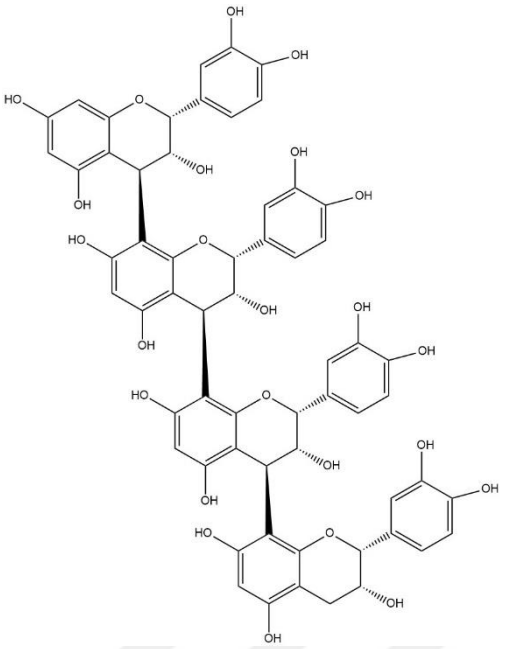


(5)

Table 17. Proanthocyanidins from *Crataegus* species.

Compounds	**Species**	**Reference**
Procyanidin B2 **(1)**	*C. monogyna* *C. pinnatifida* *C. brettscheideri* *C. laevigata* *C. scrabrifolia* *C. azarolus* *C. grayana* (fruits, leaves)	(90), (91), (92), (93), (94)
Procyanidin B4 **(2)**	*C. monogyna* *C. laevigata* (fruits, leaves)	(90), (93)
Procyanidin B5 **(3)**	*C. monogyna* *C. pinnatifida* *C. brettscheideri* *C. laevigata* *C. grayana* (fruits, leaves)	(90), (91), (92), (93)
Procyanidin C1 **(4)**	*C. monogyna* *C. pinnatifida* *C. brettscheideri* *C. laevigata* *C. scrabrifolia* *C. grayana* (fruits, leaves)	(90), (91), (92), (93)
Procyanidin D1 **(5)**	*C. monogyna* *C. pinnatifida* *C. brettscheideri* *C. laevigata* *C. scrabrifolia* *C. grayana* (fruits, leaves)	(90), (91), (92), (93)

2.4.5. Anthocyanins from *Crataegus* species

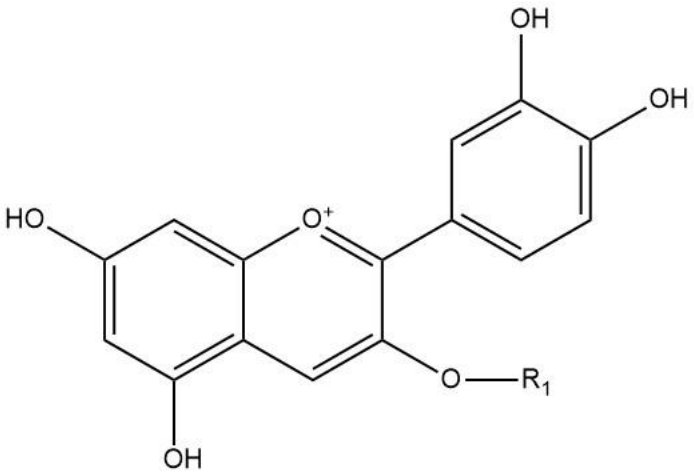
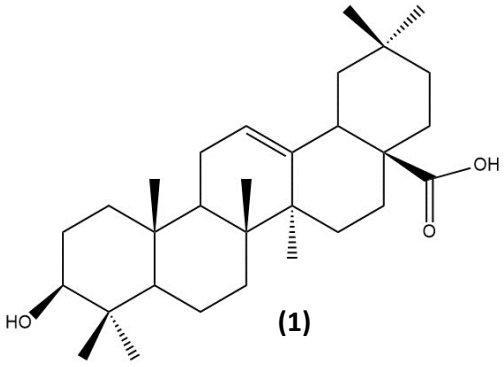


Table 18. Anthocyanins from *Crataegus* species.

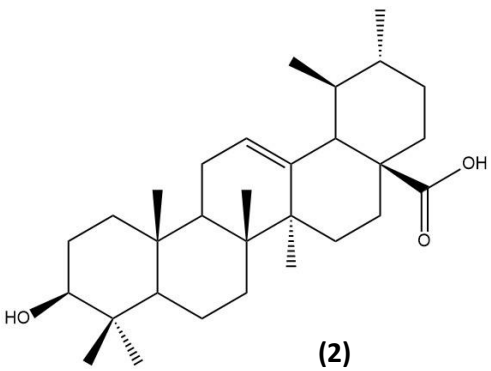
Compounds	R_1	Species	Reference
Cyanidin-3-*O*-Glucoside	Glc	*C. pinnatifida* (fruits)	(95), (96)
Cyanidin-3-*O*-Galactoside	Gal	*C. pinnatifida* (fruits)	(95), (90)

Glc: Glucose, Gal: Galactose.

2.4.6. Terpenic compounds from *Crataegus* species



(1)



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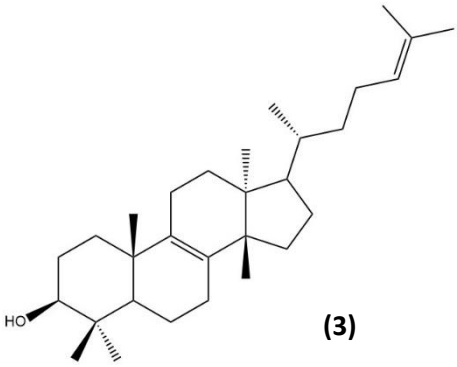

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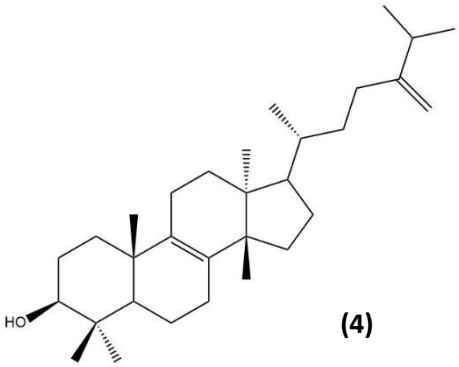

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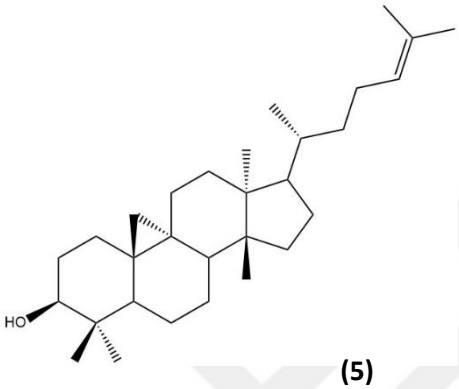

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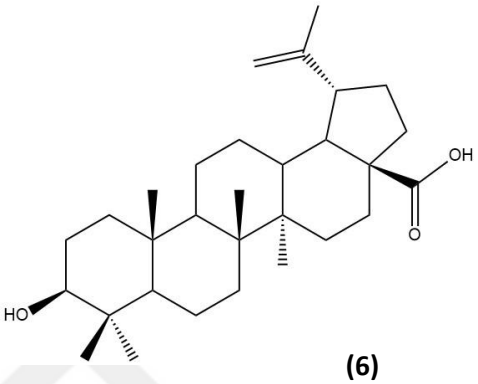

(6)

Table 19. Terpenic compounds from *Crataegus* species.

Compounds	**Species**	**Reference**
Oleanolic acid **(1)**	*C. monogyna* (leaves) *C. pinnatifida* (fruits)	(90), (97)
Ursolic acid **(2)**	*C. monogyna* *C. scrabrifolia* (leaves) *C. pinnatifida* (fruits)	(90), (97), (98)
Butyrospermol (3β-lanost 8,24-dien, 3-ol) **(3)**	*C. monogyna* (leaves)	(90)
24-Methylen-24-dihydrolanosterol (24-methylen-5α-lanost-8-en-3β-ol) **(4)**	*C. monogyna* (leaves)	(90)
Cycloartenol (9β,19-cyclo-5α,9β-lanost-24-en-3β-ol) **(5)**	*C. monogyna* (leaves)	(90)
Betulinic acid (6)	*C. monogyna* (leaves)	(90)

2.5. Inflammation

Inflammation is a necessary immune system response to various detrimental triggers, including pathogens, toxic substances, and irradiation. The main aim of the inflammation is to eradicate the harmful stimuli and commence the process of healing, thus serving as a vital defense mechanism in preserving good health (99). In general, acute inflammation is effectively controlled by cellular and molecular processes that mitigate impending injury or infection. These mechanisms aid in restoring tissue homeostasis and resolving acute inflammation. However, if not properly managed, acute inflammation can progress into chronic inflammation and contribute to diverse chronic inflammatory conditions (100). Inflammation at the tissue level is characterized by localized redness, swelling, heat, pain and impaired tissue function, resulting from local immune, vascular and inflammatory cell responses to infection or injury (101). The process of inflammation involves significant microcirculatory occurrences, which include alterations in vascular permeability the recruitment and accumulation of white blood cells and the release of mediators that promote inflammation (102). Inflammation can have either infectious or non-infectious causes. When tissues are damaged, the body triggers a chemical signaling process to promote healing, which attracts leukocytes from the bloodstream to the affected area through chemotaxis. The cytokines that are produced by these activated leukocytes cause inflammatory reactions (103).

2.5.1. Nitric Oxide

Nitric oxide (NO) was discovered in 1987, and since then, it has been the subject of extensive research and drug development efforts. This important signaling molecule in the form of gas plays a crucial role in controlling a range of physiological and pathophysiological reactions within the human body. These include the regulation of blood circulation and pressure, platelet activity, immune response and the transmission of signals in both central and peripheral nervous systems. (104).

In the late 1980s, researchers began to understand the role of NO in defense mechanism of the host and immune responses. Studies showed that mouse macrophages generate nitrite and nitrate in response to bacterial lipopolysaccharide, with NO identified as a crucial molecule in macrophage-mediated cytotoxicity (105, 106). Following that, numerous publications have emerged, detailing the impacts of NO on inflammatory reactions. NO participates in host defense in the bronchial epithelium, modulates smooth

muscle tone and acts as a neurotransmitter in NANC neurons. However, it functions as an inflammatory mediator in pathological circumstances. Although NO levels increase in reaction to inflammatory stimuli, leading to pro-inflammatory and destructive effects, it also plays a protective role in certain types of inflammatory responses (104).

2.5.2. Prostaglandin E_2

Prostaglandin E_2 (PGE_2) is a type of bioactive lipid that can cause various biological reactions related to inflammation and cancer. It is part of the prostanoid family of lipids, which is a subgroup of eicosanoids created by oxidizing essential fatty acids (EFAs) containing 20 carbons, which are commonly found in membrane phospholipids. Specific enzymes produce PGE_2 alongside other prostaniods, such as $PGF_{2\alpha}$, PGD_2, PGI_2, and thromboxane A_2 (TXA_2). The synthesis process starts with the release of free fatty acids, such as arachidonic acid (AA), from membrane lipids through phospholipases (PLAs) (107). In the initial stages of inflammation, PGE_2 and other prostanoids, such as PGI_2, function as vasodilators that allow the migration of immune cells from the bloodstream to the affected region, resulting in swelling and edema. Moreover, PGE_2 has the ability to trigger sensory nerve activation, causing an increase in pain response, and it can also act on neurons in the preoptic area to cause pyrogenic effects (108).

2.5.3. Interleukin-6

A particular class of cytokine called Interleukin-6 (IL-6) is released during inflammation and has a variety of profound impacts. Cells that produce IL-6 include fibroblasts, endothelial cells, and macrophages. B-lymphocytes are stimulated to create acute phase proteins by IL-6, which also serves as a growth and differentiation factor. Even though IL-6 is not directly responsible for tissue damage, persistently elevated levels of the protein in the blood have been associated with unfavorable outcomes in patients with sepsis or traumatic injuries (109). Furthermore, IL-6 contributes to the maturation of several cellular and humoral immune responses, such as T-cell activation, immunoglobulin secretion, and B-cell differentiation. The transition from acute to chronic inflammation requires the attraction of monocytes to the site of inflammation, and IL-6 is vital to this process (110).

2.6. Techniques for Determination of Phenolic Profile

2.6.1. Total Phenolic Content Assay

Otto Folin and Willey Glover Dennis of Harvard Medical School found in 1912 that polyphenol chemicals in human urine could be qualitatively recognized using phosphomolybdic acid and phosphotungstic acid reagents, even after many days of fasting (F-D assay) (111). Later, in 1927, Folin and Vintila Ciocalteu, introduced a method for quantifying polyphenols using the Folin-Ciocalteu (F-C) reagent under alkaline conditions to measure tyrosine and tryptophan, which are two amino acids possessing a phenolic ring and exhibiting reducing properties (112). The fundamental distinction between the F-C and F-D assays is the quantity of molybdate (Mo) employed in their production. Folin-Ciocalteu increased the Mo concentration in the F-C reagent to hinder the production of the white precipitate seen in the F-D assay (113). This use of the reagent has consequently greatly increased the sensitivity and repeatability of polyphenol quantification (114).

The phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$) constitute the components of the F-C reagent undergo reactions with both phenols and non-phenolic reducing agents to produce chromogens. As a result of this redox reaction, the formed oxotungstate and oxomolybdate exhibit a blue coloration in alkaline conditions proportional to the concentration of polyphenols, these chromogens can be detected spectrophotometrically (114). A wavelength of 765 nm is used to quantify the resulting structure (115). As a result, the F-C assay is a procedure that uses electron transfer to determine an antioxidant's reductive potential (114).

To make the F-C reagent, 700 mL of distilled water, 100 mL of concentrated hydrochloric acid, and 50 mL of 85% phosphoric acid are combined with 100 g sodium tungstate (VI) dihydrate and 25 g of sodium molybdate (VI) dihydrate. 150 g of lithium sulfate hydrate is introduced into this mixture. When protected from light, the resultant reagent is extremely stable. It also maintains its stability when diluted (115).

2.6.2. Total Flavonoid Content Assay

In the analysis of total flavonoid content, a complexation reaction occurs in the presence of Al(III). According to Barnum (116), this method of analysis was originally used to determine o-diphenols back in 1977. A widely adopted method for TFC determination is the aluminum chloride ($AlCl_3$) colorimetric assay. In this approach, $AlCl_3$ acts as a complexing agent, forming chelates with flavonoids. These chelates exhibit distinct absorption bands, particularly around 500 nm. To enhance sensitivity, sodium nitrite ($NaNO_2$) is sometimes introduced, creating flavonoid-nitroxyl derivatives. An alternative version of the $AlCl_3$ colorimetric assay introduces sodium or potassium acetate alongside $AlCl_3$. This adaptation permits TFC determination even in the absence of $NaNO_2$, typically at a wavelength falling between 410 and 440 nm. The most used flavonoid standards in this method are quercetin, catechin, and rutin. This method is commonly employed in the food, cosmetic, and pharmaceutical industries to assess the efficacy and quality of plant-based products (117).

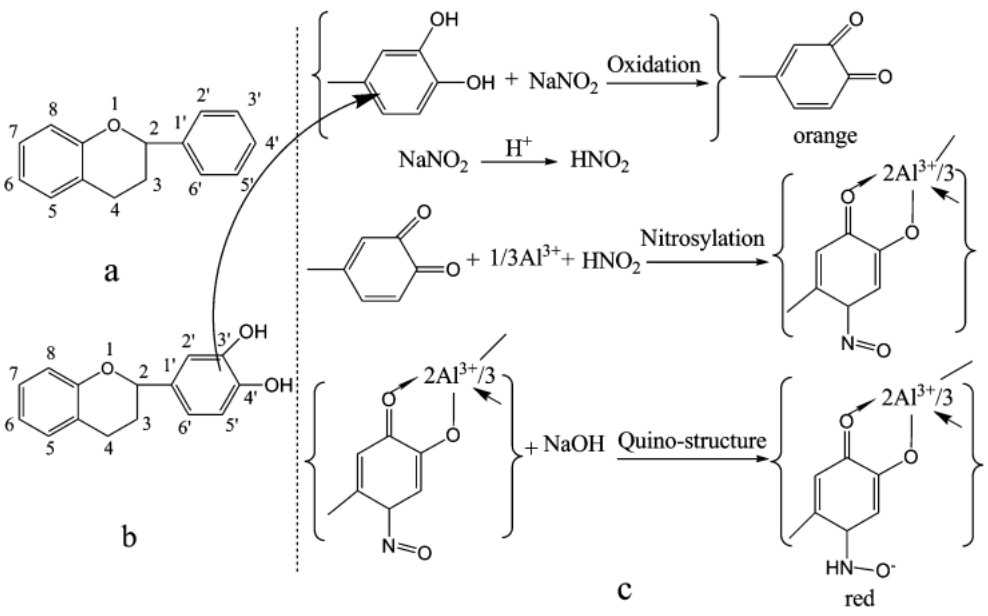

Figure 2. Total flavonoid content assay reaction (118).

2.6.3. Total Anthocyanin Content Assay

Sondheimer and Kertesz first proposed the concept of estimating the anthocyanin content of a material by measuring the change in absorbance at pH values of 3.4 and 2.0 in 1948 (119). According to this approach, anthocyanins undergo reversible structural change as a function of pH, giving the appearance of colorful oxonium and colorless hemiketal forms at pH 1.0 and pH 4.5, respectively. The pigment concentration can be determined by assessing the alteration in absorbance at the vis-max, which occurs at a wavelength of about 520 nm. The findings are presented as cyanidin-3-*O*-glucoside equivalents, the most prevalent anthocyanin pigment found in nature (120). To obtain accurate results, the samples must be clean and free of haze and sediments in order to achieve reliable findings. Any light scattering brought on by colloidal components must also be compensated for by reading at a wavelength where sample absorbance does not occur, such as 700 nm (121).

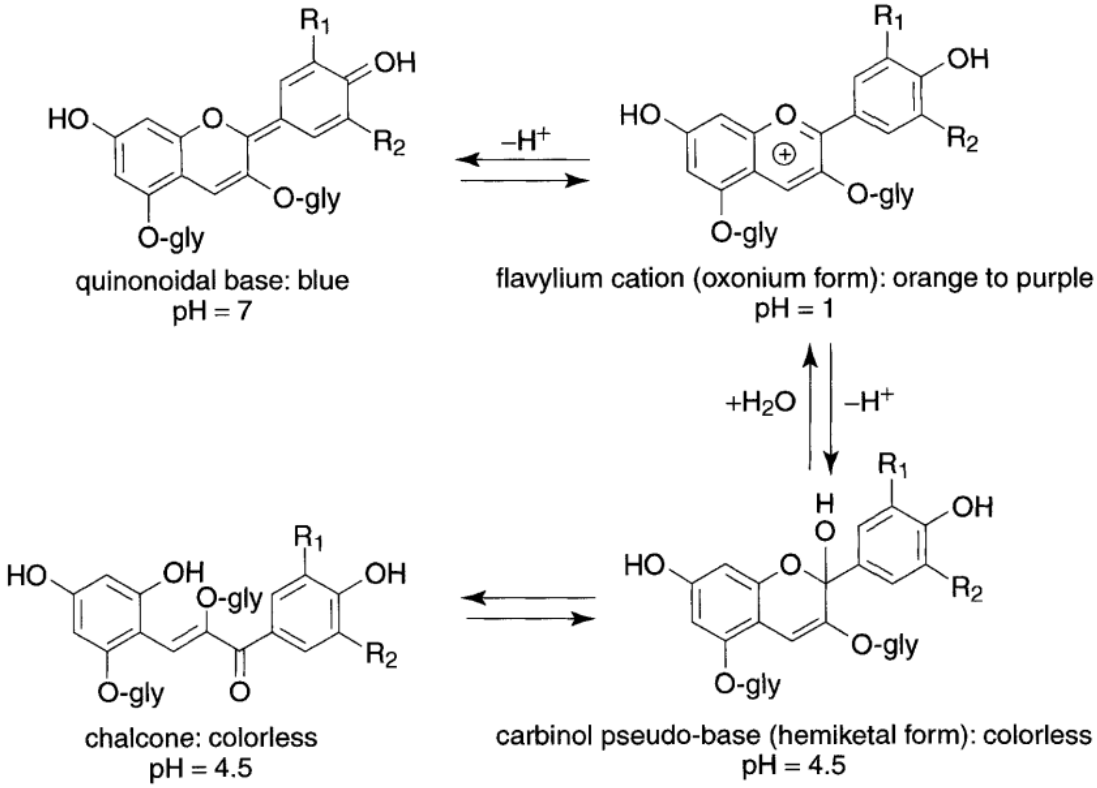

Figure 3. Primary structural forms of anthocyanins present at various pH levels (121).

2.6.4. Total Proanthocyanidin Content Assay

The vanillin-HCl assay, first proposed by Sarkar and Howarth (122), involves protonating vanillin in an acidic environment (pH 1) to create a weak electrophilic carbocation, which combines with the flavonoid ring at C_6 and C_8. Due to dehydration, this reaction results in the generation of a red-colored compound. In their study, Sun et al. (123) looked at how the amount of water and the kind of acid affected the sensitivity of the vanillin method. They found that replacing HCl with H_2SO_4 increased sensitivity and improved reproducibility (124). At the same normality, H_2SO_4 is a more effective catalyst than HCl, which is probably because their water contents differ. At the same normality, concentrated HCl has a larger water content than H_2SO_4, and this higher water content causes a quick decline in A_{500} (125). The most used standard for the vanillin-acid assay is catechin, and it is recommended to measure the absorbance at λ= 500 nm utilizing a spectrophotometer (124).

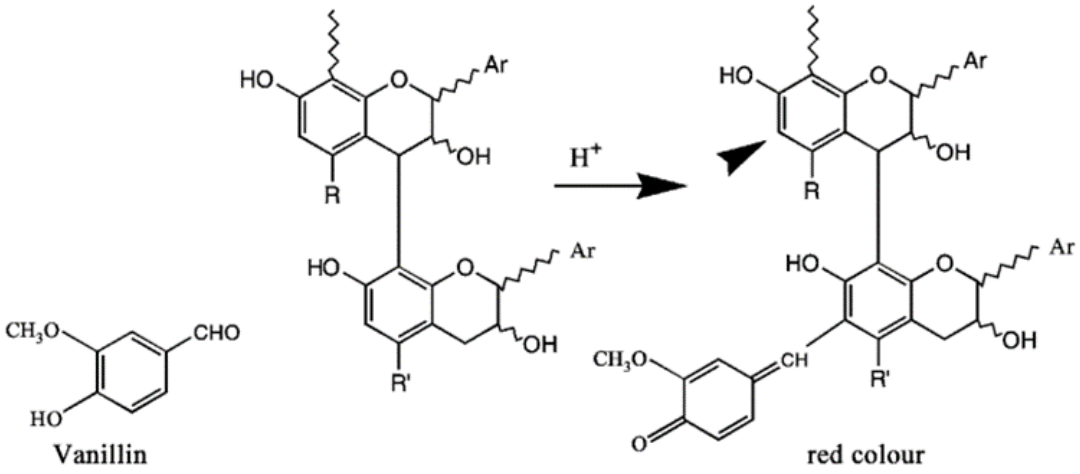

Figure 4. Chemistry of vanillin-acid assay. An additional possible reactive location is indicated by the arrowhead (125).

2.7. Techniques for Determination of Antioxidant Potential

2.7.1. Free Radical Scavenging Activity Assays

2.7.1.1. DPPH Radical Scavenging Assay

Blois (1958) developed a method for evaluating antioxidant activity using a stable free radical, α,α-diphenyl-β-picrylhydrazyl (DDPH; $C_{18}H_{12}N_5O_6$, M:394,33), which involves measuring the scavenging capacity of antioxidants towards it (126). The basic principle of the assay is that antioxidants donate a hydrogen atom to the odd electron of the nitrogen atom in DPPH, which loses its violet color when the reaction occurs. The reaction product is measured at 517 nm. This method is extensively utilized for assessing antioxidant activity, as it is a rapid, easy, and economical way to assess the potential of compounds to function as free radical scavengers or hydrogen donors. Over o duration of 120 minutes at 25°C, the absorbance of DPPH at 517 nm showed a reduction of 20% in methanol and 35% in acetone when exposed to light, while no significant change was observed in the dark for 150 minutes (127).

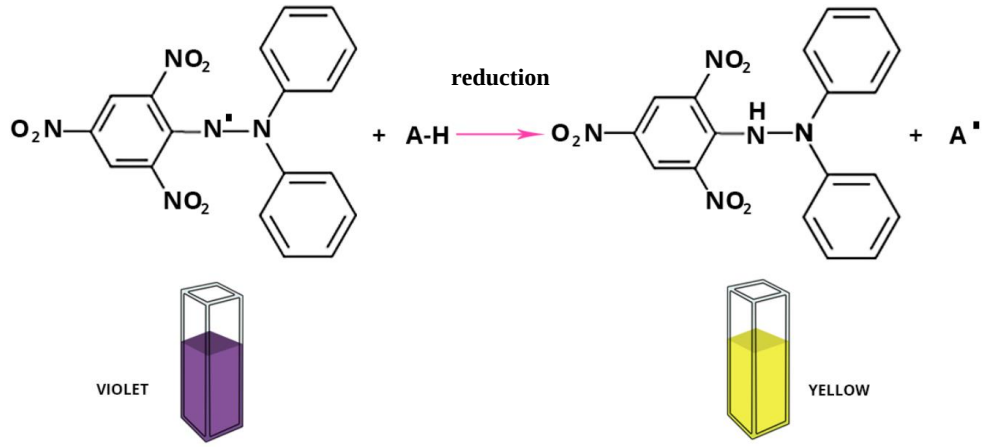

Figure 5. DPPH reaction (127).

2.7.1.2. ABTS Radical Scavenging Assay

In the method developed by Re et al. (128), the oxidation of 2,2'azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) by persulfate produces the ABTS radical, which is used to measure total radical scavenging capacity. This method is based on the ability of antioxidant compounds to cause ABTS to lose its color. In the course of this chemical reaction, the ABTS radical cation in green-blue color is transformed back to its

neutral, colorless form. The stable free radical scavenging ability of molecules is compared to Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E (129, 130)

7 mM ammonium ABTS salt is soluble in water and is treated with 2.45 mM potassium persulfate. The mixture is left at room temperature for 12-16 hours prior to utilization, resulting in a solution with a dark green-blue color. This solution is then diluted with ethanol or pH 7.4 buffer to achieve an absorbance of 0.7 at 734 nm before use (129).

The ABTS radical can be used to explore how pH affects the antioxidant process because it is stable across a wide pH range. Since the ABTS radical is soluble in both aqueous and organic solvents, it enables the evaluation of the antioxidant power of both hydrophilic and lipophilic compounds (129, 130)

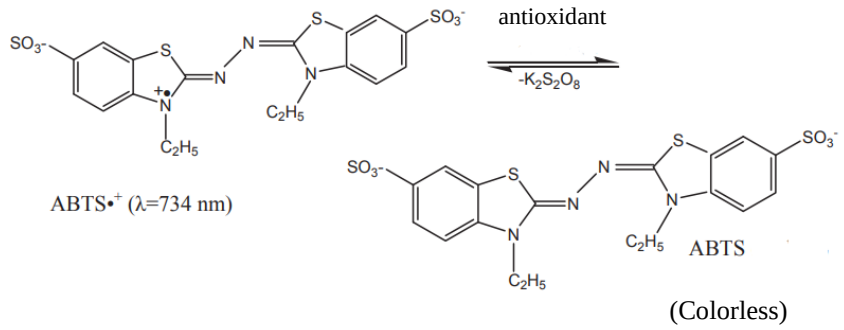

Figure 6. ABTS reaction (129).

2.7.2. Metal Reducing Activity Assays

2.7.2.1. Ferric Reducing Antioxidant Power (FRAP) Assay

The technique depends on the reduction of a complex containing ferric 2,4,6-tripyridyl-s-triazine (Fe^{3+}-TPTZ) by antioxidants, which converts it to its ferrous form (Fe^{2+}-TPTZ). The resulting change in absorbance at 595 nm is utilized to determine the $TEAC_{FRAP}$ (Trolox equivalent antioxidant capacity of FRAP) value of the antioxidant, which is calculated as the ratio of the slope of the absorbance versus concentration line for the antioxidant being tested to that of Trolox, which is measured under the same conditions as the FRAP assay (131).

In this method, the oxidizing agent is a Fe(III) salt, precisely $Fe(III)(TPTZ)_2Cl_3$. The FRAP procedure is applied in an acidic environment with a pH of 3.6 to ensure iron solubility. A dark blue complex exhibiting an absorption peak at 595 nm results from the transformation of the Fe(III)-TPTZ complex into its Fe(II) form at low pH. The determination of hydrophilic and lipophilic antioxidants is suitable for this approach. The simplicity, speed, cost-effectiveness, and stability of the FRAP approach are its most significant advantages (129).

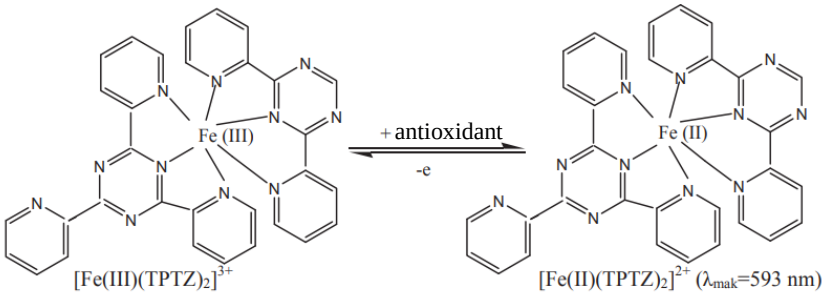

Figure 7. FRAP reaction (129).

2.7.2.2. Cupric Reducing Antioxidant Capacity (CUPRAC) Assay

Apak et al. (132) presented the Cuprac (Cupric Reducing Antioxidant Capacity) antioxidant assessment method to the international literature. The CUPRAC assay is a reliable and adaptable way to assess the antioxidant potential of different polyphenols, such as phenolic acids, hydroxycinnamic acids, flavonoids, carotenoids, and anthocyanins, alongside thiols, artificial antioxidants, and vitamin C and E. The copper(II) cation, bis(neocuprione), is employed in the CUPRAC test as a chromogenic oxidizing reagent and as an outer-sphere electron-transfer agent. By reducing this reagent with antioxidants, the resultant CUPRAC chromophore, bis(neocuprione) copper (I) cation (Cu(I)-Nc), is created. The reagent works at pH 7, and the 450 nm absorbance of the Cu(I)-chelate created by redox reaction with reducing polyphenols, vitamin C, and E are used to quantify the reagent's effectiveness. The orange-yellow color observed is due to the formation of the Cu(I)-Nc chelate (133, 134).

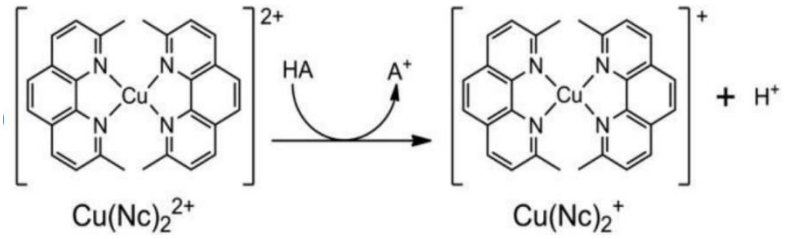

Figure 8. CUPRAC reaction (134).

2.8. Chromatography

The discovery of chromatography was made by a Russian botanist, Micheal Semyonovich Tswett, at the beginning of the 20th century. He separated the green leaf pigments into different colored bands, by passing a solvent through a column bed filled with powdered calcium carbonate. To describe the process, Micheal Semyonovich Tswett created the term chromatography. Greek words for color (chroma) and write (graphein) are the source of the word "chromatography". There were some drawbacks in the column chromatography method discovered by M.S. Tswett, and in 1930, the chromatography method was revived, and these drawbacks were eliminated (135).

The definition of chromatography, as stated by the International Union of Pure and Applied Chemistry (IUPAC), is as follows (136):

"Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction".

Table 20. Classification of Chromatography According to the Physical State of the Mobile Phase (137).

MOBILE PHASE	STATIONARY PHASE	ABBREVIATION
Gas	Solid	GSC
Gas	Liquid	GLC, GC
Liquid	Solid	LSC, LC
Liquid	Liquid	LLC
Supercritical fluid	Liquid (Solid)	SFC

Table 21. Classification of Chromatography According to the Mechanism of Separation (137).

NAME	TYPE OF INTERACTION
Adsorption chromatography	Specific interactions between functional groups present on solid surfaces and the molecules of the sample are observed. (hydrogen bonding, acid-base interactions, dipole-dipole attractions and dispersion forces etc.)
Partition chromatography	The ability of a sample to dissolve in the stationary phase depends on its lipophilic characteristics. H-bonding may also be involved.
Ion-pair chromatography	The formation of dynamic ion exchangers can be achieved by introducing counter ions into the reversed phase. Alternatively, neutral ion pairs may partition.
Ion chromatography	Ionic samples interact with the ionic sites present on the stationary phase
Hydrophobic interaction chromatography	Hydrophobic interactions with stationary phase which consist of short carbon chains
Affinity chromatography	Key-lock principle
Size-exclusion chromatography	No interaction occurs, small molecules enter the pores of stationary phase and are subsequently eluted after larger molecules

Table 22. Classification of Chromatography According to the Shape of the Chromatographic Bed (137).

APPLIED TECHNIQUE	EXAMPLES
Column chromatography	HPLC, GC, LC
Capillary chromatography	GC, LC
Planar chromatography	TLC, HPTLC

2.8.1. High-Performance Thin-Layer Chromatography (HPTLC)

Thin-layer chromatography is a multi-step process used for separating components in a sample. In the first step, a solution containing the sample is applied to a plate coated with a stationary phase. As the carrier solvent of the sample evaporates, the sample is deposited in a small spot at the base of the plate. The plate is then positioned in a sealed container containing a minimal amount of the proper solvent mixture, which then moves up the plate by capillary action. The various constituents of the sample exhibit distinct interactions with both the stationary phase and mobile phase, which leads to their separation as the solvent mixture (mobile phase) travels up the plate. This process is

known as plate development. The goal is to attain distinct spots or bands on the plate. After the plate has undergone development, it is removed from the solvent system. Typically, the sample components are rendered visible by treating them with a reagent that generates visually detectable spots, either visible or fluorescent, when exposure to regular or ultraviolet light. HPTLC utilizes 2 types of UV light which include short-wavelength UV light (with a center at 254 nm) and long-wavelength (with a center of 366 nm). The resulting pattern of the spots is called the chromatogram (138). The R_F value is a fundamental parameter used to describe the movement of the components in a chromatogram. The calculation of this value involves dividing the distance traveled by the solute (the component), which is measured from the starting point to the center of the zone, by the distance travelled by the mobile phase, which is measured from the starting point to the front edge of the solvent front (139).

HPTLC has gained widespread acceptance as a means of qualitatively and quantitatively evaluating herbs and herbal products, due to its straightforwardness, rapidity and cost-effectiveness (140). This technique represents a more sophisticated form of Thin Layer Chromatography (TLC), whereby its automated process, utilization of plates with smaller particle size and reduced developing distances contribute to improved precision and consistency of results (137). Furthermore, HPTLC has several distinctive advantages over high-performance liquid chromatography (HPLC) and Gas Chromatography (GC) including (140):

- The ability to perform parallel separations for high sample throughput.
- Simple and cost-effective.
- The capability of analyzing several samples under the same circumstances and at the same time, enabling accurate comparison.
- Short analysis time.
- It is feasible to detect visually.
- Performing each separation with fresh sorbent enhances the confirmation of identical experimental conditions.
- The two-dimensional structure of a HPTLC chromatogram enables various detection techniques.

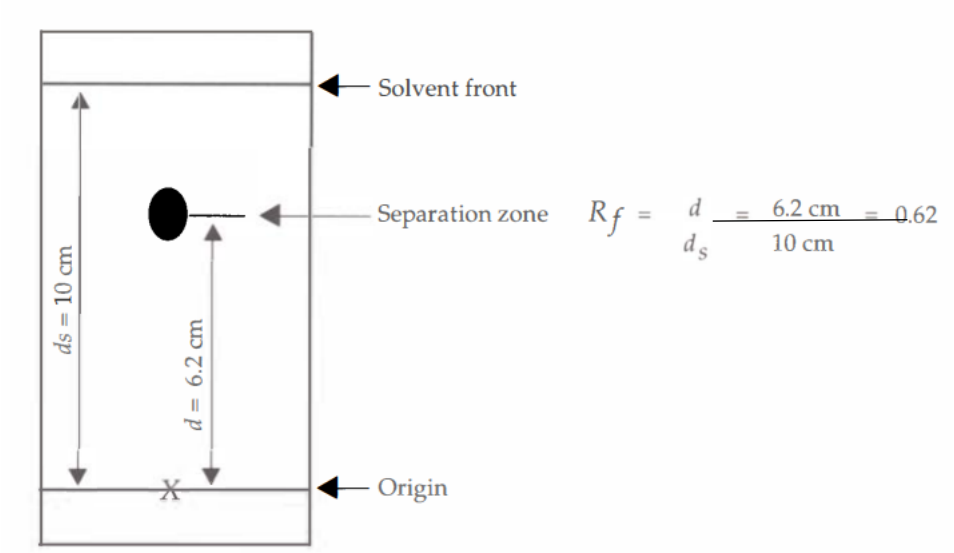

Figure 9. Calculation of the R_f value in TLC (139).

2.8.2. High-Performance Liquid Chromatography (HPLC)

The acronym HPLC was first used in 1970 to indicate the use of high pressure for generating flow in liquid chromatography with packed columns. Originally, pumps had a limited capacity of 35 bar, so this was considered high pressure. However, in the early 1970s, technology advanced rapidly and new instruments were able to generate up to 400 bar of pressure, resulting in improved injectors, detectors, and columns. This technology was known as HPLC, which later became high-performance liquid chromatography due to continued advancements in performance, such as the use of smaller particles and higher pressure. Today, HPLC is considered a highly potent analytical tool in the field of chemistry due to its capability to separate, identify, and quantify compounds in liquid-dissolved samples, even in trace concentrations (141).

Normal-Phase HPLC: For this separation method in HPLC, a stationary phase that is polar is combined with a mobile phase that is much less polar or non-polar (141).

Reversed-Phase HPLC: Reversed-phase chromatography is a chromatography mode that uses mobile phase with a polar character and a stationary phase with a non-polar character, which is in contrast to the normal phase. This method is widely used in HPLC methods, accounting for around 75% of all methods due to its reproducibility and broad applicability (141).

Validation is a laboratory process that involves verifying or establishing the accuracy, precision, and robustness of a developed method. The purpose of validating an

analytical method is to establish its suitability for its intended purpose and works consistently when carried out by different individuals, in different places, and using different chemicals. The validation process typically involves several performance parameters, including (142);

- Accuracy
- Precision
- Specificity
- Selectivity
- Linearity and range
- Limit of detection (LOD) and limit of quantification (LOQ)
- Robustness
- Stability
- System suitability

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Plant Materials

Leaves and flower-bearing branches of *C. monogyna* were collected from İstanbul, Türkiye in May 2020 and fruits of *C. monogyna* were collected in September 2020 from same place. The plant materials were authenticated by Assoc. Prof. Dr. Gizem Bulut. The leaves and flower-bearing branches of *C. orientalis* and *C. pentagyna* were gathered from Ardanuç, Artvin. Similarly, fruits of *C. orientalis* and *C. pentagyna* were collected from the same location, in October 2020. In May 2020, the leaves of *C. turcicus* were gathered from Şavşat, which is situated in Artvin. Similarly, flower-bearing branches of *C. turcicus* were obtained the same location in June 2020. Moreover, the fruits of *C. turcicus* were collected from Şavşat, Artvin in October 2020. The plant materials were collected and authenticated by Prof. Dr. Yüksel Kan. The voucher specimen of *C. monogyna* (YEF 20048) have been kept at Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Yeditepe University, İstanbul, Türkiye. The voucher specimens of *C. turcicus* (ARTH 13587) and *C. orientalis* (ARTH 13585) have been kept at Artvin Çoruh University, Forestry Faculty Herbarium, Artvin, Türkiye. Voucher specimens of *C. pentagyna* (KATO 19304) have been kept at Karadeniz Technical University, Forestry Faculty Herbarium, Trabzon, Türkiye.

3.1.2. Chemicals and Solvents

2,2-diphenyl picryl hydrazyl	Sigma Aldrich; USA 056K1147
2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ)	Honeywell Research Chemicals; USA
ABTS chemical	Sigma Aldrich; USA 10102946001
Acetic acid	Honeywell Research Chemicals; USA
Acetonitrile	J. T. Baker; USA SZBB3145V
Aluminum chloride	Merck; Germany 8.01081.1000
Ammonium acetate	Carlo Erba; France 313507
Ammonium molybdate	Honeywell Research Chemicals; USA

Caffeic acid	Sigma Aldrich; USA C0625
(+)-Catechin hydrate	Sigma Aldrich; USA C1251
Chlorogenic acid	Sigma Aldrich; USA C3878
Copper Sulphate	Carlo Erba; France 313507
Cyanidin chloride	Phytolab; Germany 80022
Cyanidin-3-*O*-glucoside	Phytolab; Germany 89616
Dulbecco's modified eagle's medium	Gibco; USA 41966-029
Dimethyl sulfoxide	Riedel de Haen; Germany
Ethanol	Sigma Aldrich; USA 46139
Ethyl acetate	Sigma Aldrich; USA SZBA113S
Ethyl methyl ketone	Merck; Germany K38981914
Epicathechin	Biosynth; Switzerland FE22720
Ferric chloride hexahydrate	Sigma Aldrich; USA 236489
Folin-Ciocalteu Reagent	Sigma Aldrich; USA F9252
Formic acid	Sigma Aldrich; USA 27001
Fetal bovine serum	Gibco; USA 10500064
Gallic acid	Sigma Aldrich; USA 67384
Glacial acetic acid	Sigma Aldrich; USA 27225
Hydrochloric acid (36.5%)	Sigma Aldrich; USA SZBA2250
Hyperoside	HWI Pharma; Germany 0018-05-85
Iron (II) chloride	J.T. Baker; USA 0125
Iron (II) sulfate heptahydrate	J.T. Baker; USA 0126
Isoquercitrin	Sigma Aldrich; USA 00140585
IL-6 ELISA Kit	Invitrogen; USA

Lipopolysaccharides from *Escherichia coli*	Sigma Aldrich; USA L4391
Methanol	Sigma Aldrich; USA SZE9365S
MTT solution	Sigma Aldrich; USA M2128
Natural products	Sigma Aldrich; USA N1501
Neocuproine	Sigma Aldrich; USA N1501
Neochlorogenic acid	Aktin; China APC-519
n-propanol	Interlab; Türkiye 961.012.2500
o-phosphoric acid	Merck; Germany
Orientin	TRC Corp.; Canada O674900
Penicillin-Streptomycin antibiotic	Gibco; USA 15140122
PBS (without Ca, Mg) solution	Gibco; USA 14190094
PGE_2 ELISA Kit	Abcam; USA
Polyethlylene glycol 400	Merck; Germany S6041785 019
potassium chloride	Sigma Aldrich; USA 12636
potassium persulphate	Sigma Aldrich; USA 216224
Protocatechuic acid	Sigma Aldrich; USA 03930590
Procyanidin B2	Sigma Aldrich; USA 29106
Quercetin	HWI Pharma; Germany 0074-05-80
RAW 264.7 Murine Macrophage Cell line	ATCC; USA
Rutin	Sigma Aldrich; USA R5143
Sodium acetate	Merck; Germany 1062680250
Sodium acetate trihydrate	Honeywell Research Chemicals; USA
Sodium carbonate	Sigma Aldrich; USA 223484
Sodium nitrite ($NaNO_2$)	Honeywell Research Chemicals; USA

Sulfanilamide	Honeywell Research Chemicals; USA
Sulfuric acid (98%)	Honeywell Research Chemicals; USA
Tripyridyltriazine	Sigma Aldrich; USA 93285
Trolox	Sigma Aldrich; USA BCBF4547V
Trypsin	Gibco; USA 25200056
Tetrahydrofuran (THF)	VWR; Singapore I 1009923132
Vanillin	Honeywell Research Chemicals; USA
Vitexin	Aktin; China APC-389
Vitexin-2"-*O*-rhamnoside	Aktin; China APC-504

3.1.3. Equipments and Instruments

96-well plate	TPP; Germany 92096
Agilent Zorbax Plus C18 column	Agilent Chemical; USA
Balance	Ohaus Explorer; USA
Beaker (50, 100, 250, 1000 mL)	Isolab; Germany
Centrifuge	Sigma Aldrich; USA 3-16 PK
Class-II Biosafety cabinette	ESCO Class-II Airstream; Singapore
Deep freezer	Arçelik; Istanbul, Türkiye
Eppendorf tubes (1,5,15 mL)	Eppendorf; Germany
Erlenmayer flask (50, 100, 250, 1000 mL)	Isolab; Germany
Falcon tubes (15- 50 mL)	Falcon; Germany
Graduated cylinder (25, 50, 100 mL)	Isolab; Germany
Hairdryer	Arcelik; Türkiye
HPTLC system	Camag; Switzerland
HPTLC Developing Chambers	Camag; Switzerland

HPTLC glass 20 cm x 10 cm, Si 60 F254	Merck; Germany
HPTLC Plates	Camag; Switzerland
Incubator	Binder CB-150; Germany
Light microscopy	Carl Zeiss Primo Vert, Germany
Lyophilizator	Christ Alpha; Germany 2-4 LD
Micropipette (100-1000 microlt)	Gilson; USA
Micropipette (500-5000 microlt)	Rainin; USA
Micropipette (20-200 microlt)	Thermo Multiskan Ascent; USA
Microplate reader	Varioskan Lux Milli Q; USA
Oven	Binder; Germany
Ultrasonic bath	Sonorex; Germany RK156BH
pH meter	Mettler-Toledo; USA MP220
RAW 264.7 mouse macrophage cells	ATCC; USA
Refrigerator	Arcelik; Türkiye
Rotatory evaporator	Heidolph; Germany
Silica gel 60 F254 Aluminium sheet	Merck; Germany
Spectrophotometer	Spekol; USA 1300
Sterile filter (0.22 μm)	GVS; Germany
Sterile serological pipette (5-10 mL)	SPL LifeScience; Korea
Sterile syringe	Hayat Lab; Türkiye
Volumetric flasks	(25, 100, 500, 1000 mL)
Water bath	GFL; England
Water device	Millipore; Germany

3.2. Methods

3.2.1. Extraction Procedure

The leaves and flower-bearing branches of *C. turcicus*, *C. monogyna*, *C. orientalis*, and *C. pentagyna* were dried at room temperature in a dark environment for two weeks and then ground into powder. The fruits, which were not deseeded, were stored at -20°C and, before extraction, dried in a lyophilizer, then ground prior to extraction. 5 g of fruits, 5 of leaves, and 5 g of flower-bearing branches of these *Crataegus* species were extracted separately with 70% ethanol (100 mL) by using an ultrasonic bath for 30 minutes. Then, hydroalcoholic extracts were filtered through 0.2 mm thick filter paper and ethanol was evaporated until complete dryness under reduced pressure by using rotatory evaporator at 45°C and 80 mbar pressure conditions. After freezing overnight at -20 °C, the remaining aqueous part was lyophilized at -55 °C and 0.2 mbar pressure. After the lyophilization process, obtained hydroalcoholic extracts were placed into amber bottles and stored in the refrigerator at +4 degrees throughout the experiment.

Figure 10. Lyophilization process of *Crataegus* extracts.

Table 23. Extraction yields of hydroalcoholic extracts from *Crataegus* species.

PLANT NAME	PART USED	YIELD
C. turcicus	Fruits	25.23%
C. turcicus	Leaves	18.50%
C. turcicus	Flower-bearing branches	18.41%
C. monogyna	Fruits	15.21%
C. monogyna	Leaves	23.41%
C. monogyna	Flower-bearing branches	19.98%
C. orientalis	Fruits	24.22%
C. orientalis	Leaves	15.48%
C. orientalis	Flower-bearing branches	17.25%
C. pentagyna	Fruits	21.48%
C. pentagyna	Leaves	18.23%
C. pentagyna	Flower-bearing branches	13.29%

3.2.2. Preparation of Sample Stock Solutions

Lyophilized sample (100 mg) was precisely weighed and dissolved in 5 mL of 70% ethanol using an ultrasonic bath for 15 min. To remove suspended particles, each sample test solution was filtered through a 0.45 µm RC-membrane filter. The final concentration of the stock sample test solution was 20 mg/mL. Furthermore, the stock solution was diluted according to the desired concentration to be used in different experiments as a sample test solution.

3.2.3. Chromatography

3.2.3.1. High-Performance Thin-Layer Chromatography

Applications to the HPTLC plates were made by semi-automatic sample spotter Linomat 5 (CAMAG, Muttenz, Switzerland). WinCATS software-controlled HPTLC system from CAMAG, which was used in this experiment, included Plate Heater III, Automatic Developing Chamber 2 (ADC2), Linomat 5 sample applicator with a 100 µL syringe, TLC Visualizer, Immersion Device, and Automatic Developing Chamber 2 (ADC2).

Analysis of phenolic acids, flavonol and flavone C-glycosides:

Sample test solution: 20 mg/mL of sample test solutions containing *Crategus* species were used.

Standard solution: Equal volumes of rutin, hyperoside, isoquercitrin, orientin, vitexin, vitexin-2''-*O*-rahmnoside, chlorogenic acid, protocatechuic acid, and neochlorogenic acid at a concentration of 50 μg/mL were mixed.

Stationary phase: 20 x 10 cm glass-backed HPTLC plates (Merck, Darmstadt, Germany) coated with silica gel 60 F_{254}

Developing solvent system: Ethyl acetate - ethyl methyl ketone - formic acid - water (5:3:1:1 *v/v/v/v*) (143)

Derivatization reagents: Natural product and PEG 400 solutions

Visualization: 254 nm and 366 nm

The standard solution (10 μL) and sample test solutions (5 μL) were applied at a speed of 100 nL/s through a syringe with 10 mm bandwidth on an HPTLC plate. After drying the application positions with a warm air, the plate was developed by using a developing solvent system by using Automatic Developing Chamber (ADC2, Camag) which was saturated for 20 min. The relative humidity was set at 46.2% using magnesium chloride hexahydrate solution ($MgCl_2.6H_2O$). The plate was developed up to 7 cm. After development, plate was dried with a cold air for 5 min. automatically by ADC2 system. Prior to derivatization process, the HPTLC plate was heated at a temperature of 105 °C for a duration of 3 minutes using the TLC plate heater (Camag). To perform derivatization, the plate was respectively dipped, by using CAMAG Immersion Device, in Natural Product reagent (1 g of the 2-aminoethyl diphenylborinate dissolved in 200 mL of ethyl acetate) and PEG 400 reagent (200 mL of dichloromethane was used to dissolve 10 g of polyethylene glycol 400), which were prepared following the method described by Güzelmeriç et al. (144). By using TLC visualizer (Camag), HPTLC plate was examined at 254 and 366 nm. Retention factor (R_F) and band colors of standards were comparatively evaluated with that of compounds for *Crataegus* species.

Analysis of flavanols:

Sample test solution: 20 mg/mL of sample test solutions containing *Crategus* species were used.

Standard solution: Equal volumes of epicatechin and procyanidin B2 at a concentration of 50 µg/mL were mixed.

Stationary phase: 20 x 10 cm glass-backed HPTLC plates (Merck, Darmstadt, Germany) coated with silica gel 60 F_{254}

Developing solvent system: ethyl acetate - formic acid - water (10:1:4 *v/v/v*) (upper phase of the mixture was used) (145)

Derivatization reagents: Vanillin-sulfuric acid reagent (5g vanillin + 475 mL ethanol +25 mL sulfuric acid) (146)

Visualization: white light

The standard solution (5 µL) and sample test solutions (5 µL) were applied at a speed of 100 nL/s through a syringe with 10 mm bandwidth on an HPTLC plate. After drying the application positions with a warm air, the plate was developed by using a developing solvent system by using Automatic Developing Chamber (ADC2, Camag) which was saturated for 20 min. The relative humidity was set at 42.7% using magnesium chloride hexahydrate solution ($MgCl_2.6H_2O$). The plate was developed up to 7 cm. After development, plate was dried with a cold air for 5 min. automatically by ADC2 system. To perform derivatization, the plate was respectively dipped, by using CAMAG Immersion Device, in vanillin-sulfuric acid reagent (5 g vanillin was mixed with 475 mL ethanol and 25 mL sulfuric acid), following the method described by Alina Pyka (146). By using TLC visualizer (Camag), HPTLC plate was examined under white light. Retention factor (R_F) and band colors of standards were comparatively evaluated with that of compounds for *Crataegus* species.

For the separation of catechin and epicatechin:

Sample test solution: 20 mg/mL of sample test solutions containing *Crategus* species were used.

Standard solution: Equal volumes of epicatechin and catechin at a concentration of 50 µg/mL were mixed.

Stationary phase: 20 x 10 cm glass-backed HPTLC plates (Merck, Darmstadt, Germany) coated with cellulose

Developing solvent system: n-propanol – water - acetic acid (4:2:1 *v/v/v/v*) (147)

Derivatization reagents: Vanillin-sulfuric acid reagent

Visualization: white light

The standard solution (5 µL) and sample test solutions (5 µL) were applied at a speed of 100 nL/s through a syringe with 10 mm bandwidth on an HPTLC plate. After drying the application positions with a warm air, the plate was developed by using a developing solvent system by using Automatic Developing Chamber (ADC2, Camag) which was saturated for 20 min. The relative humidity was set at 38% using magnesium chloride hexahydrate solution ($MgCl_2.6H_2O$). The plate was developed up to 7 cm. After development, plate was dried with a cold air for 5 min. automatically by ADC2 system. To perform derivatization, the plate was respectively dipped, by using CAMAG Immersion Device, in vanillin-sulfuric acid reagent, following the method described by Alina Pyka (146) to determine variations in the chemical composition among samples. By using TLC visualizer (Camag), HPTLC plate was examined under white light. Retention factor (R_F) and band colors of standards were used to confirm the identification of the chemical composition for *Crataegus* species.

Analysis of anthocyanins:

Sample test solution: 20 mg/mL of sample test solutions containing *Crategus* species were used.

Standard solution: Cyanidin-3-*O*-glucoside at a concentration of 160 µg/mL were mixed.

Stationary phase: 20 x 10 cm glass-backed HPTLC plates (Merck, Darmstadt, Germany) coated with silica gel 60 F_{254}

Developing solvent system: Ethyl acetate - ethyl methyl ketone - formic acid - water (5:3:1:1 *v/v/v/v*) (143)

Derivatization reagents: -

Visualization: white light

The standard solution (2 µL) and sample test solutions (applied to the plate twice, each time using a different injection volume: 5 µL and 10 µL) were applied at a speed of 100 nL/s through a syringe with 10 mm bandwidth on an HPTLC plate. After drying the application positions with a warm air, the plate was developed by using a developing solvent system by using Automatic Developing Chamber (ADC2, Camag) which was saturated for 20 min. The relative humidity was set at 40.7% using magnesium chloride hexahydrate solution ($MgCl_2.6H_2O$). The plate was developed up to 7 cm. After development, plate was dried with a cold air for 5 min. automatically by ADC2 system. By using TLC visualizer (Camag), HPTLC plate was examined under white light. Retention factor (R_F) and band colors of standards were used to confirm the identification of the chemical composition for *Crataegus* species.

Table 24. Reference compounds, mobile phase, stationary phase, and derivatization reagents.

REFERENCE COMPOUNDS	MOBILE PHASE	STATIONARY PHASE	DERIVATIZATION REAGENTS
• Cyanidin-3-*O*-glucoside	ethyl acetate - ethyl methyl ketone - formic acid - water (5:3:1:1 *v/v/v/v*) (143)	Silica gel	Derivatization reagent was not used.
• Rutin • hyperoside • isoquercitrin • orientin • vitexin • vitexin-2"-*O*-rhamnoside • protocatechuic acid • chlorogenic acid • neochlorogenic acid	ethyl acetate - ethyl methyl ketone - formic acid - water (5:3:1:1 *v/v/v/v*) (143)	Silica gel	natural product (NP)-polyethylene glycol 400 (PEG 400) (144)
• epicatechin • procyanidin B2	ethyl acetate - Formic acid - water (upper phase) (10:1:4 *v/v/v*) (145)	Silica gel	vanillin-sulfuric acid reagent (146)
• catechin • epicatechin	*n*-propanol - water- acetic acid (4:2:1 *v/v/v*) (147)	Cellulose	vanillin-sulfuric acid reagent (146)

3.2.3.2. High-Performance Liquid Chromatography

To confirm the identities of the compounds, the UV spectra of protocatechuic acid, chlorogenic acid, neochlorogenic acid, procyanidin B2, epicatechin, orientin, vitexin, vitexin-2"-*O*-rhamnoside, hyperoside, rutin and isoquercitrin were compared with the UV spectra of the sample test solutions. During the analysis, an Agilent 1260 Infinity HPLC system (Darmstadt, Germany) was utilized, which consisted of quaternary pump (G1311B), auto-sampler (G1329B), thermostatted column compartment (G1316A), diode array detector (G4212B) and Agilent ChemStation software. To achieve optimal

separation of the investigated compounds, a reversed-phase column with the specifications of Agilent Zorbax Plus C 18 (4.6 x 250 mm) and a particle size of 5 μm was employed at a temperature of 25 °C. This column configuration was selected to create ideal conditions for the separation of the compounds under study. HPLC method was applied according to previously published method by Ying et al. (148).

The mobile phase was set to flow rate of 1 mL/min and the injection volume of the analytes was 10 μL in triplicates. 2 different mobile phases were employed in the analysis. Mobile phase A was *o*-phosphoric acid-water (0.5:99.5, *v*/*v*) and B was acetonitrile–tetrahydrofuran (95:5, *v*/*v*). The mobile phases were filtered and degassed before analysis. The gradient elution in HPLC was applied as 95-5% A-B (0–20 min), 90-10% A-B (20–45 min), 86.5–13.5% A-B (45–47 min), 25–75% A-B (47–48 min), 25-75% A-B (48–50 min), and 95-5% A-B (50–55 min). The analytes were monitored using different wavelengths, which were chosen based on their Uvmax values. For quantitative analysis, protocatechuic acid, hyperoside, rutin, and isoquercitrin were detected at 260 nm, whereas procyanidin B2 and epicatechin were identified at 280 nm. Besides, 330 nm was used to monitor neochlorogenic acid and chlorogenic acid. Vitexin and vitexin-2"-*O*-rhamnoside were evaluated at 340 nm, while orientin was monitored at 350 nm.

Before HPLC analysis, stock solutions of each sample were initially diluted to concentrations of 0.5 mg/mL, 1 mg/mL and 4 mg/mL using HPLC grade MeOH. Subsequently, each prepared sample underwent HPLC analysis after being filtered through a 0.45 μm RC-membrane filter (Sartorius Stedim Biotech).

The linearity of the calibration curves was obtained by using seven different working standard solutions in between 5 to 50 μg/mL prepared from the stock solutions of protocatechuic acid, neochlorogenic acid, chlorogenic acid, procyanidin B2, epicatechin, orientin, vitexin, hyperoside, and isoquerctirin and 10 to 100 μg/mL for and vitexin-2"-*O*-rhamnoside and rutin. The regression equations and coefficient of determination (r^2) are stated in Table 25. For the calculation of the LOQ and LOD values calibration curves were obtained on consecutive days and were calculated by using the following equation 10 x (SD/S) and 3 x (SD/S), respectively, stated in Table 25.

To assess the accuracy of the method, triplicate analyses were conducted on standard solutions with varying concentrations (12.5, 6.25, 3.125 μg/mL) of protocatechuic acid, chlorogenic acid, neochlorogenic acid, procyanidin B2, epicatechin,

orientin, vitexin, vitexin-2"-*O*-rhamnoside, hyperoside, rutin and isoquercitrin. The obtained results, represented as recovery values in Table 25, exhibited a concentration difference below 5 with a relative standard deviation (RSD).

Table 25. Linearity data of the calibration curve, LOD and LOQ values.

Standards	Linearity range (µg/mL)	Calibaration equation[A]	r^2	LOQ[1] (µg/mL)	LOD[2] (µg/mL)
Protocatechuic acid	5-50	y = 34.362x+20.86	0.9995	0.50	0.15
Neochlorogenic acid	5-50	y = 21.462x-2.5439	0.9992	0.09	0.03
Chlorogenic acid	5-50	y = 24.027x-3.9582	0.9999	1.45	0.43
Procyanidin B2	5-50	y = 4.5678x-2.239	0.9977	1.19	0.36
Epicatechin	5-50	y = 6.0811x+1.6588	0.9992	0.43	0.13
Orientin	5-50	y = 25.761x+13.279	0.999	0.49	0.15
Vitexin	5-50	y = 24.314x+12.074	0.9973	0.64	0.19
Vitexin-2"-*O*-rhamnoside	10-100	y = 16.009x+8.6479	0.9995	2.51	0.75
Hyperoside	5-50	y = 18.395x-8.2302	0.9981	0.03	0.01
Rutin	10-100	y = 13.511x-3.8909	0.9995	1.08	0.32
Isoquercitrin	5-50	y = 19.323x+4.1729	0.9997	0.61	0.18

[1]LOD: Limit of detection
[2]LOQ: Limit of quantification

Table 26. The closeness of the obtained results (%) by HPLC method to their theoretical values.

Standards	**Theoretical value[1]**	**Amount found[2]**	**Recovery %**
Protocatechuic acid	12.5	12.23 ± 0.15	97.85
	6.25	6.02 ± 0.06	96.26
	3.125	2.78 ± 0.09	88.91
Neochlorogenic acid	12.5	12.99 ± 0.46	103.93
	6.25	7.00 ± 0.15	112.03
	3.125	3.36 ± 0.04	107.47
Chlorogenic acid	12.5	12.38 ± 0.42	99.04
	6.25	6.04 ± 0.13	96.64
	3.125	3.23 ± 0.09	103.38
Procyanidin B2	12.5	12.16 ± 0.37	97.27
	6.25	6.74 ± 0.15	107.79
	3.125	3.23 ± 0.02	103.25
Epicatechin	12.5	12.29 ± 0.28	98.28
	6.25	5.88 ± 0.18	94.13
	3.125	2.87 ± 0.03	91.78
Orientin	12.5	12.02 ± 0.24	96.18
	6.25	5.76 ± 0.14	92.22
	3.125	3.11 ± 0.15	99.48
Vitexin	12.5	12.23 ± 0.21	97.82
	6.25	5.76 ± 0.08	92.21
	3.125	2.58 ± 0.03	81.06
Vitexin-2"-*O*-rhamnoside	25	24.25 ± 0.89	97.02
	12.5	12.01 ± 0.03	96.07
	6.25	6.36 ± 0.16	101.73
Hyperoside	12.5	13.45 ± 0.23	107.56
	6.25	6.08 ± 0.08	97.36
	3.125	3.56 ± 0.18	113.94
Rutin	25	26.05 ± 0.77	104.19
	12.5	12.17 ± 0.19	97.40
	6.25	6.26 ± 0.05	100.21
Isoquercitrin	12.5	12.27 ± 0.34	98.17
	6.25	6.76 ± 0.09	108.14
	3.125	2.82 ± 0.12	90.13

[1]Theoretical value of the standards were expressed as μg/mL.
[2]The average found amounts were expressed as μg/mL ± SD, n=3.

3.2.4. Techniques for Determination of Phenolic Profile

3.2.4.1. Total Phenolic Content Assay

Total phenolic content of the samples was spectrophotometrically determined by Folin-Ciocalteu method explained by Singleton et al. (149) with minor alterations. The experiment was conducted in a 96-well plate. 100 µL of Na_2CO_3 (7.5% in H_2O) was added to 25 µL of each sample, blank (MeOH), and reference solutions. Then 100 µL of 10% Folin-Ciocalteu reagent in H_2O was added to this mixture. The solutions in the well plate were kept in dark environment at room temperature for a duration of 30 minutes. After that, absorbance was measured at 765 nm by using UV spectrophotometer (Multiscan Go, Thermo Scientific). To obtain calibration curve, gallic acid at between 0.004-0.125 mg/mL concentrations were used. Sample solution concentration was 0.4 mg/mL for fruit extracts, 0.1 mg/mL for the others. The results were expressed as mg gallic acid equivalents per g hydroalcoholic extract (mg GAE/g).

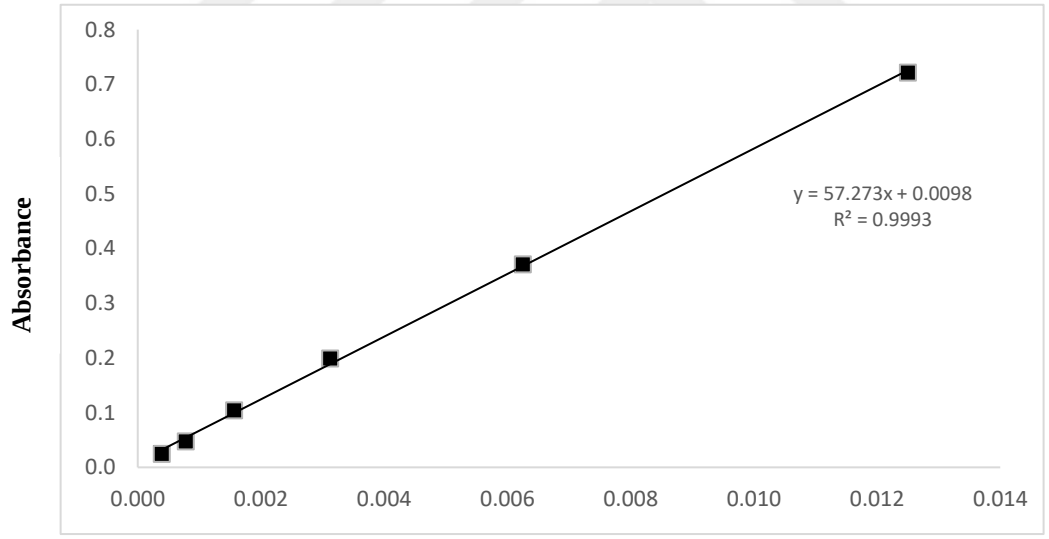

Figure 11. Calibration curve of gallic acid for determination of TPC.

3.2.4.2. Total Flavonoid Content Assay

Total flavonoid content of the samples was spectrophotometrically calculated according to the method described earlier by Güzelmeriç et al. (150) with minor changes. The experiment was carried out using a 96-well plate. 30 µL of aluminum chloride (10%

in H_2O) and 1M sodium acetate (in H_2O) and 150 µL MeOH were added to 30 µL of each sample, MeOH as a blank and reference solutions, sequentially. After the 15 minutes of incubation, absorbance was measured at 415 nm by using UV spectrophotometer (Multiscan Go, Thermo Scientific). As a standard solution, quercetin was used at between 0.994-0.125 mg/mL concentrations. Results were expressed as mg quercetin equivalents per g hydroalcoholic extract (mg QE/g). Concentration of test solutions were 2 mg/mL for *C. turcicus*, *C. orientalis* and *C. pentagyna* fruit extracts and 4 mg/mL for the other extracts.

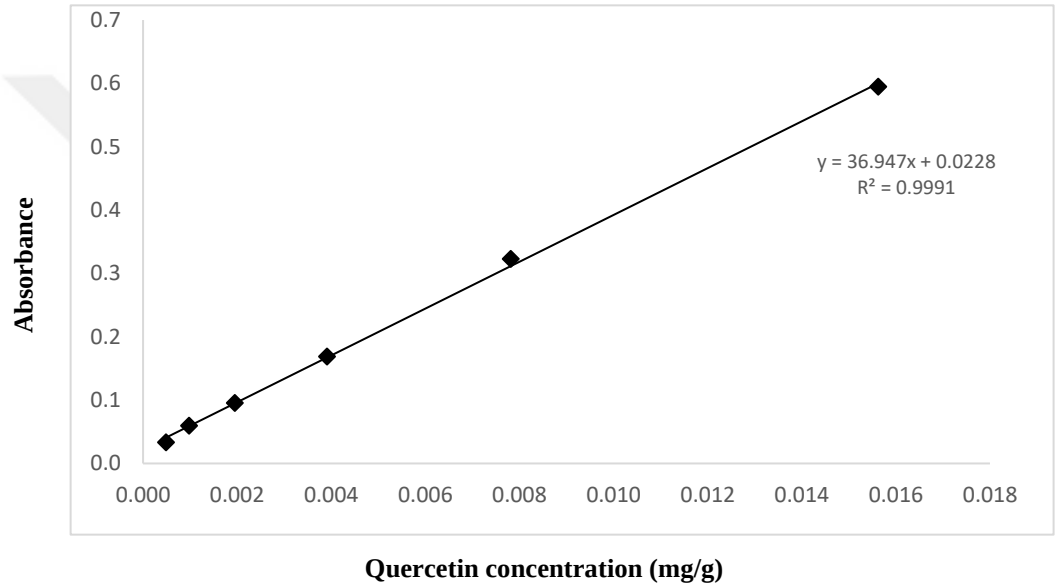

Figure 12. Calibration curve of quercetin for determination of TFC.

3.2.4.3. Total Anthocyanin Content Assay

Spectrophotometric method of total anthocyanin content assay was conducted by using the method explained earlier by Lee et al. (151). 96-well plate was used for the experiment. 160 µL of pH 1 buffer solution was added to 40 µL of each sample and reference solutions. Then absorbance was measured at 520 nm and 700 nm by using UV spectrophotometer. At the same time, 160 µL of pH 4.5 buffer solution was mixed with 40 µL sample, reference and blank solutions and absorbance of red color formation was read at 520 nm and 700 nm. Fruit extract of *C. turcicus*, *C. monogyna*, *C. orientelis* and *C. pentagyna* was used as a sample solution at 4 mg/mL concentrations. The absorbances obtained in the total anthocyanin experiment were calculated using the formula:

Absorbance (A): $(A_{520}-A_{700})_{pH1}-(A_{520}-A_{700})pH_{4.5}$. For making the calibration curve, cyanidin-3-*O*-glucoside solutions were prepared at between 0.0021-0.2mg/mL concentrations and results were given as mg cyanidin-3-*O*-glucoside equivalent per g hydroalcoholic extract (mg C3GE/g).

Buffer solutions prepared by this procedure;

pH 1 buffer: 0.025 M potassium chloride was dissolved in distilled water. After that pH was adjusted to 1 with diluted HCl.

pH 4.5 buffer: 0.4 M sodium acetate was added into distilled water and mixed. After that pH was adjusted to 4.5 with diluted HCl.

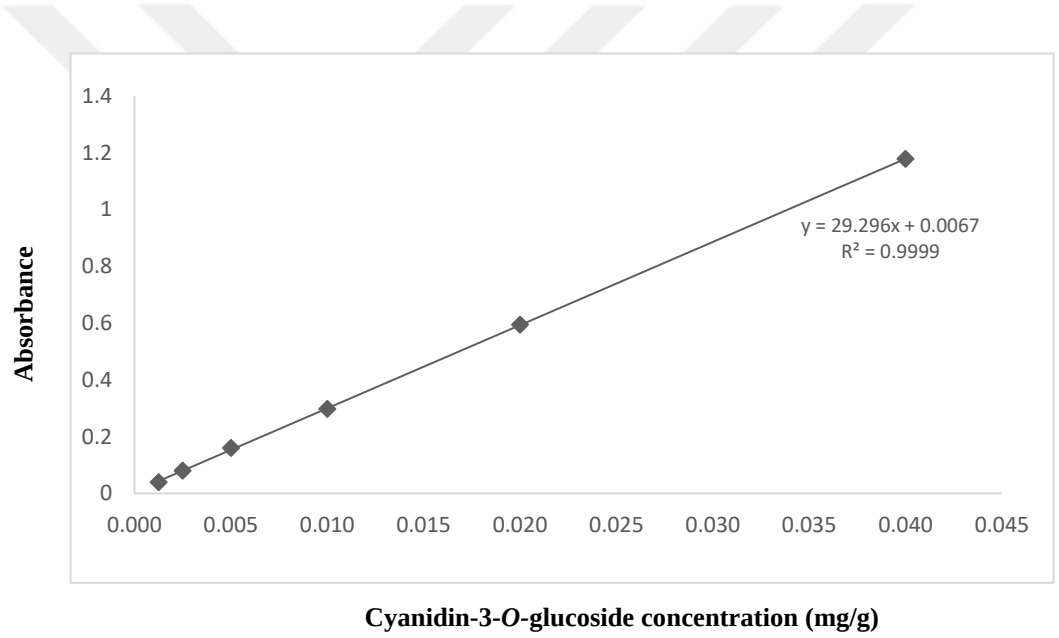

Figure 13. Calibration curve of cyanidin-3-*O*-glucoside for determination of TAC.

3.2.4.4. Total Proanthocyanidin Content Assay

Total proanthocyanidin content assay was performed with 96-well plate by using the procedure from Sun et al. (152) with small changes. In an each well plate, 50 µL sample test solutions at the concentration of 4 mg/mL, blank or reference solutions at different concentration, 125 µL 1% vanillin (in MeOH) and 125 µL 25% sulfurıc acid (in MeOH) were added in order. Then mixture was incubated 15 minutes at 30°C. When the incubation period was over, absorbance was determined at 500 nm. In this experiment,

two different references were used. Firstly, catechin was prepared at 0.2-0.002 mg/mL concentrations, then cyanidin chloride was prepared at 0.125-0.004 mg/mL concentrations to make calibration curve. Results were expressed as mg catechin equivalent per g hydroalcoholic extract (mg CE/g) and mg cyanidin chloride equivalent per g hydroalcoholic extract (mg CCE/g).

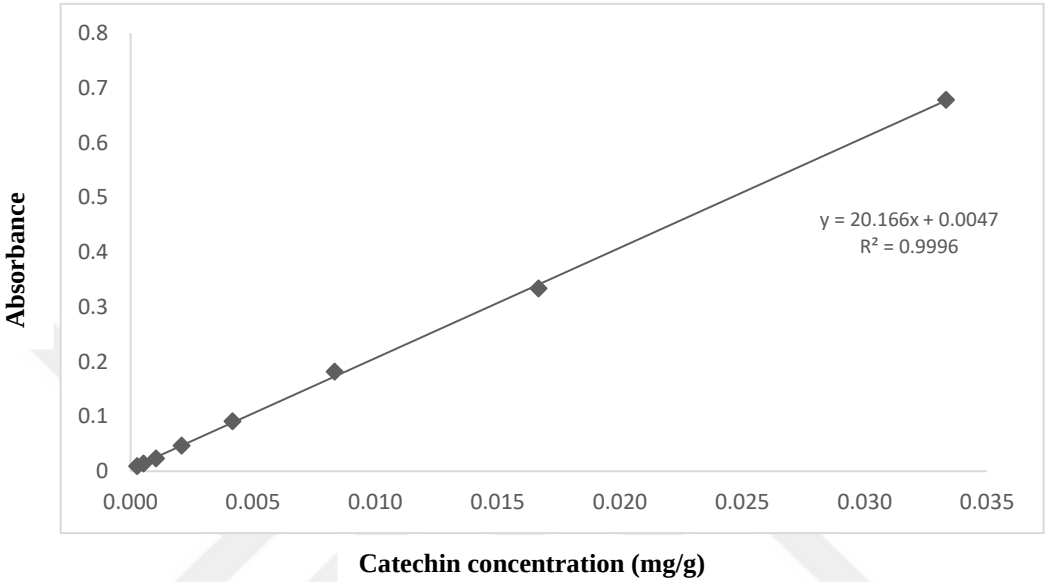

Figure 14. Calibration curve of catechin for determination of TPAC.

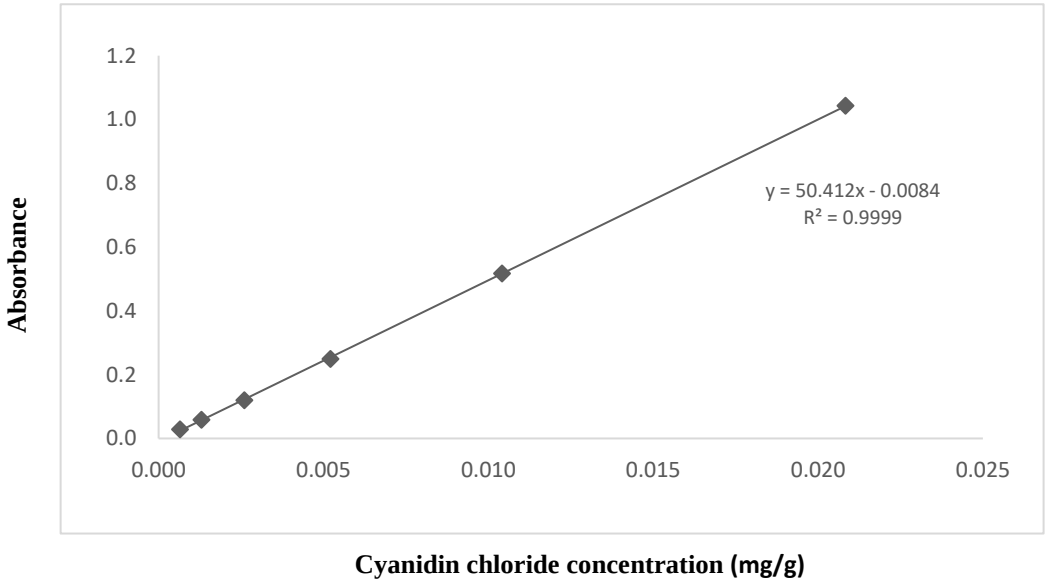

Figure 15. Calibration curve of cyanidin chloride for determination of TPAC.

3.2.5. Techniques for Determination of Antioxidant Potential

3.2.5.1. Free Radical Scavenging Activity Assays

3.2.5.1.a. DPPH Radical Scavenging Assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was performed with slight changes according to Blois (153). In this experiment, amount of each sample, methanol as a blank and reference solutions added was 20 µL. On sample, blank and reference solutions 0.1 Mm. DPPH reagent, which was prepared with 0.004 g DPPH chemical in 100 mL methanol, was added up to 300 µL. After 30 minutes of incubation in dark environment, absorbance was read at 520 nm with UV spectrophotometer. In this experiment, the concentration of test solutions were 0.4 mg/mL for hydroalcoholic extract of *C. turcicus* (fruits), *C. monogyna* (fruits and flower-bearing branches), *C. orientalis* (fruits), *C. pentagyna* (fruits, leaves and flower-bearing branches). For the other extracts, concentrations were 0.1 mg/mL. Concentrations of reference Trolox (in MeOH) solutions were between 0.003-0.200 mg/mL. Results represented as mg trolox equivalents per g hydroalcoholic extract (mg TE/g).

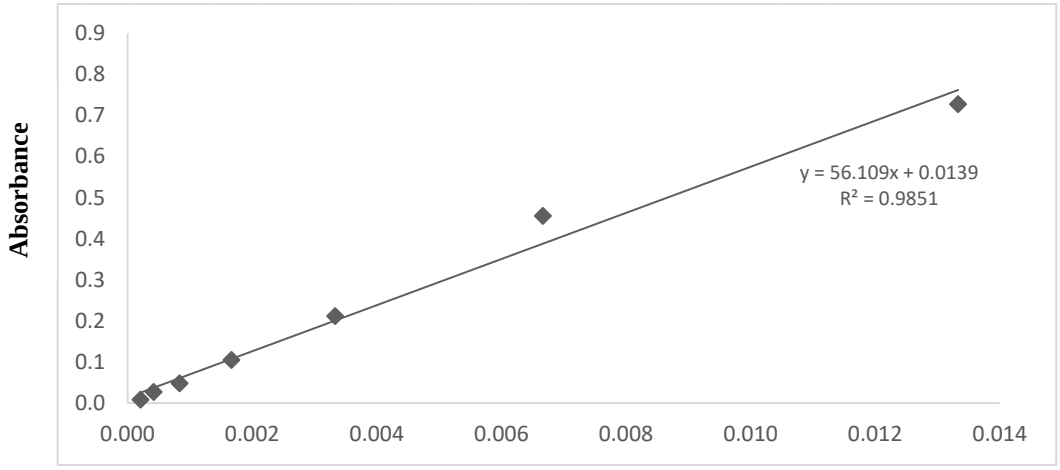

Figure 16. Calibration curve of Trolox.

3.2.5.1.b. ABTS Radical Scavenging Assay

This experiment was carried out with a 96-well plate by using the Re et al. (128) procedure with slight modifications. 20 µL sample, blank or reference solution was mixed

with 280 µL ABTS reagent. Before measuring absorbance at 734 nm, the mixture was incubated for 6 minutes. Test solutions were at 0.1 mg/mL concentration. Concentrations ranging from 0.004-0.125 mg/mL were used for reference trolox solution and results were expressed as mg trolox equivalents per g hydroalcoholic extract (mg TE/g). To make ABTS reagent, $7x10^{-3}$ M ABTS chemical, which was dissolved in distilled water, was mixed with $2.45x10^{-3}$ M potassium persulphate (in distilled water) and the ratio was 1:1 *v/v*. This mixture was kept waiting at room temperature in dark environment for 12-16 hours, then diluted with methanol at the ratio of 1:10 *v/v*.

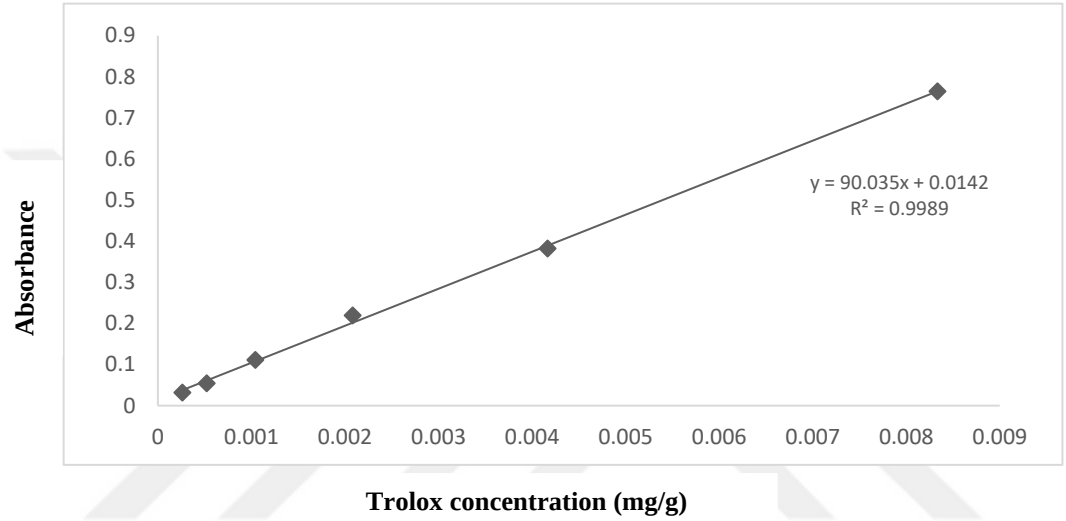

Figure 17. Calibration curve of Trolox.

3.2.5.2. Metal Reducing Activity Assays

3.2.5.2.a. Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric reducing antioxidant power assay was conducted spectrophotometrically according to Benzie et al. (154) with minor modifications. In 96-well plate, 280 µL FRAP reagent, which prepared freshly just before the analysis, was added onto 20 µL each sample, reference or blank (H_2O) solutions. Incubation period was 6 minutes. After that the absorbance of the formed blue structure was evaluated at 595 nm wavelength. Trolox was prepared at between 0.004-0.125 mg/mL concentrations with methanol, as a reference solution and results were stated as mg trolox equivalents per g hydroalcoholic extract (mg TE/g). Test solution concentrations of hydroalcoholic extract of *C. turcicus*, *C. orientalis* and *C. monogyna* was 0.1 mg/mL, other test solutions were at 0.4 mg/mL concentration. For obtaining FRAP reagent 3 different solutions were prepared: $2x10^{-2}$ M

$FeCl_3$, $1x10^{-2}$ M TPTZ and sodium acetate buffer (pH 3.6). To prepare 50 mL $2x10^{-2}$ M $FeCl_3$ solution, 0.2703 g $FeCl_3.6H_2O$ was dissolved in 1 mL 1 M HCl (8.211 mL 37% of HCl in 100 mL distilled water). After stirring, distilled water was added up to 50 mL. For the 50 mL TPTZ solution, 0.156 g TPTZ chemical was weighed, and ethanol was added up to 50 mL. Lastly, for the buffer solution, 3.1 g sodium acetatetrihydrate ($CH_3COONa.3H_2O$) was mixed with 16 mL glacial acetic acid and to complete the volume to 1 L, distilled water was added. Buffer solution's pH value was checked with pH meter. After that, these three solutions were mixed with a ratio of 1:1:10 respectively.

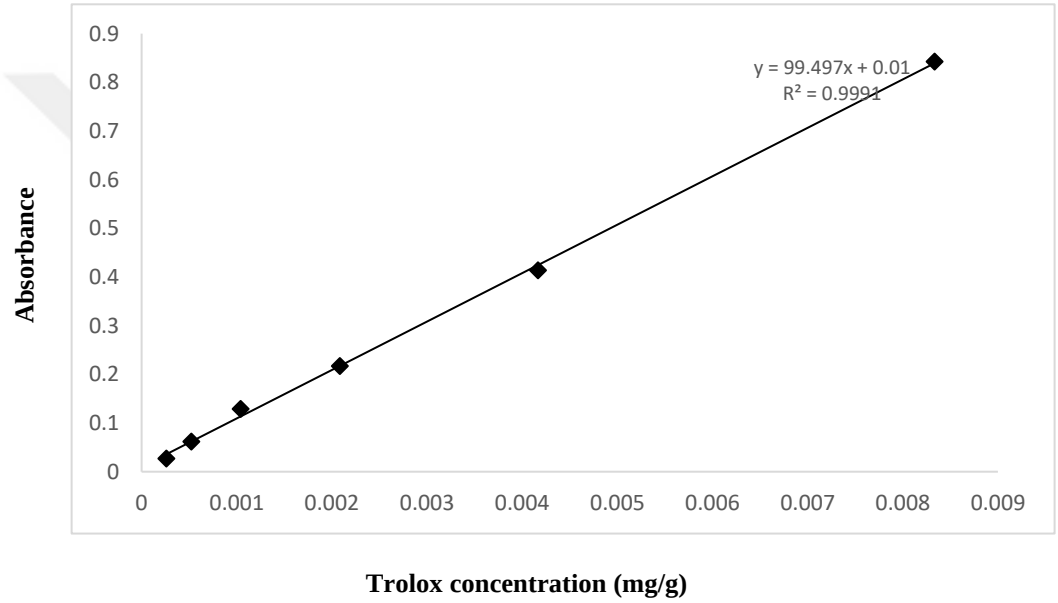

Figure 18. Calibration curve of Trolox.

3.2.5.2.b. Cupric Reducing Antioxidant Capacity (CUPRAC) Assay

CUPRAC activity assay of the samples was performed in 96-well plate according to the method of Apak et al. (155) with some modifications. Firstly 62.31 mg CuSO4.5H2O was dissolved in 25 mL distilled water to prepare 10^{-2}M CuSO4.5H2O. Secondly, 39.04 mg neocuprione was dissolved in a small amount of methanol and completed to 25 mL volume with methanol for $7.5x10^{-3}$ M neocuproine (Nc) solution. Lastly, for ammonium acetate buffer (pH 7), 1927 mg of ammonium acetate was mixed with 25 mL of distilled water. These three solutions were prepared just before the analysis. Initially, 85 µL 10^{-2}M copper (II) sulfate pentahydrate solution, 85 µL 7.5x10-3

M neocuproine (Nc) solution and 85 µL ammonium acetate buffer (pH 7) were added, sequentially into the wells, and a blue structure was formed. After that 43 µL of sample, blank (distilled water) or reference solution and 51 µL of distilled water were added to the blue color mixture. After and incubation period of 20 minutes at 50°C, the absorbance was then measured at 450 nm. As a reference, Trolox was used at concentrations in the range of 0.004-0.2 mg/mL. Sample solution concentration was 1 mg/mL. Antioxidant activities of sample solutions were stated as mg trolox equivalents per g hydroalcoholic extract (mg TE/g).

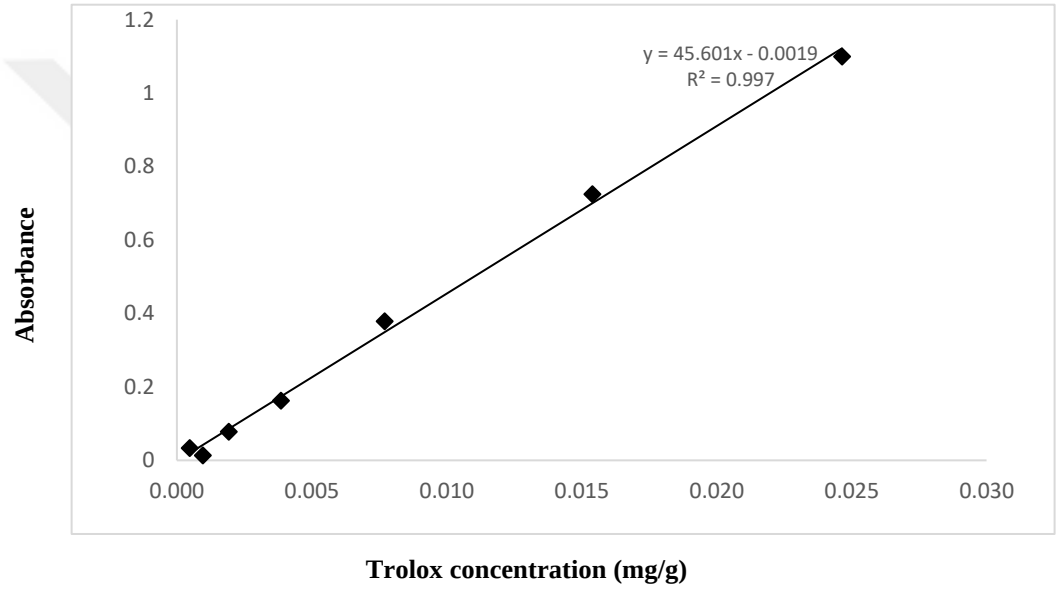

Figure 19. Calibration curve of Trolox.

3.2.6. Cell Culture Studies

The RAW 264.7 mouse macrophages (ATCC® TIB-71 TM) used in this study were acquired from ATCC (ATCC, Manassas, VA, USA). These cells were grown in a complete medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with %10 fetal bovine serum (Sigma, Aldrich, St. Louis, Missouri, USA), %10 FBS and %1 penicillin (10.000 units/mL) and streptomycin (10.000 mg/mL) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). They were maintained at a temperature of 37°C in a humidified atmosphere with 5% CO_2 (156). The cells were subcultured when they reached 80-90% confluence.

3.2.7. Cell Viability Assay

To determine the effect of Crataegus species on cell viability, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used. The RAW 264.7 cells were initially seeded in 96-well plates at a density of 5 × 104 cells per well for 24 hours in complete medium. The cells were then treated with various concentrations of the Crataegus extracts (ranging from 0.125-1 mg/mL) for 24 hours. Following this, the MTT reagent (0.5 mg/mL in PBS) (Sigma Aldrich, St. Louis, Missouri, USA) was added to the cells and incubated for 2 hours at 37°C with 5% CO_2. After removing the culture supernatants, 100 µL of isopropanol was added to each well, and the absorbance at 570 nm was measured using a microplate reader (Thermo Fisher ScientificTM, Inc., Waltham, MA, USA). The cell viability assay was conducted three times, and each assay was performed in triplicate (n=9 in three separate experiments). A cell viability of cultures treated with *Crataegus* extracts of less than 70% compared to the untreated control cultures (medium group) is considered as a cytotoxic (157). The percentage of cell viability was calculated using the following equation:

$$\text{Viability\%} = (\text{Absorbance}_{\text{Treatment group}}) / (\text{Absorbance}_{\text{Control}}) \times 100\%$$

3.2.8. Evaluation of Anti-inflammatory Activity

The inhibition of NO production was assessed by measuring nitrite levels in the cell culture medium using the Griess reagent (0.1% N-(1-Naphthyl)-ethylenediamine dihydrochloride in 5% phosphoric acid and 1% sulfanilamide) (158). RAW 264.7 cells were plated in a 96-well plate at a density of 5 × 104 cells/well and incubated for 24 hours at 37°C with 5% CO_2. After pre-treating the cells with various concentrations of the Crataegus extracts (0.125-0.25-0.5-1 mg/mL) for 2 hours and stimulating them with 1 µg/mL of LPS (Lipopolysaccharide from *Escherichia coli* 0111: B4, Sigma, USA) for an additional 22 hours, the cell culture supernatant was collected. The cell culture supernatants were collected after 24 hours for nitrite analysis. Nitrite concentrations were measured using Griess' reagent. The supernatant was mixed with an equal volume of Griess reagent in a 96-well plate for 10 minutes at room temperature in the dark. The color development corresponding to nitrite level was assessed at 540 nm using a microplate reader (Thermo Fisher ScientificTM, Inc., Waltham, MA, USA). The nitrite concentrations were determined using a sodium nitrite (Fluka Chemika-BioChemika, Ronkonkoma, N.Y., Buchs, Switzerland) standard curve. The researchers used

indomethacin (100 mM) (Sigma Aldrich, St. Louis, Missouri, USA) as a positive control (159).

3.2.9. Cytokine Analysis

Following an overnight incubation of cells in a 96-well plate at density of 5x104 cells per well, the cells were subjected to a 2-hour pre-treatment with *Crataegus* species. Subsequently, they were exposed to 1 μg/mL of lipopolysaccharide for an additional 22 hours. At the conclusion of the designated experimental period, the culture supernatant from each well was collected. To determine the concentrations of TNF-α and IL-6, ELISA kits from Invitrogen (USA) were utilized, following the instructions provided by the manufacturer. Similarly, the concentration of PGE_2 was measured using Abcam PGE_2 ELISA Kit from the UK (159).

3.2.10. Statistical Analysis

Each test was performed thrice, the average value and standard deviation (SD) were calculated, and the results were expressed as average ± SD. Microsoft Excel 2013 and Minitab 17 were used during the analyses. After performing one-way analysis of variance (one-way ANOVA), the statistical significance was considered as $p \leq 0.05$. Statistical analysis of cell culture studies was performed by GraphPadPrism 8.0 software (LaJolla, CA, USA) with one-way ANOVA.

4. RESULTS

4.1. Results of Chromatographic Analysis

4.1.1. High-Performance Thin-Layer Chromatography (HPTLC) Results

Analysis of phenolic acids, flavonol and flavone C-glycosides:

Hydroalcoholic extracts obtained from flower-bearing branches, leaves and fruits of *C. turcicus, C. monogyna, C. orientalis* and *C. pentagyna* were analyzed using HPTLC. The mobile phase used for the analysis contained ethyl acetate, ethyl methyl ketone, formic acid, and water in a ratio of 5:3:1:1 (*v/v/v/v*). Figure 20 depicts the HPTLC chromatogram of the samples under examination. After being derivatized with Natural Product and PEG 400, the R_F values and band color of the standards were measured at 366 nm.

The HPTLC chromatogram of the hydroalcoholic extracts revealed the following retention factors (R_F) for the standards: rutin ($R_F \approx 0.36$), vitexin-2"-*O*-rhamnoside ($R_F \approx 0.40$), chlorogenic acid ($R_F \approx 0.48$), neochlorogenic acid ($R_F \approx 0.53$), hyperoside ($R_F \approx 0.56$), isoquercitrin ($R_F \approx 0.59$), orientin ($R_F \approx 0.59$), vitexin ($R_F \approx 0.66$), and protocatechuic acid ($R_F \approx 0.88$). These values were determined from bottom to top examination of standard mixture. As depicted in Figure 20, rutin ($R_F \approx 0.36$, orange band color) was observed in all *Crataegus* samples. More occurrences of rutin were observed in samples of flower-bearing branches and leaves compared to fruit samples. Among the flower-bearing branches, notable presence of rutin was found in *C. turcicus, C. orientalis* and *C. pentagyna.* In leaves, rutin was mainly observed in *C. turcicus, C. orientalis* and *C. pengayna.* Vitexin-2"-*O*-rhamnoside ($R_F \approx 0.40$, green band color) was visually detected in all samples of *Crataegus species* except for fruit samples, and it was predominantly observed in *C. monoyna* leaves and flower-bearing branches, and *C. orientalis* leaves. While chlorogenic acid ($R_F \approx 0.48$, blue band color) was detected in all *Crataegus* samples, it was mainly found in *C. turcicus* and *C. monoygna* flower-bearing branches, *C. turcicus* leaves and *C. monogyna leaves.* Neochlorogenic acid ($R_F \approx 0.53$, blue band color) was detected in all *Crataegus* samples except *C. turcicus leaves*. It was mainly found in *C. turcicus, C. monogyna* and *Cratageus pentagyna* flower-bearing branches. Among the fruits, it was notably observed in *Cratagues pentagyna*. Hyperoside ($R_F \approx 0.56$, orange band color) was slightly observed in all fruit samples compared to flower-bearing branches and leaves of *Crataegus* species. It was notably found in *C.*

monogyna flower-bearing braches and *C. orientalis* leaves. Isoquercitrin and orientin had the same R_F value as they co-migrated in the stationary phase. Orientin ($R_F \approx 0.59$, yellow band color) was predominantly detected in *C. pentagyna* leaves. Besides, it was also found in *C. turcicus* leaves and *C. pentagyna* flower-bearing branches. Isoquercitrin ($R_F \approx 0.59$, orange band color) was detected in all samples, whereas it was notably observed in flower-bearing branches and leaves of *Crataegus* species. Vitexin ($R_F \approx 0.66$, green band color) was not clearly observed on the HPTLC plate. This could be due to the blue-colored compound at the same RF value with vitexin, potentially a phenolic acid, co-migrated with it. All Crataegus species may possibly have a protocatechuic acid (RF ≈ 0.88, dark blue band color).

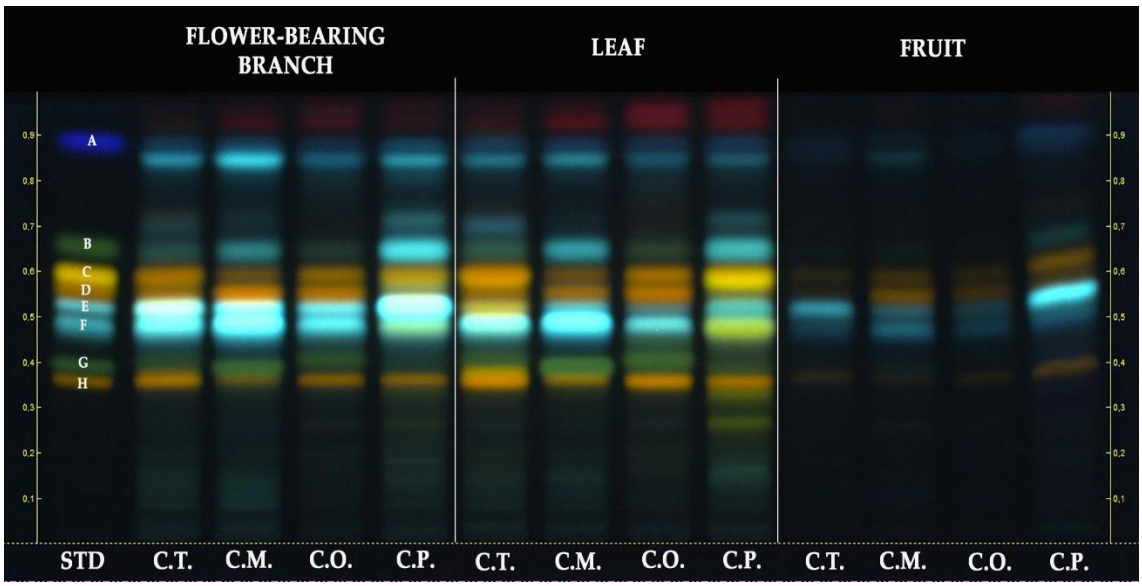

Figure 20. HPTLC chromatogram of different parts of Crataegus species at 366 nm.
(C.T.: *Crataegus turcicus*, C.M.: *Crataegus monogyna*, C.O.: *Crataegus orientalis*, C.P.: *Cratageus pentagyna*, A: protocatechuic acid, B: vitexin, C: isoquercitrin+orientin, D: hyperoside, E: neochlorogenic acid, F: chlorogenic acid, G: vitexin-2''-*O*-rhamnoside, H: rutin)

Analysis of flavanols:

Epicatechin and procyanidin B2 in hydroalcoholic extracts of flower-bearing branches, leaves, and fruits of *C. turcicus, C. monogyna, C. orientalis* and *C. pentagyna* were investigated using HPTLC. Ethyl acetate, formic acid and water were the components of the mobile phase used in the analysis in the proportions of 10:1:4 (*v/v/v*). Figure 21 shows the chromatogram of the examined samples. After HPTLC plate treated

with vanillin-sulfuric acid reagent, R_F values and band color of the standards were determined at white light.

The HPTLC chromatogram of the hydroalcoholic extracts showed that the retention factors (R_F) for procyanidin B2 ($R_F \approx 0.6$) and epicatechin ($R_F \approx 0.8$) were determined by analyzing the standard mixture from bottom to top. As shown in Figure 21, epicatechin ($R_F \approx 0.8$, red band color) was found in all samples and mainly detected in flower-bearing branches of *C. orientalis*, *C. pentagyna* and *C. monogyna* and as well as *C. monogyna* fruits. Procyanidin B2 ($R_F \approx 0.6$, red band color) was found in all samples and notably detected in *C. monogyna* fruits.

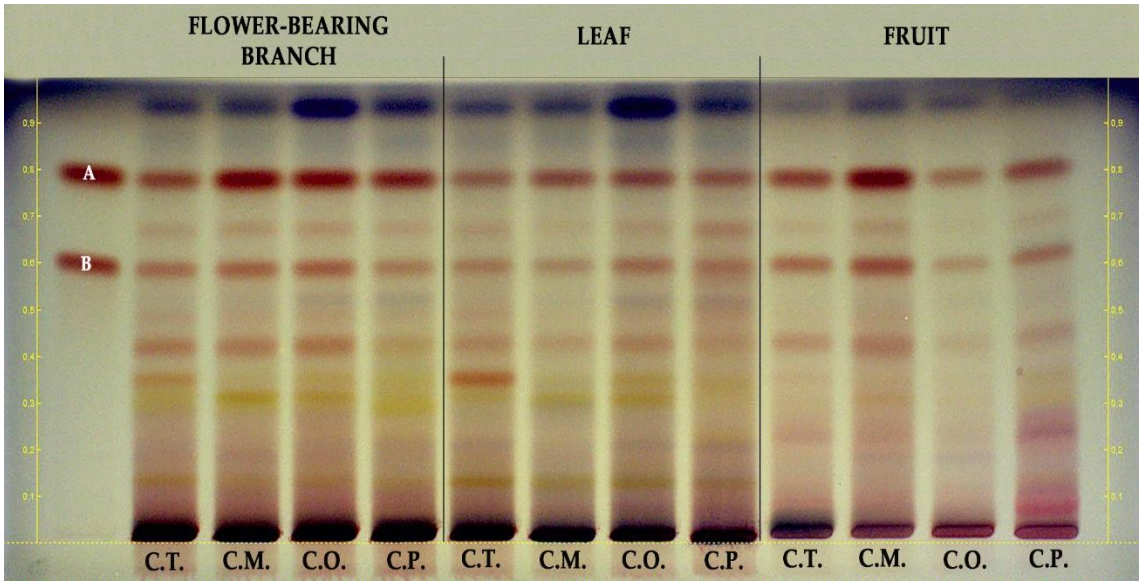

Figure 21. HPTLC chromatogram of different parts of Crataegus species at white light. (C.T.: *Crataegus turcicus*, C.M.: *Crataegus monogyna*, C.O.: *Crataegus orientalis*, C.P.: *Cratageus pentagyna*, A: epicathechin, B: procyanidin B2)

<u>For the separation of catechin and epicatechin:</u>

The presence of catechin and epicatechin was examined in the hydroalcoholic extracts of flower-bearing branches, leaves and fruits of *C. turcicus, C. monogyna, C. orientalis* and *C. pentagyna* using HPTLC. The mobile phase used in the analysis consisted of n-propanol, water and acetic acid (4:2:1 *v/v/v*). Figure 22 displays the chromatogram of the samples that were investigated. After derivatizing the HPTLC plate with vanillin-sulfuric acid reagent, the R_F values and band color of the standards were determined under white light.

The HPTLC chromatogram of the hydroalcoholic extracts indicated the following retention factors (R_F) for the standard compounds from bottom to top: epicatechin ($R_F \approx$ 0.69,), catechin ($R_F \approx 0.77$, red band color). As seen in Figure 22, epicatechin ($R_F \approx 0.69$, red band color) was detected in all samples with dominance in *C. monogyna* fruits, and flower-bearing branches of *C. monogyna* and *C. orientalis*.

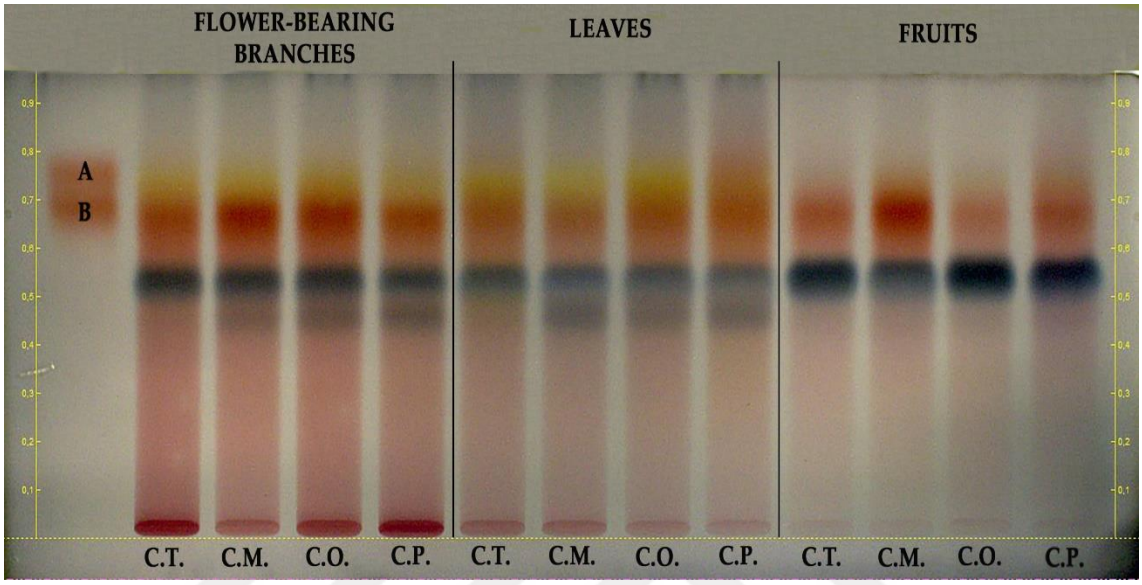

Figure 22. HPTLC chromatogram showing epicatechin and catechin in different parts of Crataegus species at white light.

(C.T.: *Crataegus turcicus*, C.M.: *Crataegus monogyna*, C.O.: *Crataegus orientalis*, C.P.: *Cratageus pentagyna*, A: catechin, B: epicathechin)

Analysis of anthocyanins:

HPTLC was employed to examine anthocyanins in hydroalcoholic extracts of fruit part of *C. turcicus, C. monogyna, C. orientalis* and *C. pentagyna.* The mobile phase used in the analysis consisted of ethyl acetate, ethyl methyl ketone, formic acid and water in a ratio of 5:3:1:1 (*v/v/v/v*). Figure 23 illustrates the HPTLC chromatogram of the examined samples. The R_F values and band color of the standards were provided at white light without undergoing any derivatization process.

According to the visual results of HPTLC plate (shown in Figure 23), cyanidin-3-*O*-glucoside ($R_F \approx 0.31$) was detected in the fruits of *C. turcicus, C. monogyna* and *C. pentagyna.* It is predominantly present in the *C. pentagyna* compared to the others. Other reddish band colors may confirm the presence of other anthocyanins in the fruits.

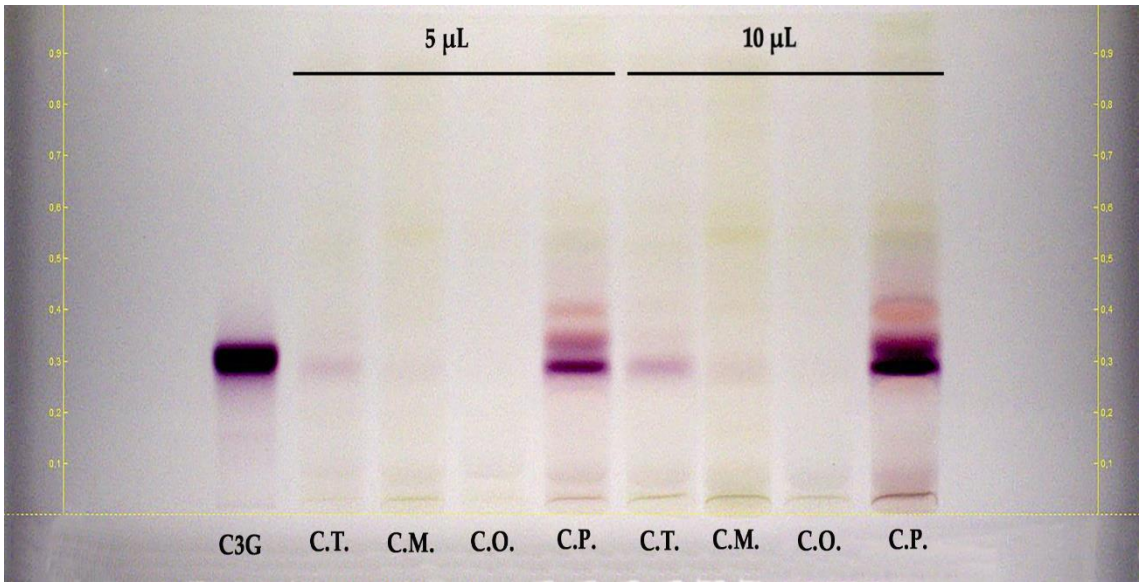

Figure 23. HPTLC chromatogram of fruits belonging to *Crataegus* species at white light. (C3G: cyanidin-3-*O*-glucoside, C.T.: *Crataegus turcicus*, C.M.: *Crataegus monogyna*, C.O.: *Crataegus orientalis*, C.P.: *Cratageus pentagyna*)

4.1.2. High-Performance Liquid Chromatography (HPLC) Results

HPLC method was used to determine the amounts of various compounds including protocatechuic acid, chlorogenic acid, neochlorogenic acid, procyanidin B2, epicatechin, orientin, vitexin, vitexin-2"-*O*-rhamnoside, hyperoside, rutin and isoquercitrin in hydroalcoholic extracts of flower-bearing branches, leaves and fruits from *C. turcicus, C. monogyna, C. orientalis* and *C. pentagyna*. Figure 24-36 displays the HPLC chromatograms of the standard mixture and samples, along with the retention time (tR) values of the standards.

Table 27 displays the HPLC results. According to these results, protocatechuic acid was present in all samples except *C. monogyna* and *C. orientalis* fruits. The most dominant sample in terms of protocatechuic acid content was *C. pentagyna leaves* (0.88 ± 0.0 mg/g), followed by *C. monogyna* leaves (0.77 ± 0.0 mg/g). The sample with the lowest protocatechuic acid concentration was *C. turcicus* fruits (0.04 ± 0.01 mg/g). Neochlorogenic acid was present in all samples except for *C. turcicus* leaves. It was predominantly found in *C. pentagyna* flower-bearing branches (50.89 ± 4.02 mg/g). Also neochlorogenic acid presence in *C. turcicus* (18.18 ± 0.56 mg/g) and *C. monogyna* (21.9 ± 0.06 mg/g) flower-bearing branches was notably. Among all samples, the one with the

lowest neochlorogenic acid content was *C. orientalis* fruits (0.86 ± 0.04 mg/g). Chlorogenic acid was detected in all samples and found predominantly in *C. monogna* flower-bearing branches (45.52 ± 0.5 mg/g), followed by *C. monogyna* leaves (40.89 ± 0.44 mg/g). Chlorogenic acid was detected in lower amounts in fruits compared to leaves and flower-bearing branches. It was determined that the lowest content of chlorogenic acid was found in *C. orientalis* fruits (0.55 ± 0.0 mg/g). Procyanidin B2 was detected in all samples. It was predominantly found in *C. monogyna* fruits (11.65 ± 0.17 mg/g), whereas its lower content was found in *C. turcicus* leaves (1.81 ± 0.09 mg/g). Epicatechin was identified in all samples, with a predominant presence observed in *C. monogyna* flower-bearing branches (17.56 ± 0.27 mg/g). Conversely, *C. turcicus* leaves (2.83 ± 0.1 mg/g) exhibited the lowest content of epicatechin. Most of the samples did not show the presence of orientin; however, it was detected in flower-bearing branches and leaves of *C. turcicus* and *C. pentagyna*. In addition, orientin was found predominantly in *C. pentagyna* leaves (13.49 ± 0.03 mg/g). Vitexin was detected in leaves and flower-bearing branches of *C. turcicus* and *C. orientalis*, flower-bearing branches of *C. pentagyna* and predominantly in leaves of *C. pentagyna* (4.98 ± 0.02 mg/g). Vitexin-2''-*O*-rhamnoside was not detected in fruit samples. It was found predominantly in *C. mononyna* leaves (76.03 ± 0.36 mg/g). Hyperoside was detected in all samples except for *C. pentagyna* leaves. It was predominantly found in *C. monogyna* flower-bearing branches (17.29 ± 0.01 mg/g), followed by *C. orientalis* leaves (16.25 ± 0.29 mg/g), and flower-bearing branches (11.54 ± 0.31 mg/g), respectively. Except for fruits and flower-bearing branches of *C. monogyna* and *C. orientalis* fruits, rutin was detected in all samples. It was predominantly found in *C. pentagyna* leaves (6.03 ± 0.37 mg/g), followed by leaves of *C. orientalis* (5.82 ± 0.25 mg/g) and *C. turcicus* (5.27 ± 0.32 mg/g). Isoquercitrin was found in all samples and hydroalcoholic extract of *C. pentagyna* leaves (12.94 ± 0.06 mg/g) had the highest amount of isoquercitrin. In samples belonging to *C. turcicus*, the peak of isoquercitrin overlapped with another co-eluted compound, so its quantity could not be determined.

Table 27. Quantitative results of the investigated standards in *Crataegus* species by HPLC.

	FLOWER-BEARING BRANCHES				LEAVES				FRUITS			
	C.T.	**C.M.**	**C.O.**	**C.P.**	**C.T.**	**C.M.**	**C.O.**	**C.P.**	**C.T.**	**C.M.**	**C.O.**	**C.P.**
Protocatechuic acid	0.15 ± 0.01^{H}	0.18 ± 0.01^{H}	0.39 ± 0.01^{F}	0.43 ± 0.03^{E}	0.35 ± 0.01^{G}	0.77 ± 0.00^{B}	0.55 ± 0.01^{C}	0.88 ± 0.00^{A}	0.04 ± 0.01^{I}	n.d.	n.d.	0.49 ± 0.01^{D}
Neochlorogenic acid	18.18 ± 0.56^{C}	21.9 ± 0.06^{B}	7.82 ± 0.22^{DE}	50.32 ± 4.02^{A}	n.d.	4.64 ± 0.04^{EF}	3.26 ± 0.07^{FG}	4.64 ± 0.03^{EF}	6.9 ± 0.02^{DE}	1.26 ± 0.01^{FG}	0.86 ± 0.04^{G}	9.17 ± 0.16^{D}
Chlorogenic acid	21.61 ± 0.61^{E}	45.52 ± 0.50^{A}	12.12 ± 0.13^{G}	15.69 ± 0.75^{F}	29.6 ± 0.04^{D}	40.89 ± 0.44^{B}	13.73 ± 0.39^{G}	32.35 ± 0.02^{C}	0.93 ± 0.01^{H}	2.14 ± 0.07^{H}	0.55 ± 0.00^{H}	0.71 ± 0.01^{H}
Procyanidin B2	3.67 ± 0.07^{F}	7.15 ± 0.05^{B}	5.48 ± 0.19^{D}	6.44 ± 0.04^{C}	1.81 ± 0.09^{I}	2.03 ± 0.09^{HI}	2.92 ± 0.17^{G}	1.97 ± 0.10^{HI}	4.56 ± 0.05^{E}	11.65 ± 0.17^{A}	3.24 ± 0.14^{G}	2.29 ± 0.16^{H}
Epicatechin	3.59 ± 0.03^{FG}	17.56 ± 0.27^{A}	11.78 ± 0.75^{C}	13.83 ± 0.11^{B}	2.83 ± 0.10^{G}	5.95 ± 0.11^{E}	3.82 ± 0.51^{F}	7.3 ± 0.02^{D}	7.92 ± 0.06^{D}	13.24 ± 0.18^{B}	2.89 ± 0.12^{G}	13.29 ± 0.21^{B}
Orientin	0.08 ± 0.07^{D}	n.d.	n.d.	1.54 ± 0.02^{B}	0.47 ± 0.03^{C}	n.d.	n.d.	13.49 ± 0.03^{A}	n.d.	n.d.	n.d.	n.d.
Vitexin	0.9 ± 0.03^{E}	n.d.	1.09 ± 0.08^{D}	0.3 ± 0.05^{F}	3.61 ± 0.14^{B}	n.d.	1.77 ± 0.01^{C}	4.98 ± 0.02^{A}	n.d.	n.d.	n.d.	n.d.
Vitexin-2''-*O*-rhamnoside	4.92 ± 0.18^{G}	26.5 ± 0.7^{C}	13.93 ± 0.1^{E}	1.7 ± 0.09^{H}	12.83 ± 0.14^{F}	76.03 ± 0.36^{A}	49.41 ± 0.92^{B}	19.63 ± 0.02^{D}	n.d.	n.d.	n.d.	n.d.
Hyperoside	7.81 ± 0.22^{E}	17.29 ± 0.01^{A}	11.54 ± 0.31^{C}	2.84 ± 0.03^{H}	4.41 ± 0.22^{G}	10.09 ± 0.55^{D}	16.25 ± 0.29^{B}	n.d.	0.45 ± 0.0^{J}	5.61 ± 0.15^{F}	2.23 ± 0.01^{HI}	1.68 ± 0.02^{I}
Rutin	1.83 ± 0.08^{C}	n.d.	1.33 ± 0.11^{DE}	1.8 ± 0.01^{CD}	5.27 ± 0.32^{B}	1.67 ± 0.10^{CD}	5.82 ± 0.25^{A}	6.03 ± 0.37^{A}	0.84 ± 0.0^{F}	n.d.	n.d.	0.96 ± 0.02^{EF}
Isoquercitrin	n.d.	3.07 ± 0.11^{F}	6.3 ± 0.24^{C}	5.12 ± 0.18^{D}	n.d.	4.03 ± 0.02^{E}	11.09 ± 0.23^{B}	12.94 ± 0.06^{A}	n.d.	2.18 ± 0.00^{G}	1.28 ± 0.45^{H}	2.02±0.02G
Total Phenolics	62.74^{ABC}	139.51^{A}	71.78^{ABC}	100.58^{ABC}	61.18^{ABC}	148.35^{A}	108.84^{AB}	104.21^{ABC}	21.64^{BC}	36.08^{BC}	11.05^{C}	30.61^{BC}
Total Phenolics in Parts	374.61^{A}				422.58^{A}				99.38^{B}			

Results were given as mg/g hydroalcoholic extract, C.T.: *C. turcicus*, C.M.: *C. monogyna*, C.O.: *C. orientalis*, C.P.: *C. pentagyna*, n.d.: not detected

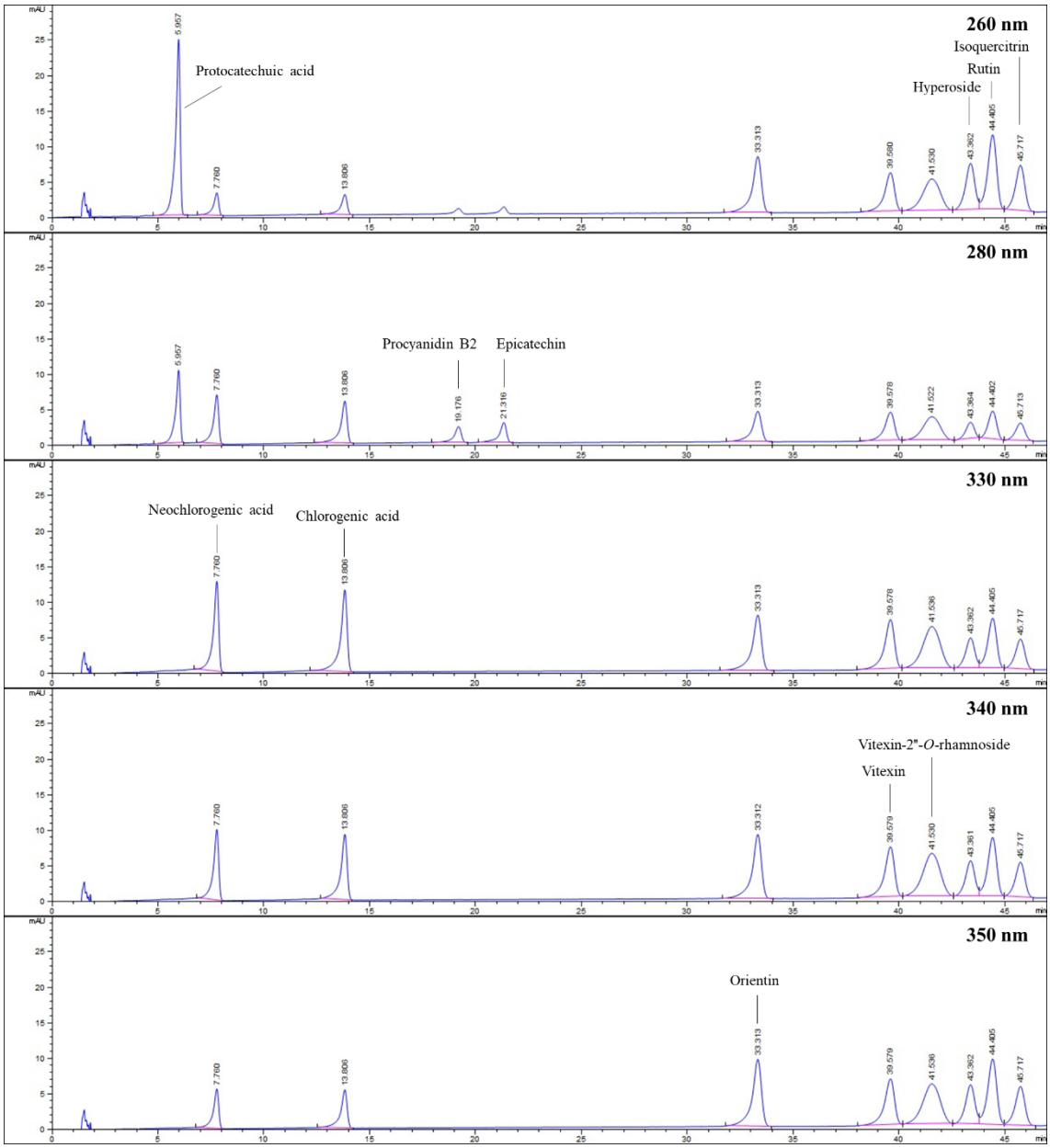

Figure 24. HPLC chromatograms of standards analyzed at different wavelenghts.

The t_R values were as follows: protocatechic acid (5.93 ± 0.03), neochlorogenic acid (7.72 ± 0.02), chlorogenic acid (13.74 ± 0.01), procyanidin B2 (19.08 ± 0.01), epicathechin (21.22 ± 0.01), orientin (33.19 ± 0.01), vitexin(39.43 ± 0.02), vitexin-2"-*O*-rhamnoside (41.40 ± 0.02), hyperoside (43.20 ± 0.03), rutin (44.24 ± 0.03) and isoquercitrin (45.55 ± 0.02)

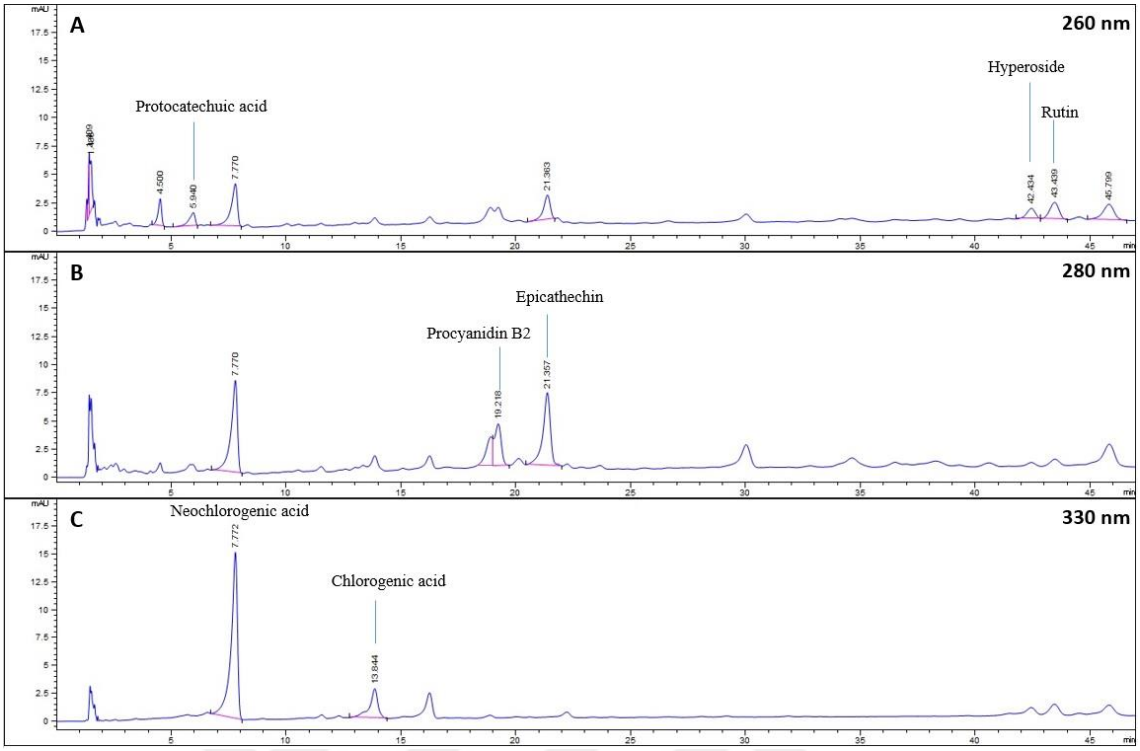

Figure 25. HPLC chromatograms of hydroalcoholic extract belonging to *C. turcicus* fruits at 260 nm (A), at 280 nm (B) and at 330 nm (C).

The t_R values were as follows: protocatechic acid (5.94 ± 0.01), neochlorogenic acid (7.77 ± 0.01), chlorogenic acid (13.82 ± 0.01), procyanidin B2 (19.18 ± 0.01), epicathechin (21.32 ± 0.01), orientin (n.d.), vitexin(n.d.), vitexin-2"-*O*-rhamnoside (n.d.), hyperoside (42.44 ± 0.01), rutin (43.44 ± 0.01) and isoquercitrin (n.d.)

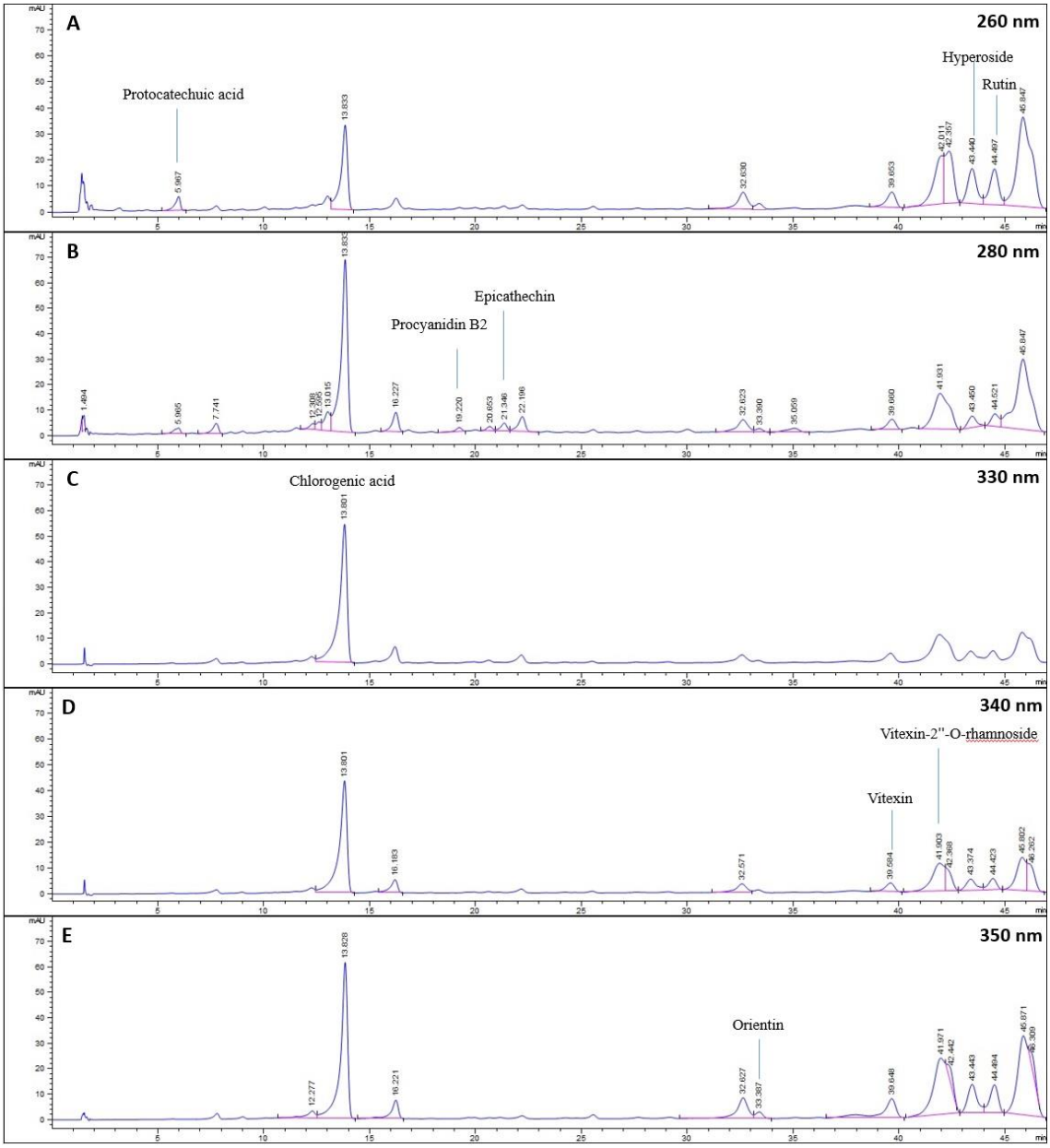

Figure 26. HPLC chromatograms of hydroalcoholic extract belonging to *C. turcicus* leaves at 260 nm (A), at 280 nm (B), at 330 nm (C), at 340 nm (D) and at 350 nm (E).

The t_R values were as follows: protocatechic acid (5.94 ± 0.02), neochlorogenic acid (n.d.), chlorogenic acid (13.82 ± 0.02), procyanidin B2 (19.23 ± 0.01), epicathechin (21.35 ± 0.01), orientin (33.39 ± 0.01), vitexin(39.65 ± 0.01), vitexin-2''-*O*-rhamnoside (41.92 ± 0.02), hyperoside (43.45 ± 0.01), rutin (44.50 ± 0.00), and isoquercitrin (n.d.)

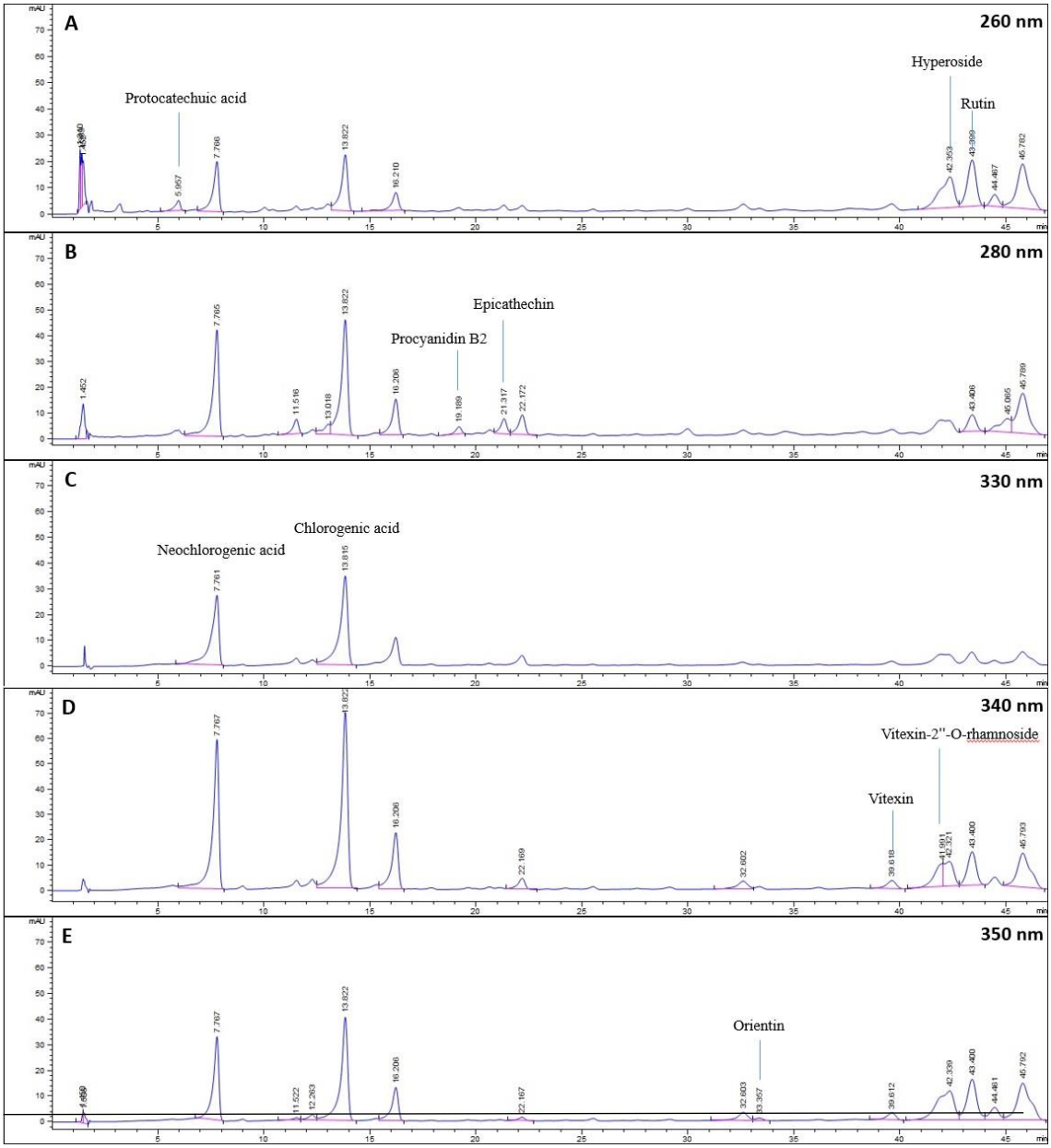

Figure 27. HPLC chromatograms of hydroalcoholic extract belonging to *C. turcicus* flower-bearing branches at 260 nm (A), at 280 nm (B), at 330 nm (C), at 340 nm (D) and at 350 nm (E).

The t_R values were as follows: protocatechic acid (5.95 ± 0.01), neochlorogenic acid (7.77 ± 0.02), chlorogenic acid (13.82 ± 0.01), procyanidin B2 (19.19 ± 0.01), epicathechin (21.32 ± 0.01), orientin (33.37 ± 0.02), vitexin(39.63 ± 0.02), vitexin-2''-*O*-rhamnoside (41.87 ± 0.03), hyperoside (43.36 ± 0.04), rutin (44.48 ± 0.01) and isoquercitrin (n.d.)

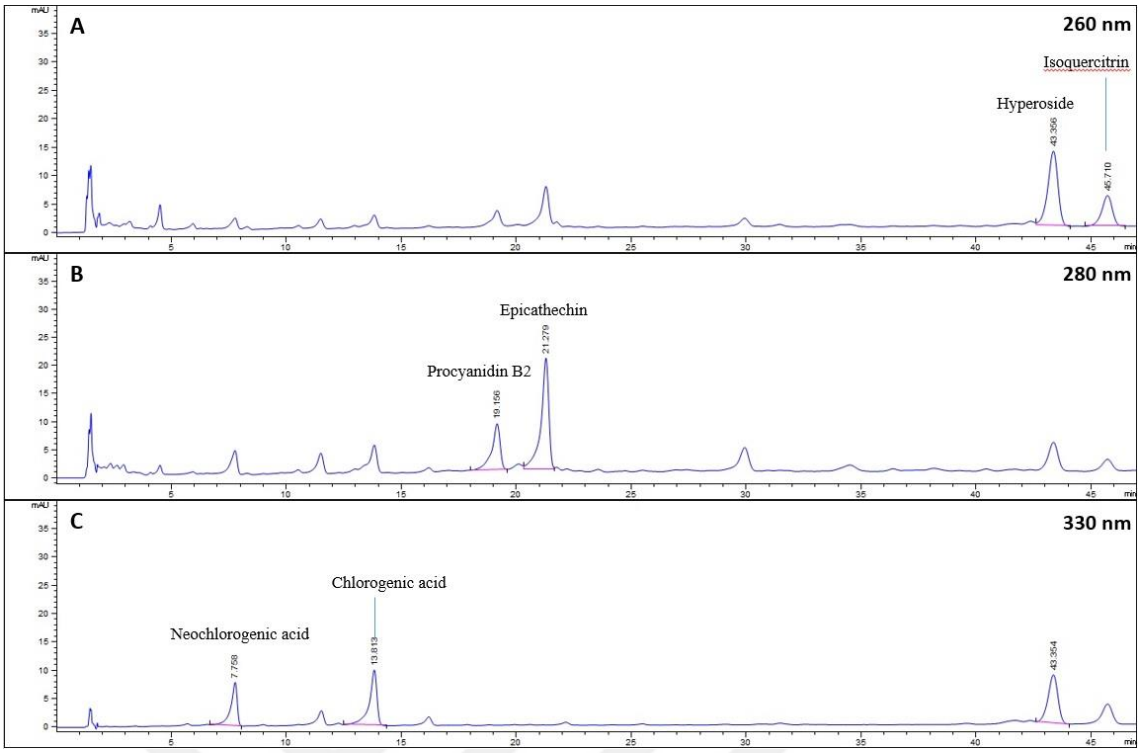

Figure 28. HPLC chromatograms of hydroalcoholic extract belonging to *C. monogyna* fruits at 260 nm (A), at 280 nm (B) and at 330 nm (C).

The t_R values were as follows: protocatechic acid (n.d), neochlorogenic acid (7.77 ± 0.01), chlorogenic acid (13.85 ± 0.09), procyanidin B2 (19.17 ± 0.01), epicathechin (21.2 ± 0.02), orientin (n.d.), vitexin (n.d.), vitexin-2''-*O*-rhamnoside (n.d.), hyperoside (43.50 ± 0.21), rutin (n.d.) and isoquercitrin (45.73 ± 0.02).

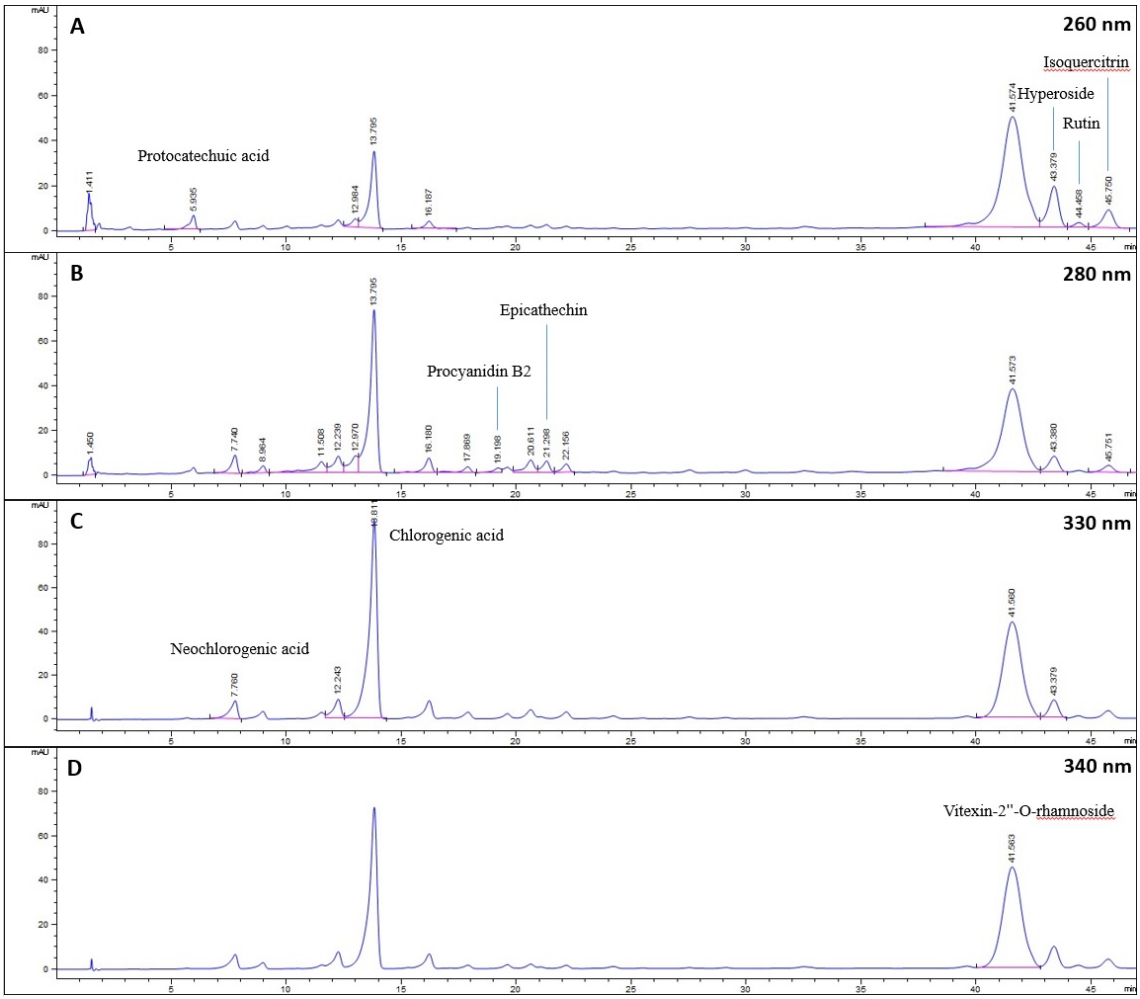

Figure 29. HPLC chromatograms of hydroalcoholic extract belonging to *C. monogyna* leaves at 260 nm (A), at 280 nm (B), at 330 nm (C) and at 340 nm (D).

The t_R values were as follows: protocatechic acid (5.95 ± 0.01), neochlorogenic acid (7.76 ± 0.01), chlorogenic acid (13.94 ± 0.02), procyanidin B2 (19.21 ± 0.01), epicathechin (21.31 ± 0.01), orientin (n.d.), vitexin(n.d.), vitexin-2''-*O*-rhamnoside (41.81 ± 0.04), hyperoside (43.38 ± 0.02), rutin (44.46 ± 0.02) and isoquercitrin (45.75 ± 0.02).

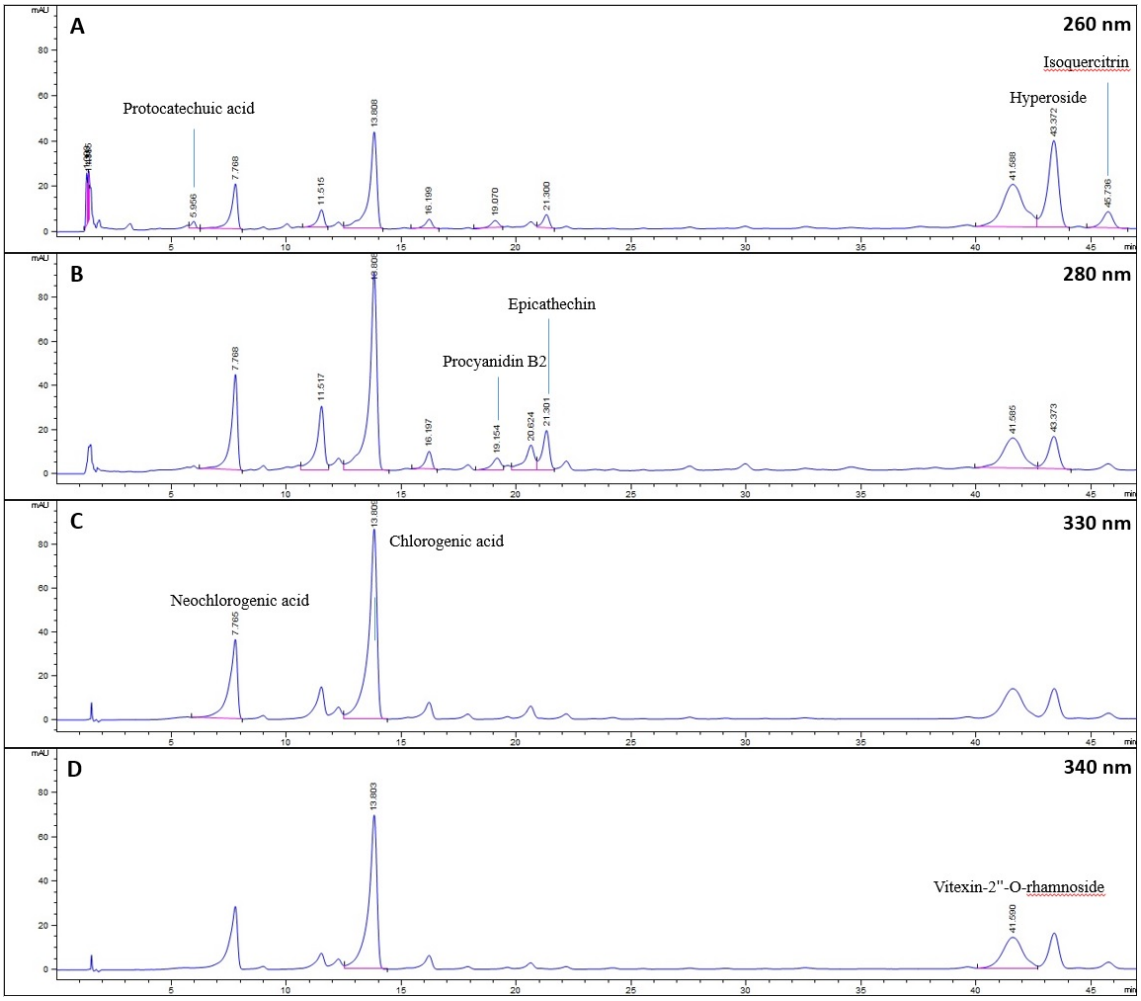

Figure 30. HPLC chromatograms of hydroalcoholic extract belonging to *C. monogyna* flower-bearing branches at 260 nm (A), at 280 nm (B), at 330 nm (C) and at 350 nm (D).

The t_R values were as follows: protocatechic acid (5.95 ± 0.02), neochlorogenic acid (7.77 ± 0.01), chlorogenic acid (13.96 ± 0.10), procyanidin B2 (19.16 ± 0.01), epicathechin (21.32 ± 0.01), orientin (n.d.), vitexin(n.d.), vitexin-2''-*O*-rhamnoside (41.79 ± 0.17), hyperoside (43.64 ± 0.20), rutin (n.d.) and isoquercitrin (45.76 ± 0.02).

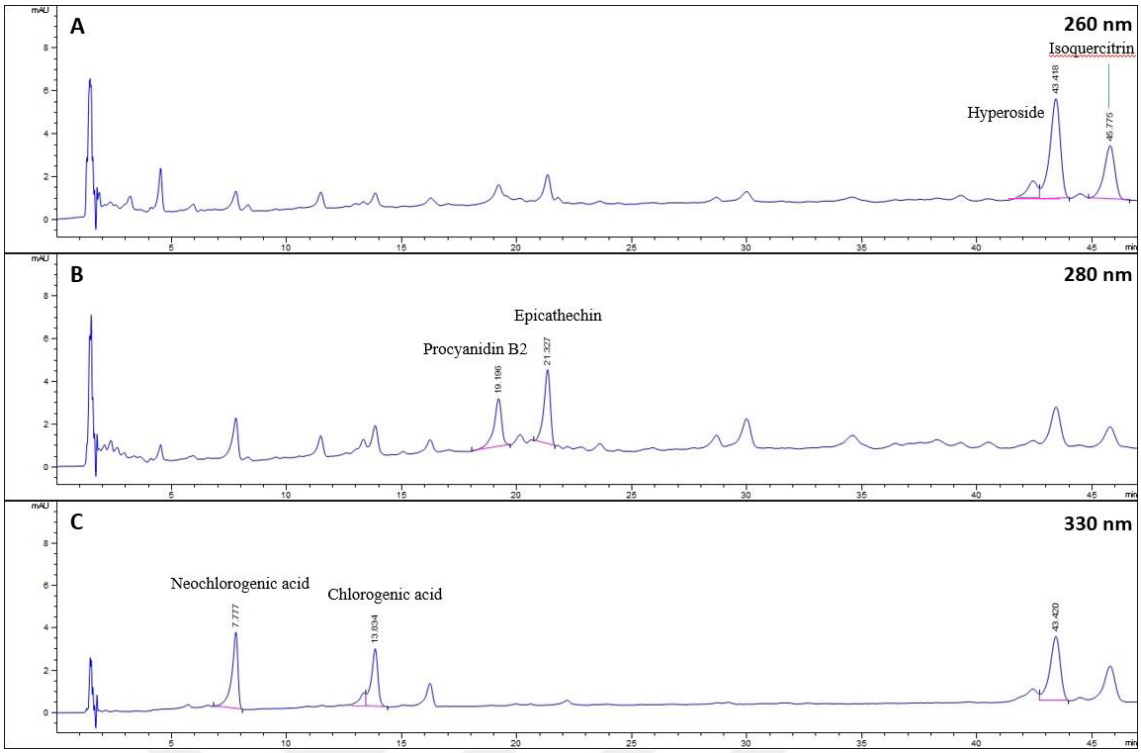

Figure 31. HPLC chromatograms of hydroalcoholic extract belonging to *C. orientalis* fruits at 260 nm (A), at 280 nm (B) and at 330 nm (C).

The t_R values were as follows: protocatechic acid (n.d.) neochlorogenic acid (7.78 ± 0.02), chlorogenic acid (13.83 ± 0.01), procyanidin B2 (19.21 ± 0.01), epicathechin (21.34 ± 0.01), orientin (n.d.), vitexin(n.d.), vitexin-2"-*O*-rhamnoside (n.d.), hyperoside (43.44 ± 0.01), rutin (n.d.) and isoquercitrin (45.80 ± 0.02).

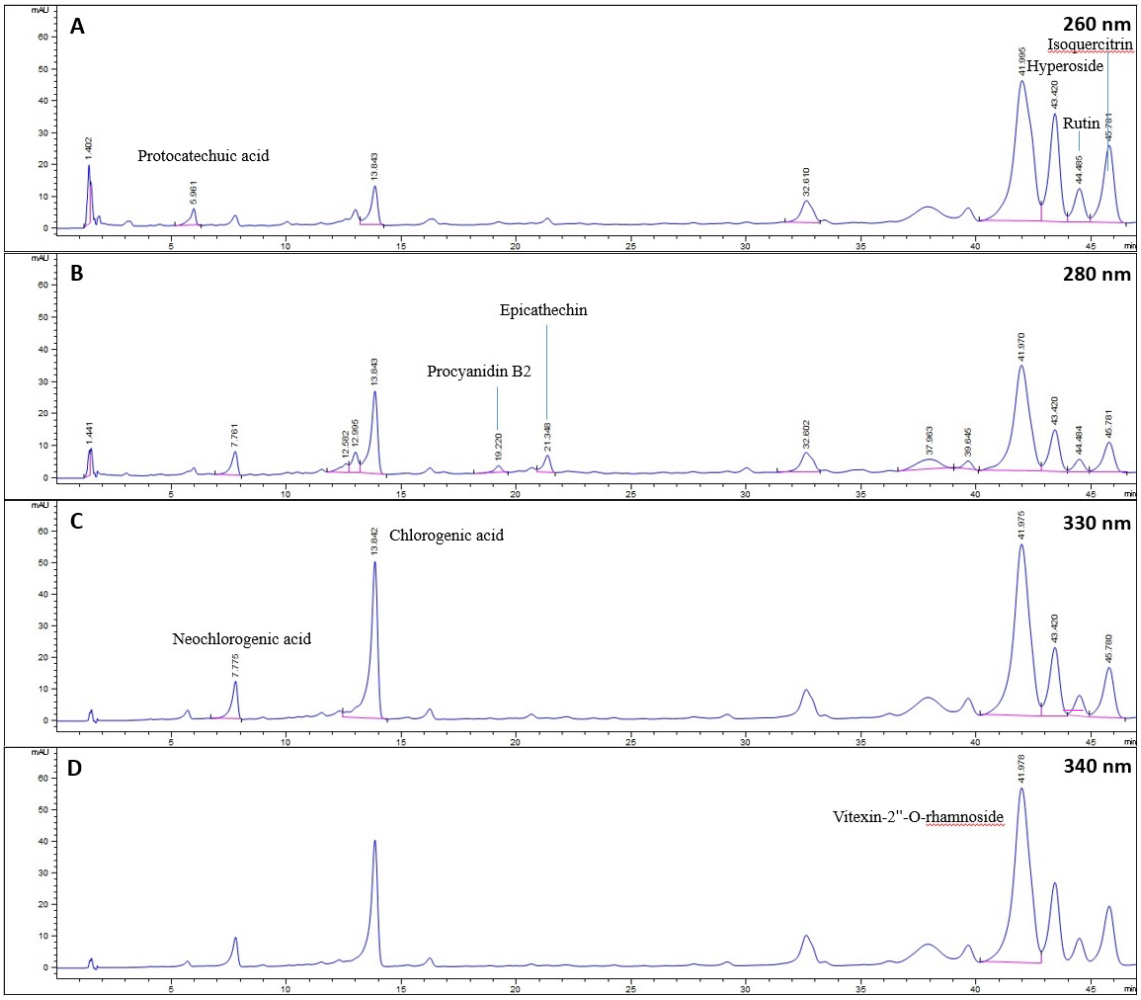

Figure 32. HPLC chromatograms of hydroalcoholic extract belonging to *C. orientalis* leaves at 260 nm (A), at 280 nm (B), at 330 nm (C) and at 340 nm (D).

The t_R values were as follows: protocatechic acid (5.96 ± 0.01), neochlorogenic acid (7.77 ± 0.01), chlorogenic acid (13.83 ± 0.01), procyanidin B2 (19.22 ± 0.01), epicathechin (21.31 ± 0.01), orientin (n.d.), vitexin(39.59 ± 0.01), vitexin-2''-*O*-rhamnoside (41.97 ± 0.01), hyperoside (44.45 ± 0.01), rutin (43.44 ± 0.01) and isoquercitrin (45.75 ± 0.02).

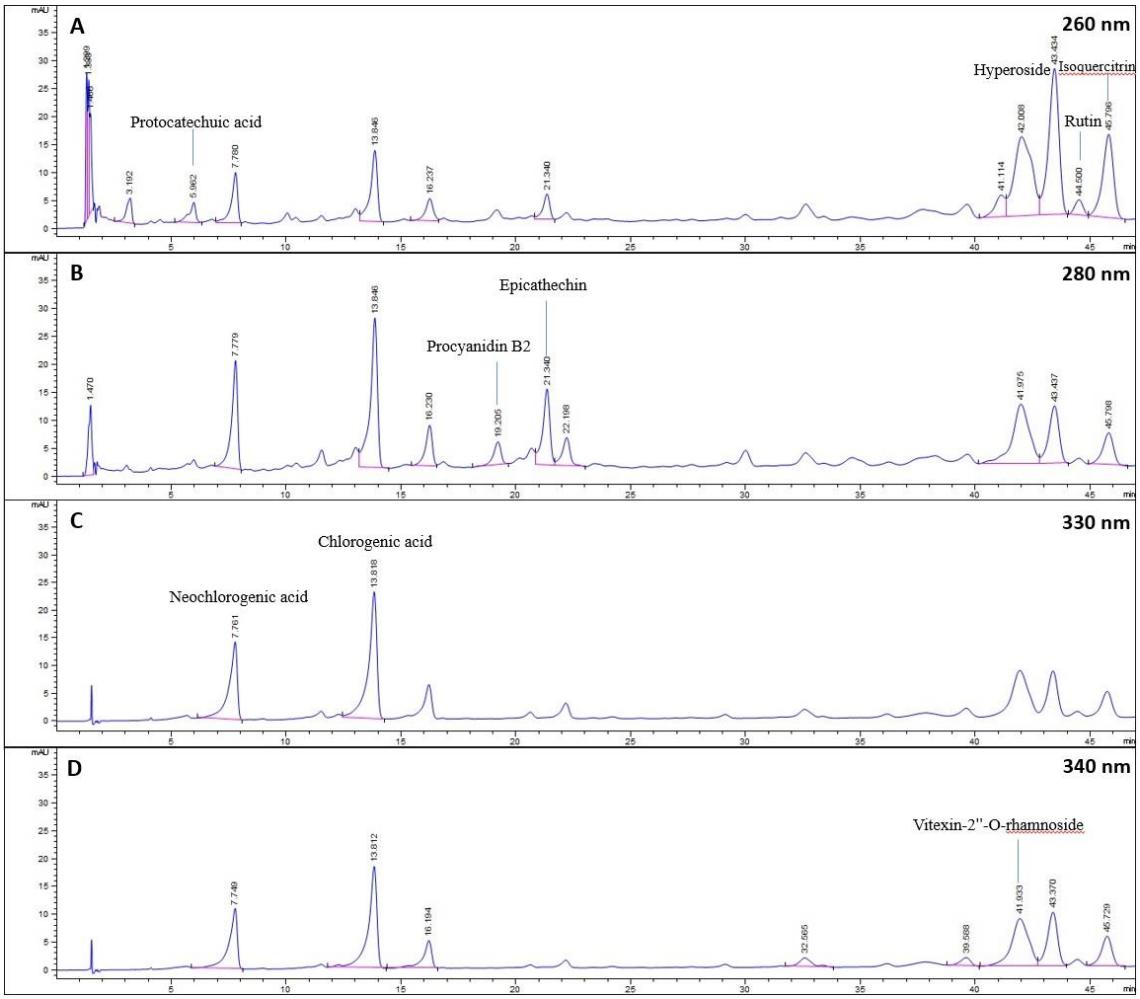

Figure 33. HPLC chromatograms of hydroalcoholic extract belonging to *C. orientalis* flower-bearing branches at 260 nm (A), at 280 nm (B), at 330 nm (C) and at 340 nm (D).

The t_R values were as follows: protocatechic acid (5.96 ± 0.02), neochlorogenic acid (7.77 ± 0.01), chlorogenic acid (13.83 ± 0.01), procyanidin B2 (19.17 ± 0.01), epicathechin (21.31 ± 0.02), orientin (n.d.), vitexin(39.64 ± 0.01), vitexin-2''-*O*-rhamnoside (41.93 ± 0.01), hyperoside (43.44 ± 0.00), rutin (44.50 ± 0.01) and isoquercitrin (45.80 ± 0.01).

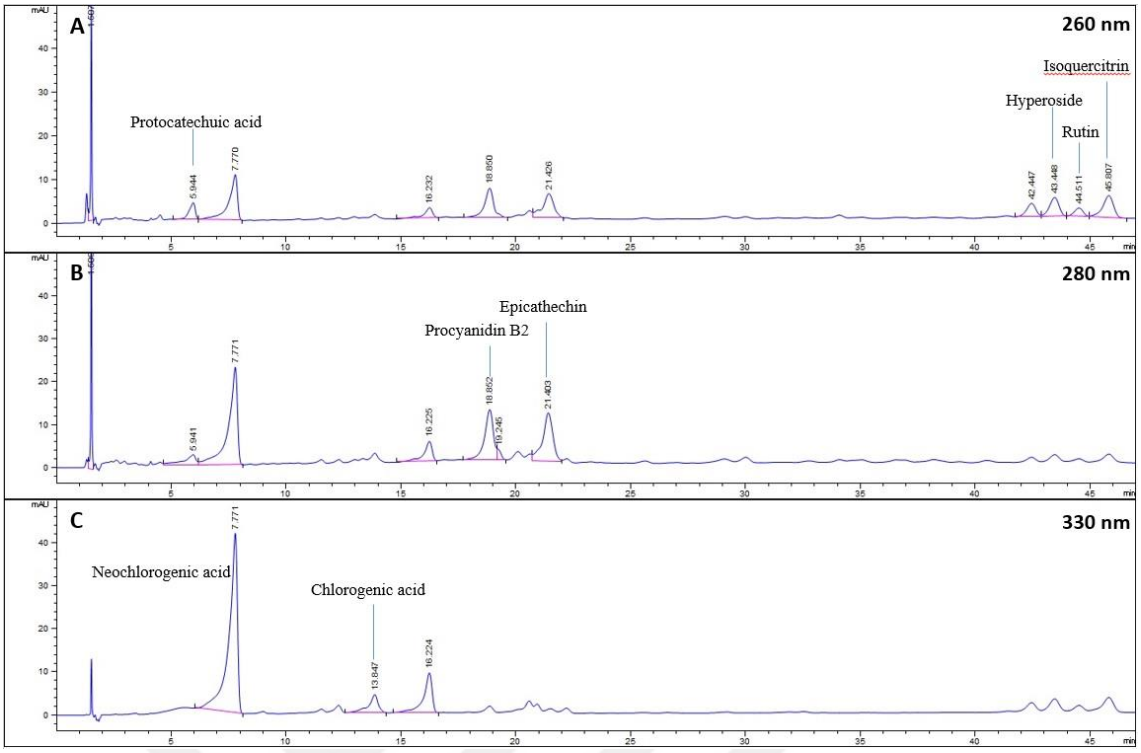

Figure 34. HPLC chromatograms of hydroalcoholic extract belonging to *C. pentagyna* fruits at 260 nm (A), at 280 nm (B) and at 330 nm (C).

The t_R values were as follows: protocatechic acid (5.94 ± 0.02), neochlorogenic acid (7.78 ± 0.01), chlorogenic acid (13.82 ± 0.00), procyanidin B2 (19.18 ± 0.01), epicathechin (21.40 ± 0.01), orientin (n.d.), vitexin(n.d.), vitexin-2"-*O*-rhamnoside (n.d.), hyperoside (43.44 ± 0.01), rutin (44.51 ± 0.01) and isoquercitrin (45.80 ± 0.01).

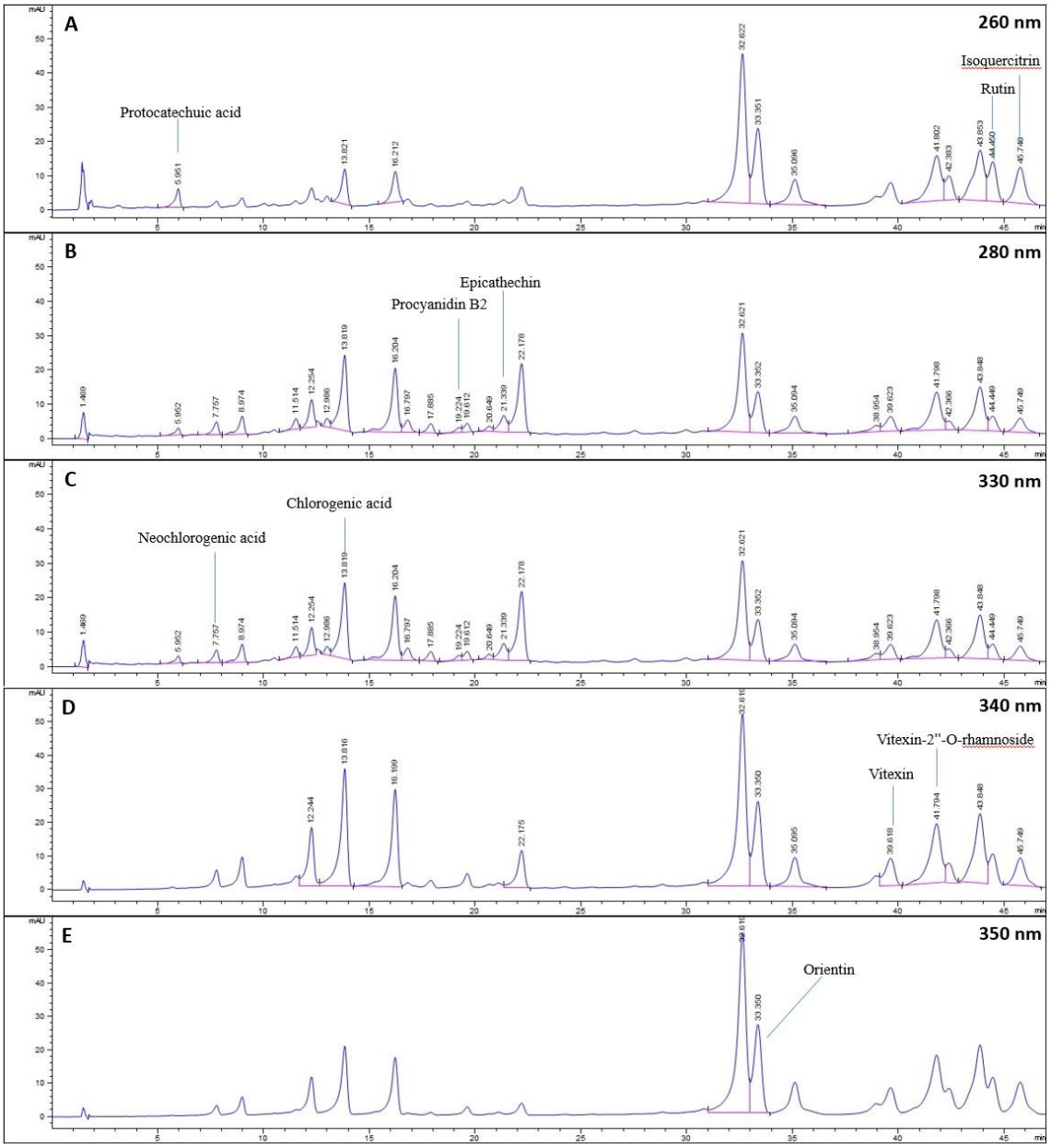

Figure 35. HPLC chromatograms of hydroalcoholic extract belonging to *C. pentagyna* leaves at 260 nm (A), at 280 nm (B), at 330 nm (C), 340 nm (D) and at 340 nm (E).

The t_R values were as follows: protocatechic acid (5.94 ± 0.02), neochlorogenic acid (7.78 ± 0.01), chlorogenic acid (13.85 ± 0.02), procyanidin B2 (19.21 ± 0.01), epicathechin (21.32 ± 0.01), orientin (33.35 ± 0.01), vitexin(39.62 ± 0.01), vitexin-2"-*O*-rhamnoside (41.79 ± 0.01), hyperoside (n.d.), rutin (44.45 ± 0.03) and isoquercitrin (45.82 ± 0.01).

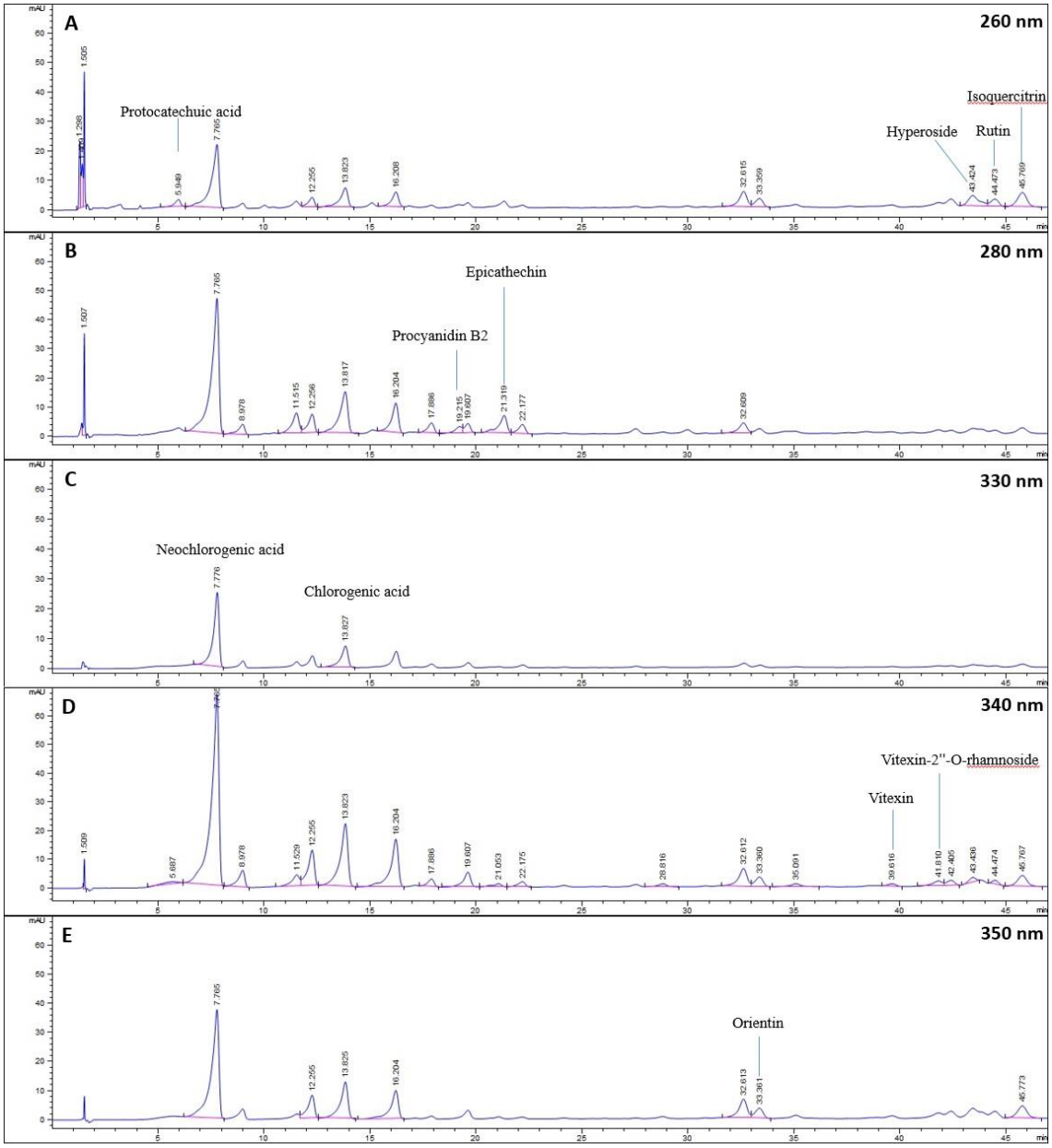

Figure 36. HPLC chromatograms of hydroalcoholic extract belonging to *C. pentagyna* flower-bearing branches at 260 nm (A), at 280 nm (B), at 330 nm (C), at 340 nm (D) and at 350 nm (E).

The t_R values were as follows: protocatechic acid (5.94 ± 0.01), neochlorogenic acid (7.77 ± 0.01), chlorogenic acid (13.83 ± 7.98), procyanidin B2 (19.22 ± 0.01), epicathechin (21.32 ± 0.01), orientin (33.37 ± 0.01), vitexin (39.68 ± 0.19), vitexin-2''-*O*-rhamnoside (41.88 ± 0.19), hyperoside (43.43 ± 0.01), rutin (44.48 ± 0.02) and isoquercitrin (45.85 ± 0.24).

4.2. Results of Phenolic Content Determination

4.2.1. Results of Total Phenolic Content Assay

Total phenolic content of hydroalcoholic extracts of flower-bearing branches, leaves and fruits of four different *Crataegus* species were demonstrated in Table 28 *C. orientalis* leaves showed the highest total phenolic content (874.060 ± 74.94 mg GAE/g). Among fruits, highest TPC was detected in *C. monogyna* (116.24 ± 10.83 mg GAE/g) and *C. pentagyna* (90.99 ± 2.76 mg GAE/g). Among flower-bearing branches, *C. pentagyna* (368.93 ± 7.77 mg GAE/g) had the highest result of total phenolic content followed by *C. monogyna* (301.77 ± 21.80 mg GAE/g) and *C. orientalis* (287.92 ± 37.02 mg GAE/g) which had statistically same phenolic content. When plant parts compared to each other, fruits of four *Crataegus* species had the lowest content of phenolics than leaves and flower-bearing branches.

4.2.2. Results of Total Flavonoid Content Assay

Results of total flavonoid content of hydroalcoholic extracts belonging to four *Crataegus* species were shown in Table 28 *C. pentagyna* (63.67 ± 4.71 mg QE/g) leaves demonstrated greater results than the samples of other *Crataegus* species. Second highest results were observed in *C. turcicus* (34.25± 2.77 mg QE/g) leaves and *C. orientalis* leaves. In general view, fruit of *Crataegus* species had the lowest flavonoid content. Considering that flower-bearing branches, *C. pentagyna* (26.92 ± 1.17 mg QE/g) showed the highest flavonoid content. Among fruits of *Crataegus* species, *C. pentagyna* (9.87 ± 0.67 mg QE/g) and *C. orientalis* (8.37 ± 0.97 mg QE/g) showed similar TFC values and statistically greater than the other *Crataegus* species.

4.2.3. Results of Anthocyanin Content Assay

According to the total anthocyanin content assay results which were showed in Table 28, *C. pentagyna* (15.45 ± 0.28 mg C3GE/g) demonstrated the highest anthocyanin content followed by *C. turcicus* (6.47 ± 0.45 mg C3GE/g). *C. turcicus* showed statistically more significant results than *C. monogyna* (2.02 ± 0.20 mg C3GE/g) and *C. orientalis*, while *C. orientalis* (0.24 ± 0.07 mg C3GE/g) had the lowest content of anthocyanin.

4.2.4. Results of Total Proanthocyanidin Content Assay

Based on the outcomes of the Total Proanthocyanidin Content Assay, flower-bearing branches of *C. turcicus* (72.498 ± 1.359 mg CE/g and 30.727 ± 0.540 mg CCE/g),

C. monogyna (70.961 ± 2.838 mg CE/g and 30.112 ± 1.135 mg CCE/g) and *C. orientalis* (67.415 ± 8.153 mg CE/g and 28.694 ± 3.261 mg CCE/g) showed statistically same and the highest results. Fruits of *C. orientalis* (14.331 ± 1.248 mg CE/g and 7.459 ± 0.499 mg CCE/g) demonstrated the lowest proanthocyanidin content results in all samples. Considering hydroalcoholic extracts of fruits, *Cratagues pentagyna* (42.572 ± 1.265 mg CE/g and 18.755 ± 0.506 mg CCE/g) possessed the highest proanthocyanidin content. Among hydroalcoholic extracts of leaves, the highest total proanthocyanidin content results were detected in *C. turcicus* (48.126 ± 0.129 mg CE/g and 20.977 ± 0.520 mg CCE/g), followed by *C. pentagyna* (46.117±3.009 mg CE/g and 20.174 ± 1.204 mg CCE/g) and *C. orientalis* (45.696 ± 1.177 mg CE/g and 20.005 ± 0.471 mg CCE/g) which showed statistically same results. According to the catechin and cyanidin chloride references used in this experiment, the statistical rankings of proanthocyanidin contents of all samples were the same. Results are shown in Table 28.

Table 28. Total phenolic, flavonoid, proanthocyanidin and anthocyanin contents of different parts of *Crataegus* species[1].

SAMPLES	PARTS	TPC[1] mg GAE/g	TFC[2] mg QE/g	TPAC[3] mg CCE/g	TPAC[4] mg CE/g	TAC[5] mg C3GE/g
C. turcicus	fruits	69.43 ± 5.9^{G}	1.54 ± 0.17^{F}	12.96 ± 0.62^{F}	28.09 ± 1.55^{F}	6.47 ± 0.45^{B}
	leaves	227.57 ± 12.05^{DE}	34.26 ± 2.77^{B}	20.98 ± 0.05^{C}	48.13 ± 0.13^{C}	-
	flower-bearing branches	239.09 ± 9.77^{DE}	22.15 ± 1.37^{CD}	30.73 ± 0.54^{A}	72.50 ± 1.35^{A}	-
C. monogyna	fruits	116.24 ± 10.83^{FG}	6.08 ± 0.53^{EF}	15.16 ± 0.43^{EF}	33.57 ± 1.08^{EF}	2.02 ± 2.02^{C}
	leaves	169.66 ± 12.34^{EF}	20.54 ± 1.33^{D}	17.22 ± 1.31^{DE}	38.73 ± 3.28^{DE}	-
	flower-bearing branches	301.77 ± 21.8^{CD}	20.49 ± 0.69^{D}	30.11 ± 1.14^{A}	70.96 ± 2.84^{A}	-
C. orientalis	fruits	42.12 ± 4.71^{G}	8.37 ± 0.98^{E}	7.46 ± 0.5^{G}	14.33 ± 1.25^{G}	0.24 ± 0.24^{D}
	leaves	874.06 ± 74.94^{A}	37.47 ± 3.48^{B}	20.01 ± 0.47^{CD}	45.70 ± 1.78^{CD}	-
	flower-bearing branches	287.92 ± 37.02^{CD}	20.72 ± 0.53^{D}	28.69 ± 3.26^{A}	67.41 ± 8.15^{A}	-
C. pentagyna	fruits	91.00 ± 2.77^{FG}	9.87 ± 0.67^{E}	18.76 ± 0.51^{CD}	42.57 ± 1.26^{CD}	15.45 ± 15.45^{A}
	leaves	394.66 ± 35.05^{B}	63.67 ± 4.71^{A}	20.17 ± 1.2^{CD}	46.12 ± 3.01^{CD}	-
	Flower-bearing branches	368.94 ± 7.78^{BC}	26.92 ± 1.18^{C}	24.67 ± 1.07^{B}	57.35 ± 2.68^{B}	-

1: TPC: Total Phenolic Content; mg GAE/g: mg gallic acid equivalent per g hydroalcoholic extract, 2: TFC: Total Flavonoid Content; mg QE/g: mg quercetin equivalent per g hydroalcoholic extract, 3 and 4: TPAC: Total Proanthocyanidin Content; mg CCE/g: mg cyanidin chloride equivalent per g hydroalcoholic extract and mg CE/g: mg catechin equivalent per g hydroalcoholic extract, 5: TAC: Total Anthocyanin Content; mg C3GE/g: mg cyanidin-3-*O*-glucoside equivalent per g hydroalcoholic extract. Different letters in the same column indicate significantly different values at $p \leq 0.05$.

4.3. Results of Antioxidant Potential Determination

4.3.1. Results of Free Radical Scavenging Activity Assays

4.3.1.1. Results of DPPH Radical Scavenging Assay

According to the DPPH radical scavenging activity results of hydroalcoholic extract of flower-bearing branches belonging to *C. turcicus* demonstrated the highest antioxidant activity and this results statistically significant (466.59 ± 8.96 mg TE/g). From 4 different hydroalcoholic extract of leaves belonging to *Crataegus* species, *C. turcicus* (346.64 ± 7.80 mg TE/g) showed the highest radical scavenging activity. While *C. monogyna* (132.88 ± 20.31 mg TE/g) fruits, *C. pentagyna* (160.02 ± 14.21 mg TE/g) fruits and *C. turcicus* (129.92 ± 4.16 mg TE/g) fruits possessed statistically similar results, *C. orientalis* (70.04 ± 2.69 mg TE/g) fruits showed the lowest antioxidant capacity among fruits and among all samples.

4.3.1.2. Results of ABTS Radical Scavenging Assay

According to ABTS radical scavenging activity, *C. monogyna* (457.26 ± 38.03 mg TE/g) flower-bearing branches showed the highest antioxidant activity followed by flower-bearing branches of *C. pentagyna* (442.72 ± 32.10 mg TE/g) and *C. orientalis* (400.34 ± 14.59 mg TE/g), respectively. Among hydroalcoholic extracts of fruits, *C. monogyna* (119.45 ± 11.95 mg TE/g) had statistical significant difference and demonstrated the highest activity. The lowest activity among all samples was in *C. pentagyna* (148.88 ± 5.33 mg TE/g), *C. turcicus* (130.78 ± 4.62 mg TE/g) and *C. orientalis* (119.45 ± 11.95 mg TE/g) fruits, which had similar radical scavenging capacity.

4.3.2. Results of Metal Reducing Activity Assays

4.3.2.1. Results of Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric reducing antioxidant potential of *C. monogyna* flower-bearing branches possessed the highest value (372.51 ± 9.03 mg TE/g), followed by *C. turcicus* flowering branches (304.48 ± 6.89 mg TE/g). Among all hydroalcoholic samples, *C. orientalis* (66.52 ± 3.27 mg TE/g) fruits had the lowest ferric reducing antioxidant activity. Considering leaves of 4 types of *Crataegus* species, *C. turcicus* (226.50 ± 8.66 mg TE/g) demonstrated the highest antioxidant activity.

4.3.2.2. Results of Cupric Reducing Antioxidant Capacity (CUPRAC) Assay

According to Cupric-reducing antioxidant activity of hydroalcoholic extracts of flower-bearing branches, leaves and fruits belonging to *Crataegus* species, flower-bearing branches showed the highest activity followed by leaves and fruits respectively. Among all samples, *C. monogyna* flowering branches (852.09 ± 21.15 mg TE/g) had the highest antioxidant activity whereas, *C. pentagyna* fruits (180.87 ± 0.60 mg TE/g) possess the lowest activity. Among leaves, antioxidant results of *C. turcicus* was the highest (692.83 ± 39.21 mg TE/g), other three extracts of leaves (*C. monogyna* (568.09 ± 10.40 mg TE/g), *C. orientalis* (596.72 ± 32.51 mg TE/g) and *C. pentagyna* (553.50 ± 25.26 mg TE/g) had similar antioxidant potential.

Table 29. Total antioxidant contents of different parts of *Crataegus* species[1].

SAMPLES	PARTS	DPPH	ABTS	FRAP	CUPRAC
		mg TE/g[1]			
C. turcicus	fruits	129.93±4.17^{F}	130.78±4.63^{G}	105.51±6.3^{H}	277.03±20.28^{G}
	leaves	346.65±7.80^{C}	365.47±25.53^{CD}	226.50±8.66^{D}	692.83±39.22^{D}
	flower-bearing branches	466.59±8.97^{A}	380.780±20.11^{BCD}	304.48±6.89^{B}	755.42±16.76^{CD}
C. monogyna	fruits	132.89±20.32^{F}	215.97±24.11^{F}	128.81±11.67^{G}	359.33±11.57^{F}
	leaves	243.63±8.18^{E}	289.05±10.26^{E}	162.66±3.33^{F}	568.10±10.41^{E}
	flower-bearing branches	319.29±12.60^{CD}	457.27±38.03^{A}	371.52±9.04^{A}	852.09±21.16^{A}
C. orientalis	fruits	70.04±2.70^{G}	119.45±11.95^{G}	66.52±3.28^{I}	222.13±11.82^{GH}
	leaves	313.59±13.11^{CD}	334.43±15.68^{DE}	198.72±7.68^{E}	596.73±32.51^{E}
	flower-bearing branches	383.98±19.93^{B}	400.34±14.59^{ABC}	271.72±5.84^{C}	779.56±41.05^{BC}
C. pentagyna	fruits	160.02±14.22^{F}	148.89±5.34^{G}	163.89±2.24^{F}	180.87±0.61^{H}
	leaves	296.23±10.91^{D}	397.07±29.02^{ABCD}	217.48±11.91^{DE}	553.50±28.91^{E}
	flower-bearing branches	411.07±3.34^{B}	442.72±32.10^{AB}	272.860±9.44^{C}	847.94±30.557^{AB}

[1] mg TE/g: mg Trolox equivalent per g hydroalcoholic extract
Different letters in the same column indicate significantly different values at $p \leq 0.05$

4.4. Effects of *Crataegus* extracts on the Cell Viability of RAW 264.7 Macrophages

As an initial step, non-toxic concentrations of extracts with a cell viability of more than 70% were determined before evaluating anti-inflammatory and analgesic activity. Based on the MTT assay results, it was observed that all extracts, up to concentrations of 1 mg/mL at 24h, did not display any cytotoxic effects when compared to the control cells that received no treatment, as shown in Table 30.

Table 30. Effects of *Crataegus* hydroalcoholic extracts on the viability of RAW 264.7 macrophage cells and the effects of hydroalcoholic extracts on nitrite levels and % nitrite inhibition in RAW 264.7 cells stimulated with 1 μg/mL LPS.

Groups	Concentration (mg/mL)	Cell Viability (%)	Nitrite Level (μM)	Nitrite Inhibition (%)
Ctrl		117.13 ± 1.75	2.15 ± 2.27	-
Ctrl+LPS		101.12 ± 2.14	64.17 ± 0.84	-
Indomethacin	100 μM	92.92 ± 1.47	32.20 ± 0.95*	49.83 ± 1.39
C. turcicus flower-bearing branches	0.125	115.61 ± 0.73	63.49 ± 1.18	1.06 ± 0.88
	0.25	107.82 ± 1.93	61.33 ± 0.81	4.42 ± 0.58
	0.5	104.06 ± 1.47	55.04 ± 1.03	14.22 ± 2.23
	1	102.15 ± 1.47	13.86 ± 1.41*	78.39 ± 2.31
C. turcicus leaves	0.125	107.50 ± 0.71	61.15 ± 1.77	4.71 ± 2.90
	0.25	95.32 ± 2.83	54.11 ± 0.49	15.67 ± 1.18
	0.5	83.70 ± 1.07	48.01 ± 1.15*	25.19 ± 1.62
	1	79.97 ± 3.61	13.99 ± 1.30*	78.19 ± 2.20
C. turcicus fruits	0.125	117.56 ± 0.94	61.58 ± 1.98	4.05 ± 2.13
	0.25	110.97 ± 1.72	58.62 ± 2.53	8.65 ± 1.05
	0.5	102.83 ± 1.64	43.06 ± 0.88*	32.90 ± 0.98
	1	96.66 ± 4.04	32.75 ± 2.26*	48.94 ± 3.99
C. monogyna flower-bearing branches	0.125	108.40 ± 1.18	58.99 ± 1.37	8.09 ± 1.12
	0.25	99.89 ± 0.63	55.41 ± 1.70	13.66 ± 2.45
	0.5	93.50 ± 1.21	50.22 ± 3.70	21.77 ± 1.93
	1	86.20 ± 0.65	21.27 ± 2.08*	66.87 ± 2.13
C. monogyna leaves	0.125	106.40 ± 0.23	59.67 ± 0.98	7.02 ± 0.48
	0.25	96.79 ± 0.98	52.51 ± 1.57	18.16 ± 2.33
	0.5	89.85 ± 1.44	43.00 ± 0.32*	32.98 ± 1.32
	1	84.30 ± 2.10	13.80 ± 0.47*	78.49 ± 0.92

Ctrl: Control group treated with DMEM; Ctrl+LPS: Control group only stimulated with LPS; LPS: Lipopolysaccharides from *E. coli*; IND: Indomethacin (100 μM). Statistically significant differences were indicated for each compound vs. LPS (* $p<0.0001$).

Table 30. Cont.

Groups	Concentration (mg/mL)	Cell Viability (%)	Nitrite Level (µM)	Nitrite Inhibition (%)
C. monogyna fruits	0.125	107.77 ± 1.53	57.69 ± 1.23	10.09 ± 2.50
	0.25	101.11 ± 0.84	47.63 ± 3.80*	25.75 ± 1.42
	0.5	91.91 ± 1.48	37.51 ± 2.68*	41.52 ± 1.83
	1	85.39 ± 2.06	23.12 ± 1.49*	63.98 ± 2.05
C. orientalis flower-bearing branches	0.125	114.39 ± 1.55	56.64 ± 1.19	11.71 ± 2.95
	0.25	110.37 ± 1.04	51.52 ± 0.67	19.71 ± 1.21
	0.5	103.35 ± 2.97	44.54 ± 0.60*	30.58 ± 1.23
	1	95.53 ± 0.95	11.09 ± 2.23*	82.71 ± 2.59
C. orientalis leaves	0.125	108.88 ± 0.69	63.12 ± 0.60	1.63 ± 1.42
	0.25	102.43 ± 1.15	58.06 ± 2.15	9.51 ± 2.13
	0.5	98.61 ± 1.63	54.67 ± 1.03	14.81 ± 1.32
	1	93.55 ± 1.12	10.65 ± 0.57*	83.40 ± 0.78
C. orientalis fruits	0.125	110.72 ± 0.89	60.72 ± 2.19	5.40 ± 2.55
	0.25	104.69 ± 1.64	52.57 ± 0.65	18.07 ± 1.86
	0.5	100.34 ± 1.76	51.02 ± 0.47	20.48 ± 1.04
	1	90.10 ± 0.26	45.96 ± 1.85*	28.38 ± 2.62
C. pentagyna flower-bearing branches	0.125	112.26 ± 1.32	58.49 ± 0.75	8.84 ± 1.97
	0.25	109.34 ± 2.51	53.06 ± 1.60	17.33 ± 1.47
	0.5	99.33 ± 0.92	48.62 ± 2.51*	24.23 ± 2.06
	1	83.23 ± 0.52	27.44 ± 1.28*	57.23 ± 2.05
C. pentagyna leaves	0.125	115.10 ± 1.54	48.68 ± 3.06	24.18 ± 1.78
	0.25	108.45 ± 1.64	43.62 ± 1.19	32.02 ± 2.14
	0.5	99.85 ± 1.98	30.22 ± 1.77*	52.88 ± 2.28
	1	88.03 ± 1.29	8.19 ± 1.79*	87.24 ± 2.81
C. pentagyna fruits	0.125	106.00 ± 0.84	63.19 ± 0.67	1.54 ± 0.42
	0.25	100.88 ± 1.77	60.41 ± 1.47	5.87 ± 1.67
	0.5	96.09 ± 0.88	54.48 ± 1.34	15.10 ± 2.01
	1	85.62 ± 1.37	41.70 ± 0.49*	35.01 ± 0.99

Ctrl: Control group treated with DMEM; Ctrl+LPS: Control group only stimulated with LPS; LPS: Lipopolysaccharides from *E. coli*; IND: Indomethacin (100 µM). Statistically significant differences were indicated for each compound vs. LPS (* $p<0.0001$).

4.5. Nitric Oxide Production in LPS-Stimulated RAW 264.7 Cells

The ability of hydroalcoholic extracts of Crataegus species to inhibit LPS-induced nitrite production in RAW 264.7 cells was evaluated in this study, with indomethacin, a widely used anti-inflammatory agent, serving as a reference compound. Treatment with LPS (1 µg/mL) caused a significant increase in nitrite levels in the cell culture supernatant (Table 4). NO production after LPS stimulation increased to 64.17 ± 0.84 µM in the untreated cells. However, all of the extracts exhibited a concentration-dependent reduction in LPS-induced nitrite production, as shown in Table 4. Comparative analysis of anti-inflammatory activity of the hydroalcoholic extracts with reference molecule, indomethacin (100 µM), were also shown in Table 4. The treatment of indomethacin (100 µM) significantly inhibited nitrite oxide production %50 (p<0.0001). In particular, C. pentagyna leaves showed remarkably higher anti-inflammatory activity at the highest concentration (1 mg/mL) (p<0.0001) on LPS-stimulated RAW 264.7 cells when compared to the control group. Moreover, nitrite inhibition of C. orientalis leaves and C. orientalis flower-bearing branches at their highest studied concentrations was 83%. As a result, all extracts at 1 mg/mL concentration have shown a significant anti-inflammatory activity in comparison to LPS control (Figure 37).

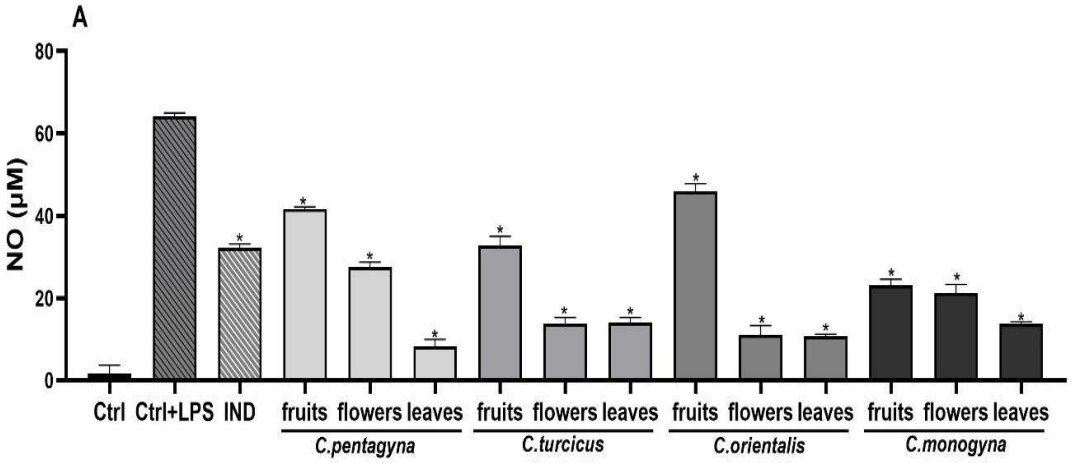

Figure 37. The effects of hydroalcoholic extracts of *Crataegus* species at 1 mg/mL concentration on nitrite production on LPS stimulated RAW 264.7 cell.

Ctrl: Control group treated with DMEM; Ctrl+LPS: Control group only stimulated with LPS; LPS: Lipopolysaccharides from *E. coli*; IND: Indomethacin (100 µM). Statistical significant differences were indicated for each compound vs. LPS (* p<0.0001).

4.6. Evaluation of Analgesic Activity (PGE_2 Assay)

The analgesic activity of hydroalcoholic *Crataegus* extracts was assessed using the highest non-toxic dose (1 mg/mL), which exhibited the highest anti-inflammatory activity. Figure 38 illustrates that the production of PGE_2 production was significantly increased in LPS-stimulated RAW 264.7 macrophages compared to unstimulated negative controls. According to the results of PGE_2 level, hydroalcoholic extract of *C. monogyna* fruits showed the most remarkable decline in PGE_2 level (37.1 ± 10.81 pg/mL) among the other *Crataegus* species ($p < 0.0001$). However, experimental positive control indomethacin (31.65 ± 11.42 pg/mL) exhibited the most potent PGE_2 inhibition compared to the LPS-activated control group (282.85 ± 46.13 pg/mL) (Figure 38).

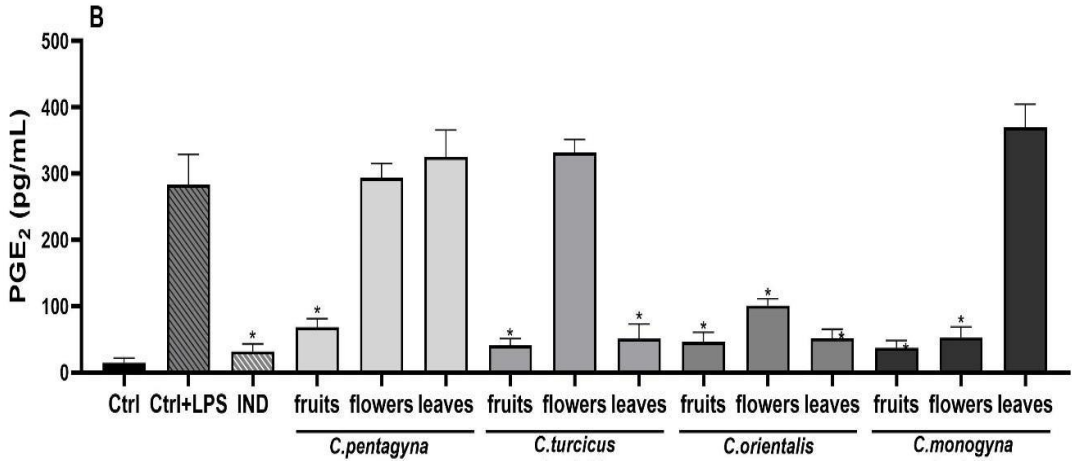

Figure 38. The effects of hydroalcoholic extracts of *Crataegus* species at 1 mg/mL concentration on PGE_2 production on LPS stimulated RAW 264.7 cell.

Ctrl: Control group treated with DMEM; Ctrl+LPS: Control group only stimulated with LPS; LPS: Lipopolysaccharides from *E. coli*; IND: Indomethacin (100 µM). Statistical significant differences were indicated for each compound vs. LPS (* $p<0.0001$).

4.7. Results of IL-6 Releasing Inhibition Assay

RAW 264.7 cells inflammation reflection was activated when exposed to LPS, as IL-6 secretion in the supernatants considerably increased after LPS stimulation for 24 h, and pre-treatment with 1 mg/mL of *Crataegus* hydroalcoholic extracts in prior to LPS challenge dominantly reduced the upregulation of this cytokine secretion. The IL-6

production was higher in the LPS treated group than in the control group. As shown in Figure 39, all compounds suppressed the levels of IL-6 at 1 mg/mL concentrations when compared to the LPS treated group. According to these findings, LPS-activated release of IL-6 significantly declined with 1 mg/mL concentration of *C. orientalis* flower-bearing branches pre-treatment, while *C. pentagyna* was the second most active extract compared to control (515.77 ± 20.63 pg/mL; 525.43 ± 23.06 pg/mL, respectively) ($p < 0.0001$).

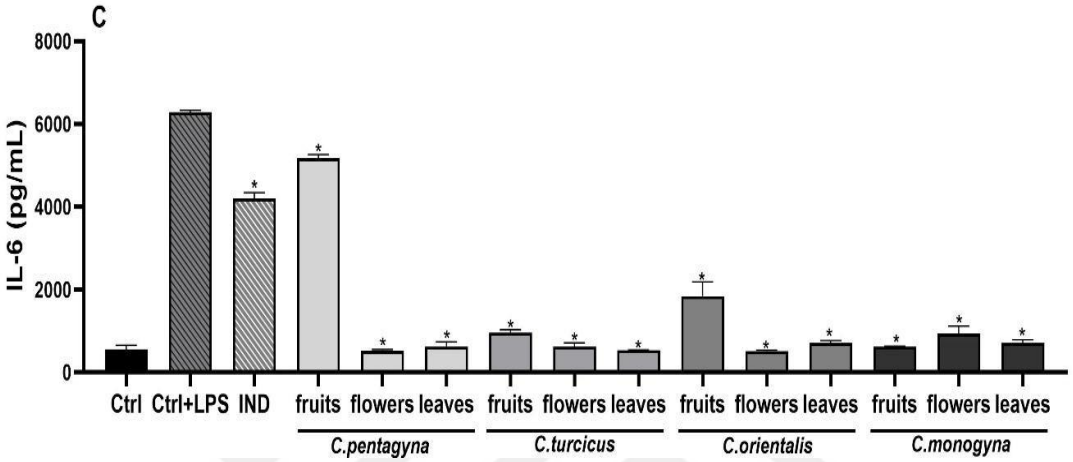

Figure 39. The effects of hydroalcoholic extracts of *Crataegus* species at 1 mg/mL concentration on IL-6 production on LPS stimulated RAW 264.7 cell.

Ctrl: Control group treated with DMEM; Ctrl+LPS: Control group only stimulated with LPS; LPS: Lipopolysaccharides from *E. coli*; IND: Indomethacin (100 µM). Statistical significant differences were indicated for each compound vs. LPS (* $p<0.0001$).

4.8. Tumour Necrosis Factor-alpha (TNF- α)

The inflammatory cytokine TNF-α, which is produced by macrophages and monocytes during acute inflammation, is responsible for a broad range of internal cell signaling processes that cause necrosis or apoptosis (160). LPS stimulation markedly increased the concentrations of pro-inflammatory cytokine TNF-α (Figure 40). On the other hand, treatment with *Crataegus* species at high concentrations (1 mg/mL) dramatically reduced the levels of TNF-α that were induced by LPS. *C. turcicus* leaves, *C.monogyna* fruits and *C. orientalis* leaves reduced LPS-stimulated TNF-α production significantly (93%, 93% and 89%, respectively). These extracts reduced TNF-α levels almost to medium control levels. Moreover, these extracts are more potent than the positive controls indomethacin (57%).

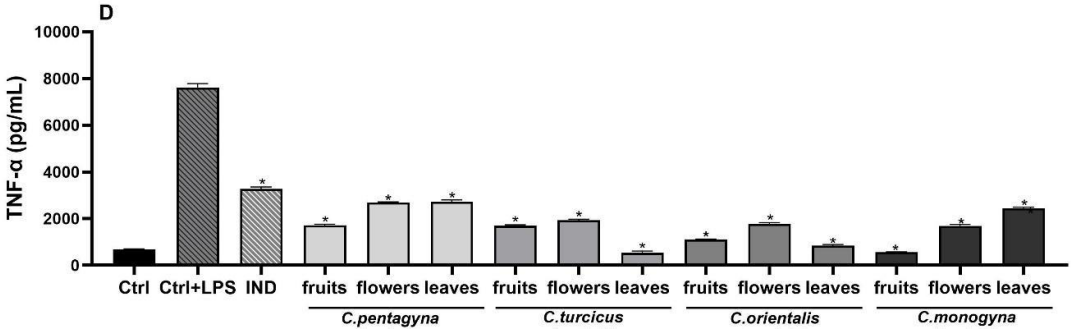

Figure 40. The effects of extracts at 1 mg/mL concentration on TNF-α production on LPS stimulated RAW 264.7 cell.

Ctrl: Control group treated with DMEM; Ctrl+LPS: Control group only stimulated with LPS; LPS: Lipopolysaccharides from *E. coli*; IND: Indomethacin (100 µM). Statistically significant differences were indicated for each compound vs. LPS (* $p<0.0001$).

5. DISCUSSION

Oxidative stress occurs when there is an excessive production of reactive oxygen species (ROS) or oxidants, which are free radicals and reactive metabolites, in organisms. This oxidative stress has a profound impact on the entire, leading to damage of vital cellular molecules. Excessive ROS production can damage cellular components, including DNA, proteins, and lipids, leading to various diseases such as cardiovascular disorders, neurodegenerative diseases, and cancer (24, 25). The organism activates its own defense mechanisms to prevent the toxic effects of oxidative stress. However, in cases where the organism's natural protective system is insufficient, antioxidants play a crucial role in neutralizing the toxic effects of reactive oxygen species by converting them into non-radical metabolites (26). The presence of reactive oxygen species can contribute to the development of numerous diseases, including inflammation (24). Inflammation is a natural biological response of the organism to pathogens and is relevant to various illnesses, such as microbial and viral infections, exposure to allergens, radiation and toxic chemicals, autoimmune and chronic diseases, obesity, alcohol consumption, smoking, and a high-calorie diet (25).

Evidence of medicinal plant use can be traced back as far as 60.000 years ago, encompassing various cultures and countries. Initially, with the advent of Chemistry, the focus shifted towards isolating and identifying biologically active compounds from plants, as they were deemed more valuable therapeutically. These compounds also served as the basis for synthesizing new and potentially more valuable compounds in laboratories. Over time, there has been revived interest in the medicinal benefits of plants, giving rise to a new category of therapeutic agents known as "Nutraceuticals" coined in 1989 by DeFelice, the term refers to foods or food components that provide health benefits, including disease prevention and treatment. According to the World Health Organization (WHO), approximately 4 billion people, equivalent to 80 % of the world's population, incorporate herbal medicines into their primary healthcare practices (161).

Plants possess the ability to synthesize various organic molecules known as secondary metabolites. These secondary metabolites, characterized by unique carbon skeletal structures, are not essential for a cell or organism's survival but play a crucial role in its interaction with the surrounding environment, ensuring its continued existence within ecosystems. Some compounds derived from plants, such as terpenoids, alkaloids,

and flavonoids, are currently utilized as drugs or dietary supplements for the prevention and treatment of various diseases. Notably, certain compounds have shown promising effectiveness in preventing and inhibiting different types of cancer. Research indicated that an estimated 14-28% of higher plant species are employed for medicinal purposes, with 74% of pharmacologically active components derived from plants being discovered through the exploration of their ethnomedicinal use (162).

The species *Crataegus* is widely utilized as an herb for the prevention and treatment of cardiovascular diseases. Additionally, it has been traditionally used as an antispasmodic, cardiotonic, anti-inflammatory, diuretic, hypotensive, and anti-atherosclerotic agent. Various studies conducted thus far have confirmed the rich phenolic content of *Crataegus* species (36). There are also various antioxidant and anti-inflammatory studies have been conducted using *Crataegus* species. However, there is currently no study comparing the flower-bearing branches, leaves and fruits of *Crataegus turcicus* with those of other *Crataegus* species. Considering this knowledge, the antioxidant and anti-inflammatory activities of *C. turcicus, C. orientalis, C. monogyna* and *C. pentagyna* were examined, and their phenolic contents were qualitatively analyzed using HPTLC and quantitatively analyzed using HPLC.

In the current study, hydroalcoholic extracts of flower-bearing branches, leaves and fruits belonging to *C. turcicus, C. monogyna, C. orientalis* and *C. pentagyna* were analyzed for their total phenolic content using the Folin-Ciocalteu method, and *C. turcicus* was compared with these three species. According to the results, the hydroalcoholic extract of *C. turcicus* leaves (227.57 ± 12.05 mg GAE/g) showed statistically higher total phenolic content than hydroalcoholic extract of *C. monogyna* leaves (169.66 ± 12.34 mg GAE/g). The hydroalcoholic extracts of flower-bearing branches belonging to *C. monogyna* (301.77 ± 21.8 mg GAE/g), *C. orientalis* (287.92 ± 37.02 mg GAE/g) and *C. pentagyna* (368.94 ± 7.78 mg GAE/g) exhibited higher phenolic content compared to hydroalcoholic extract of *C. turcicus* flower-bearing branches (239.09 ± 9.77 mg GAE/g). Additionally, the hydroalcoholic extract of *C. turcicus* fruits (69.43 ± 5.9 mg GAE/g) exhibited statistically lower total phenolic content compared to hydroalcoholic extracts of leaves and flower-bearing branches belonging to *C. turcicus.* Furthermore, the hydroalcoholic extracts of fruits from *C. monogyna* (116.24 ± 10.83 mg GAE/g) and *C. pentagyna* (91.00 ± 2.77 mg GAE/g) showed superior TPC results than hydroalcoholic extract of *C. turcicus* fruits, while hydroalcoholic extract of *C. orientalis*

fruits (42.12 ± 4.71 mg GAE/g) showed statistically equal total phenolic content compared to the *C. turcicus* fruits.

In a study conducted by Bardakçı et al. (89), the fruit extract of *C. turcicus* was compared to the fruit extracts of *C. monogyna* and *C. orientalis* in terms of total phenolic content value. In contrast to this study, their results showed that the hydralcoholic extract of *C. turcicus* fruits (398.48 ± 0.98 mg GAE/g extract) had statistically equal total phenolic content compared to the hydralcoholic extract of *C. monogyna* fruits (391.97 ± 2.30 mg GAE/g extract) and statistically superior total phenolic content than the hydralcoholic extract of *C. orientalis* fruits (380.93 ± 3.78 mg GAE/g extract). The difference in results could be attributed to the time of plant collection and variations in the extraction methods used in the experiments. Due to the lack of other studies on *C. turcicus*, the leaves and flower-bearing branches of the plant could not be compared with other research findings in terms of TPC. In a prior investigation conducted by Bedreag et al. (163), the Performed Folin-Ciocalteu technique was employed to determine total phenolic content (TPC) value of 70% methanolic extracts obtained from leaves and flowers of *C. pentagyna.* According to their results, the extract of *C. pentagyna* leaves (206.94 ± 4.86 mg GAE/g extract) exhibited a higher total phenolic content compared to flower extract (184.62 ± 1.71 mg GAE/g extract). Similarly, in the present study, the hydroalcoholic extract of leaves belonging to *C. pentagyna* (394.66 ± 35.05 mg GAE/g) also exhibited higher total phenolic content compared to the hydroalcoholic extract of flower-bearing branches belonging to *C. pentagyna* (368.94 ± 7.78 mg GAE/g). Barros et al. (164) investigated petrolium ether extract of *C. monogyna* by Folin-Ciocalteu technique to determine total phenolic content (TPC) value. Compatible with the present study flower extracts of *C. monogyna* (303.32 ± 1.82 mg GAE/g extract) showed higher total phenolic content compared to fruit extract of *C. monogyna* (274.27 ± 5.91 mg GAE/g extract). In the current investigation, the hydroalcoholic extract of flower-bearing branches from *C. monogyna* (301.77 ± 21.80 mg GAE/g) demonstrated higher total phenolic content in comparison to the hydroalcoholic extract obtained from fruits of *C. monogyna* (116.24 ± 10.83 mg GAE/g). Froehlicher et al. (165) utilized Folin-Ciocalteu technique to examine 80% hydroalcoholic (80% hydroalcoholic extract was obtained after a maceration process) extract of *C. monogyna* flowers and fruits in order to determine the total phenolic content value. Consistent with the present study the flower extract of *C. monogyna* (56.29 ± 201.70 mg GAE/g extract) displayed a higher total

phenolic content compared to the fruit extract of *C. monogyna* (12.82 ± 76.1 mg GAE/g extract). In another study, to calculate the total phenolic content value, Šavikin et al. (61) used the Folin-Ciocalteu technique on %96 hydroalcoholic extract of *C. orientalis* leaves and fruits. According to the study, extract of *C. orientalis* leaves (94.2 ± 0.1 mg GAE/g extract) has greater total phenolic content than the fruit extract of *C. orientalis* (77.6 ± 0.8 mg GAE/g extract). Similarly in the current study, hydroalcoholic extract obtained from *C. orientalis* leaves (874.06 ± 74.94 mg GAE/g) showed higher total phenolic content than the hydroalcoholic extract of *C. orientalis* fruits (42.12 ± 4.71 mg GAE/g). Belabdelli et al. (166) employed a hydroalcoholic extract of *C. monogyna* leaves using the Folin-Ciocalteu procedure to calculate the total phenolic content value. According to their results, 473.4 mg GAE/g extract was found. Based on the finding of this study, total phenolic content value of the hydroalcoholic extract of *Cratategus monogyna* leaves was found 169.66 ± 12.34 mg GAE/g. Bor et al. (59) studied total phenolic content value of *C. orientalis* leaves using the Folin-Ciocalteu technique. Total phenolic content value of 50% hydroalcoholic extract of the leaves was found 98.24 ± 2.4 mg GAE/g extract. In addition according to Çalışkan et al., the total phenolic content of *C. orientalis* fruits in a 60% methanolic extract was reported to be 51.2 mg GAE/g extract. The total phenolic content of *C. orientalis* leaves found in this present study was 874.06 ± 74.94 mg GAE/g. In the study conducted by Dekıć et al. (167), the ethanolic extracts of the leaves and fruits of the *C. monogyna* were compared in terms of total phenolic content. According to their findings, the extract of leaves (188.21 ± 0.18 mg GAE/g extract) was found to be superior to the extract of fruits (101.01 ± 0.12 mg GAE/g extract). This study, similar to their research, also found that the leaves of *C. monogyna* (169.66 ± 12.34 mg GAE/g) had a higher total phenolic content compared to the fruits of *C. monogyna* (116.24 ± 10.83 mg GAE/g).

The present study examined the total flavonoid content of hydroalcoholic extracts of *C. turcicus, C. monogyna, C. orientalis* and *C. pentagyna*. It was found that the hydroalcoholic extract obtained from *C. turcicus* leaves (34.26 ± 2.77 mg QE/g) statistically surpassed the hydroalcoholic extract obtained from *C. monogyna* leaves (20.54 ± 1.33 mg QE/g) in terms of total flavonoid content. It was also determined that the hydroalcoholic extract of leaves belonging to *C. orientalis* (37.47 ± 3.48 mg QE/g) had statistically same total flavonoid content. Additionally, the flower-bearing branches of *C. turcicus* (22.15 ± 1.37 mg QE/g) also exhibited a higher total phenolic content

compared to the flower-bearing branches of *C. monogyna* (20.49 ± 0.69 mg QE/g). In this study, the TFC ranged from 1.54 ± 0.17 mg QE/g to 63.67 ± 4.71 mg QE/g, with the lowest result obtained from the hydroalcoholic extract obtained from *C. turcicus* fruits (1.54 ± 0.17 mg QE/g).

In a study conducted by Bardakçı et al. (89) on the hydroalcoholic extracts obtained from fruits of *C. turcicus, C. monogyna* and *C. orientalis,* it was found that, in contrast to this study, the hydroalcoholic extract of *C. turcicus* fruits (23.87 ± 2.74 mg QE/g extract) had a higher total flavonoid content than hydroalcoholic extracts of *C. orientalis* (380.93 ± 3.78 mg QE/g extract) and *C. monogyna* (391.97 ± 2.30 mg QE/g extract) fruits. The reason for this discrepancy may be the different extraction methods used in both studies. Because there are no other existing studies on *C. turcicus,* it was not possible to compare the extracts of leaves and flower-bearing branches from it with previous research findings regarding (TFC). Bedreag et al. (163) conducted a study to determine the total flavonoid content of 70% methanolic extracts of *C. pentagyna* flowers and leaves. The study revealed that the flower extract (67.04 ± 0.52 mg QE/g extract) had a higher total flavonoid content compared to the leaves extract (57.08 ± 0.21 mg QE/g extract). Similarly, in the present study, hydroalcoholic extract of *C. pentaygna* flower-bearing branches (26.92 ± 1.18 mg QE/g) was found to statistically surpass the hydroalcoholic extract of *C. pentaygna* leaves (63.67 ± 4.71 mg QE/g) in terms of total flavonoid content. In another study total flavonoid content of ethanolic extracts from *C. monogyna* fruits and leaves was determined by Dekıć et al. (167). They found that the extract of *C. monogyna* leaves (89.78 ± 0.13 mg rutin equivalents per g of dried weight) had a higher TFC compared to the fruit extract of *C. monogyna* (48.27 ± 0.26 mg rutin equivalents per g of dried weight). Similarly, in this study, the hydroalcoholic extract of *C. monogyna* leaves (20.54 ± 1.33 mg QE/g) was found to statistically surpass the hydroalcoholic extract of *C. monogyna* fruits (20.49 ± 0.69 mg QE/g) in term of TFC. Barros et al. (164) conducted a study to determine total flavonoid content of petroleum ether extract from *C. monogyna* flowers and fruits. It was found that flowers of *C. monogyna* (103.78 ± 7.65 mg CE/g extract) had higher TFC value compared to fruit extract (21.70 ± 0.82 mg CE/g extract). Likewise, the current study revelaed that TFC value of hydroalcoholic extract belonging to flower-bearing branches of *C. monogyna* (30.11 ± 1.14 mg QE/g) was higher than the hydroalcoholic extract belonging to fruits of *C. monogyna* (6.08 ± 5.53 mg QE/g extract).

In the present study, based on the experimental results of total proanthocyanidin content conducted on hydroalcoholic *Crataegus* extracts, the flower-bearing branches of the *C. turcicus* (30.73 ± 0.54 mg CCE/g) yielded the highest results. The hydroalcoholic extract of flower-bearing branches belonging to *C. turcicus* showed statistically equal results with the hydroalcoholic extract of flower-bearing branches belonging to *Cratageus monogyna* (30.11 ± 1.14 mg CCE/g) and *C. orientalis* (28.69 ± 3.26 mg CCE/g). Additionally, among the hydroalcoholic extracts of leaves, it was determined that the *C. turcicus* (20.98 ± 0.05 mg CCE/g) had the highest total proanthocyanidin content. Furthermore, based on the results of the fruit extracts, the *C. turcicus* (12.96 ± 0.62 mg CCE/g) exhibited a statistically lower total proanthocyanidin content compared to the *C. monogyna* 15.16 ± 0.43 mg CCE/g), while it had a higher value compared to the *C. orientalis* (7.46 ± 0.5 mg CCE/g).

Bardakçı et al. (89) conducted a study on TPAC of fruits belonging to *C.turcicus*, *C. monogyna* and *C. orientalis*, which is also correlated with the current study, they found that *C. monogyna* (175.65 ± 10.59 mg epigallocatechin equivalents (EGCG-E) per g dried extract) had the highest TPAC value, followed by *C. turcicus* (59.76 ± 4.81 mg EGCG-E per g dried extract) and *C. orientalis* (31.73 ± 1.36 mg EGCG-E per g dried extract), respectively. Since there is scarcity of existing research on *C. turcicus*, it was not possible to compare the extracts of leaves and flower-bearing branches from this plant with the findings of other studies in terms of TPAC. According to a study conducted by Bedreag et al. (163) total proanthocyanidin content of flower extract from *C. pentagyna* (97.70 ± 3.81 mg cyanidin/g extract) was higher than the extract obtained from *C. pentagyna* leaves (68.92 ± 1.81 mg cyanidin/g extract). This study also determined that *C. pentagyna* flower-bearing branches (24.67 ± 1.07 mg cyanidin/g extract) had statistically superior total proanthocyanidin results compared to *C. pentagyna* leaves (20.17 ± 1.2 mg cyanidin/g extract). Froehlicher et al. (165) determined the total proanthocyanidin content of *C. monogyna* flowers and fruits. Their results showed that flower extract of plant (5.97 ± 44.7 mg cyanidin/g extract) showed statistically higher result than the fruit extract (0.96 ± 4.8 mg cyanidin/g extract). Same with the present study, TPAC result of extract obtained from *C. monogyna* flower-bearing branches (30.11 ± 1.14 mg cyanidin/g extract) was higher compared to extract obtained from *C. monogyna* fruits (15.16 ± 0.43 mg cyanidin/g extract).

Total anthocyanin content of hydroalcoholic fruit extracts from *C. turcicus, C. monogyna, C. orientalis* and *C. pentagyna* were determined in this study and results showed that anthocyanin content of *C. pentagyna* (15.45 ± 15.45 mg C3GE/g) fruits had significantly higher results than the other species examined, followed by *C. turcicus* fruits (6.47 ± 0.45 mg C3GE/g). Since there is no study available on the total anthocyanin content of *C. turcicus*, *C. orientalis* and *C. pentagyna* fruits, the obtained results could not be compared. However, in a study conducted by Froehlicher et al. (165) on 80% hydroalcoholic fruit extract of *C. monogyna,* the total anthocyanin content found to be 0.15 mg eq. C3GE/ g dry weight. In another study by Stanković et al. (168), the total anthocyanin content of 75% hydroalcoholic extract of *C. monogyna* fruits was found to be 0.03 ± 0.004 mg malvinidin-3-glucoside equivalent/ g extract. The differences in the obtained values could possibly be attributed to the different geographical regions where the plant was collected (169).

In the present study, the phenolic profiles of the samples were visually investigated using HPTLC analysis. While there have been several HPTLC studies on *Crataegus* species, this study is the first to visually examine and compare protocatechuic acid, neochlorogenic acid, chlorogenic acid, procyanidin B2, epicatechin, orientin, vitexin , vitexin-2"-*O*-rhamnoside, hyperoside, rutin and isoquercitrin compounds in the flower-bearing branches, leaves and fruits of *C. turcicus* with those *C. monogyna, C. orientalis* and *C. pentagyna.*

Bardakçı et al. (170) conducted HPTLC analysis to examine the hyperoside content of two different extracts of *C. monogyna.* Hyperoside was detected in the both *C. monogyna* extracts they examined. Similarly, according to the results of the HPTLC analysis in the current study, hyperoside was visually detected in the extracts of flower-bearing branches, leaves and fruits belonging to *C. monogyna.* Bubueanu et al. (171) conducted HPTLC analysis to determine the presence of hyperoside, chlorogenic acid and rutin standards in the hydroalcoholic extract containing both leaves and flowers of the *C. monogyna.* According to their results, only hyperoside was detected in the extract. In the current study, the leaves and flower-bearing branches of the *C. monogyna* were separately examined and hyperoside, chlorogenic acid and rutin were visually detected in both extract of leaves and flower-bearing branches of *C monogyna.* Cretu et al. (172) qualitatively examined the 70% hydroalcoholic extract of the *C. monogyna,* which included both the flowers and leaves, using HPTLC analysis with rutin, chlorogenic acid,

hyperoside and caffeic acid standards. According to the results, rutin and hyperoside were detected in the extract. As mentioned before in the current study the leaves and flower-bearing branches of the *C. monogyna* were separately examined as individual extracts and rutin, hyperoside and chlorogenic acid were visually detected in both extracts. However, caffeic acid was not investigated in the current study. Bardakçı et al. (89) conducted HPTLC analysis in a study that included fruit extracts of *C. turcicus, C. monogyna* and *C. orientalis.* In this analysis, the detection of hyperoside and chlorogenic acid standards was performed. According to the results, it was observed that both hyperoside and chlorogenic acid were present in the fruit extracts of all three *Crataegus* species. Similarly, in the current study, visual detection of hyperoside and chlorogenic acid was observed in these three *Crataegus* species as well. Khoklova et al. (173) conducted HPTLC study on methanolic fruit extracts of certain *Crataegus* species using ethyl acetate, ethyl methyl ketone, formic acid and water (5:3:1:1 *v/v/v/v*) as the mobile phase and the silica as the stationary phase. The study utilized rutin, hyperoside and chlorogenic acid standards. Among the *Crataegus* species they investigated, both *C. monogyna* and *C. pentagyna*, which were also used in the current study, exhibited presence of chlorogenic acid, hyperoside and rutin. Similarly, in this study, same mobile and stationary phase was used and chlorogenic acid, rutin and hyperoside were visually detected in both *C. monogyna* and *C. pentagyna* fruit extracts.

In the current study, HPLC analysis was performed to quantify the compounds present in the hydroalcoholic extracts of flower-bearing branches, leaves and fruits of *C. turcicus, C. monogyna, C. orientalis* and *C. pentagyna.* According to results, extracts of the flower-bearing branches have the highest phenolic compound content, followed by leaves and then fruits. Furthermore, when examining the total detected phenolic compound amount in the plant parts individually, *C. monogyna* (139.51 mg/g extract) exhibited the highest content. In the fruit extract of *C. turcicus,* epicatechin (7.92 ± 0.06 mg/g extract) was found to be the most abundant compound, followed by neochlorogenic acid (6.9 ± 0.02 mg/g extract). In the hydroalcoholic extracts of *C. turcicus* leaves, it was determined that chlorogenic acid was the most abundant phenolic compound (29.6 ± 0.04 mg/g extract), followed by vitexin-2"-*O*-rhamnoside (12.83 ± 0.14 mg/g extract). Similarly in the flower-bearing branches of the *C. turcicus,* neochlorogenic acid was identified as the compound with the highest content (18.18 ± 0.56 mg/g extract). Isoquercitrin was detected in all samples, but its quantity could not be determined in the

C. turcicus extracts due to its peak overlapping with another compound. Chlorogenic acid, procyanidin B2 and epicatechin were detected in all samples. Orientin was not detected in the samples of *C. monogyna* and *C. orientalis*, while it was found in low amounts in the hydroalcoholic extracts of flower-bearing branches (0.08 ± 0.07 mg/g extract) and leaves (0.47 ± 0.03 mg/g extract) of the *C. turcicus*. Vitexin was detected in the leaves (3.61 ± 0.14 mg/g extract) and flower-bearing branches (0.9 ± 0.03 mg/g extract) of the *C. turcicus* but was not found in any of the extracts from *C. monogyna*. Since this was the first HPLC study conducted on the *C. turcicus*, the results could not be compared with any other study.

Alirezalu et al. (174) conducted a study on the fruits of *C. monogyna, C. pentagyna* and *C. orientalis*, where they quantitatively analyzed the chlorogenic acid, vitexin, vitexin-2"-*O*-rhamnoside, rutin, hyperoside and isoquercetin contents of 80% methanolic extracts using HPLC-DAD method. The results revealed that the *C. pentagyna* had the highest content of chlorogenic acid (0.50 ± 0.06 mg/g extract), followed by the *C. monogyna* (0.40 ± 0.04 mg/g extract) and *C. orientalis* (0.77 ± 0.06 mg/g extract), respectively. Vitexin-2"-*O*-rhamnoside was not detected in the extracts, while vitexin was absent in the *C. orientalis* but found in the *C. monogyna* (0.18 ± 0.05 mg/g extract) in highest amounts. Rutin was not detected in *C. monogyna* but showed highest quantity in the *C. pentagyna* (1.27 ± 0.08 mg/g extract). *C. pentagyna* exhibited the highest hyperoside content (2.41 ± 0.04 mg/g extract), followed by *C. orientalis* (1.62 ± 0.04 mg/g extract) and *C. monogyna* (0.15 ± 0.07 mg/g extract), respectively. Likewise, isoquercitrin was found most abundant in *C. pentagyna* (1.18 ± 0.07 mg/g extract) and least abundant in *C. monogyna* (0.68 ± 0.04 mg/g extract). In the current study, based on the results of fruit extracts from *C. monogyna, C. orientalis* and *C. pentagyna* in terms of chlorogenic acid, vitexin, vitexin-2"-*O*-rhamnoside, rutin, hyperoside and isoquercetin contents, the concentration of chlorogenic acid was found to be highest in the *C. monogyna* (2.14 ± 0.07 mg/g extract), and it was 5 times higher compared to the previous study. It was followed by *C. pentagyna* (0.71 ± 0.01 mg/g extract) and *C. orientalis* (0.55 ± 0.0 mg/g extract), respectively. Vitexin on the other hand could not be detected in the fruit extracts of *C. monogyna, C. orientalis* and *C. pentagyna*. The quantity of hyperoside was highest in the *C. monogyna* (5.61 ± 0.15 mg/g extract), and it was 5 times higher compared to the previous study, followed by *C. orientalis* (2.23 ± 0.01 mg/g extract) and *C. pentagyna* (1.68 ± 0.02 mg/g extract), respectively. The content of isoquercitrin was

also highest in *C. monogyna* (2.18 ± 0.00 mg/g extract), and it was 3 times higher compared to the previous study. The content of isoquercitrin was lowest in *C. orientalis* (1.28 ± 0.45 mg/g extract). Rutin was not detected in *C. orientalis* and *C. monogyna,* while it was found to be 3 times higher compared to the previous study in *C. pentagyna* (0.96 ± 0.02 mg/g extract). Consistent with the previous study, vitexin-2"-*O*-rhamnoside was not detected in fruit extract of *C. monogyna, C. pentagyna* and *C. orientalis.*

Stoenescu et al. (175) analyzed the methanolic extracts of fruits, leaves and flowers of *C. monogyna* and *C. pentagyna* for their phenolic content using HPLC. They quantified several compounds, including chlorogenic acid, neochlorogenic acid, epicatechin and rutin, which were also found in this study. According to their results, epicatechin was not detected in the leaves and flowers of *C. monogyna* and *C. pentagyna,* however, it was found 4 times higher amounts in the fruit extract of *C. monogyna* (1.43 ± 10.64 mg/g extract) compared to the fruit extract of *C. pentagyna* (0.37 ± 1.25 mg/g extract). Rutin was detected in the highest amounts in the extracts of both *C. monogyna* leaves (1.53 ± 4.56 mg/g extract) and *C. pentagyna* leaves (2.33 ± 10.47 mg/g extract), followed by flower and fruit extracts, respectively. Chlorogenic acid was found in the highest amounts in the extract of *C. monogyna* leaves (5.08 ± 24.74 mg/g extract) among other *C. monogyna extracts,* while it was detected in the highest amounts in the flower extracts of *C. pentagyna* (2.46 ± 15.23 mg/g extract) among other *C. pentagyna extracts.* Neochlorogenic acid was found in the highest amounts in flower extracts of both *C. monogyna* (2.03 ± 15.2 mg/g extract) and *C. pentagyna* (6.33 ± 35.1 mg/g extract). According to results of this study in terms of chlorogenic acid, neochlorogenic acid, epicatechin and rutin content, unlike this research, epicatechin was detected in all extracts. Similar to previous study, rutin content was found to be highest in extract of both *C. monogyna* leaves (1.67 ± 0.1 mg/g extract) and *C. pentagyna* leaves (6.03 ± 0.37 mg/g extract) among their other extracts (fruits and flower-bearing branches). In the present study, rutin was not detected in the fruits and flower-bearing branches of *C. monogyna,* and rutin was found 3 times higher in the extract of *C. pentagyna* leaves compared to the other study. Chlorogenic acid was detected in the highest amounts in the flower-bearing branches of *C. monogyna* (45.52 ± 0.5 mg/g extract), and in the leaves of *C. pentagyna* (32.35 ± 0.02 mg/g extract). The current study similarly found that neochlorogenic acid quantity was highest in the flower-bearing branches compared to the other parts.

In the study conducted by Pavlovic et al. (176), they investigated different parts of *C. pentagyna* in different harvesting seasons as May (flowers and leaves) and September (fruits). Accordingly, %80 acetone extract, neochlorogenic acid, chlorogenic acid, rutin, hyperoside, isoquercitrin, procyanidin B2 and epicatechin components were quantified using HPLC. According to the results, epicatechin was found to be the most abundant compound in the fruit extract (13.1 ± 0.1 mg/g extract), followed by procyanidin B2 (10.6 ± 0.1 mg/g extract). In the flower extract, neochlorogenic acid was found in the highest amount (5.20 ± 0.30 mg/g extract), followed by chlorogenic acid (4.85 ± 0.03 mg/g extract). In the leaves, it was determined that epicatechin (9.75 ± 0.11 mg/g extract) was present in the highest amount, among other components. While rutin (1.23 ± 0.03 and hyperoside (4.54 ± 0.05 mg/g extract) were found more abundant in the extract of leaves, isoquercitrin (3.14 ± 0.05 mg/g extract) was found in higher quantity in the flower extract. Epicatechin and procyanidin B2 were found to be present in higher amounts in the fruit extract compared to the other samples. In terms of the compounds examined in the previous study, it was determined that, consistent with the previous study, epicatechin was found more abundant in the *C. pentagyna* fruit extracts (13.29 ± 0.21 mg/g extract), and neochlorogenic acid was determined as a most abundant phenolic compound in the extract of flower-bearing branches belonging to *C. pentagyna* (50.89 ± 4.02 mg/g extract), followed by chlorogenic acid (15.69 ± 0.75. Additionally, it was found that the extract of *C. pentagyna* leaves (6.03 ± 0.37 mg/g extract) had the highest content of rutin. However, unlike the previous study, the amount of chlorogenic acid was higher in the extract of *C. pentagyna* leaves (32.35 ± 0.02 mg/g extract) compared to the other components we considered. Furthermore, hyperoside was not detected in the *C. pentagyna* leaves extract.

Sagaradze et al. (177) performed HPLC analysis on the 70% hydroalcoholic flower extracts of *C. monogyna* and *C. pentagyna*. Among the compounds they quantified, according to the results of vitexin, rutin and hyperoside, which were also relevant to this study, hyperoside was the most abundant component in both *C. monogyna* (12.58 ± 1.28 mg/g extract) and *C. pentagyna* (4.41 ± 0.02 mg/g extract) flower extracts. Vitexin and rutin were detected in higher amounts in the *C. pentagyna* extract compared to *C. monogyna*, while the amount of hyperoside was higher in *C. monogyna*. Considering only the results of vitexin, rutin and hyperoside, this study similarly found hyperoside to be the abundant compound in the hydroalcoholic extract *C. pentagyna* flower-bearing branches (2.84 ± 0.03 mg/g extract), with a quantity 1.5 times lower than the other study.

Additionally, consistent with the previous study, the content of hyperoside in the hydroalcoholic extract of *C. monogyna* flower-bearing branches (17.29 ± 0.01 mg/g extract) was higher compared to the hydroalcoholic extract of *C. pentagyna* flower-bearing branches (2.84 ± 0.03 mg/g extract). Vitexin and rutin were not detected in the *C. monogyna* flower-bearing branches.

Luis et al. (178) conducted an HPLC study using the methanolic extracts of the leaves, fruits and flowers of *C. monogyna*. Among the phenolic compounds they quantified using HPLC, focusing on the results of chlorogenic acid, which was relevant to current study, it was determined that chlorogenic acid was present in higher amounts in the extract of leaves (10.3 ± 1.2 mg/g extract), followed by the fruit extract (3.0 ± 0.3 mg/g extract), then flower extract (1.5 ± 0.3 mg/g extract). In the present study, however, the flower-bearing branches of *C. monogyna* (45.52 ± 0.5 mg/g extract) were found to have the highest amount of chlorogenic acid. The chlorogenic acid content in the hydroalcoholic extract belonging to leaves of *C. monogyna* (40.89 ± 0.44 mg/g extract) was found to be 4 times higher compared to the other study. The abundance and concentration of secondary metabolites found in plants can be affected by abiotic and biotic factors in their environment such as animals, fungi, bacteria, water soil, temperature and chemicals (179).

The antioxidant activities of hydroalcoholic extracts of the flower-bearing branches, leaves and fruits belonging to *C. monogyna*, *C. turcicus*, *C. orientalis* and *C. pentagyna* were examined using four different experiments: DPPH radical scavenging activity, ABTS radical scavenging activity, FRAP metal-reducing activity and CUPRAC assays. According to the results of the DPPH assay, hydroalcoholic extract of *C. turcicus* flower-bearing branches exhibited the highest antioxidant activity statistically among all the extracts (466.59 ± 8.97 mg TE/g), which may be attributed to the high levels of neochlorogenic acid and hyperoside in the extract, as observed in the HPLC analysis. Among the hydroalcoholic extracts of leaves, *C. turcicus* showed the highest antioxidant activity statistically (346.65 ± 7.80 mg TE/g). Additionally, the fruit extract of *C. turcicus* (129.93 ± 4.17 mg TE/g) exhibited higher antioxidant activity than the fruit extract of *C. orientalis* (70.04 ± 2.70 mg TE/g).

Froehlicher et al. (165) conducted a study on the DPPH radical scavenging activity of the 80% hydroalcoholic extract of *C. monogyna* fruits and flowers. The results

indicated that the flower extracts (87.10 ± 1.1 mg TE/g extract) exhibited greater antioxidant activity compared to the fruit extract (15.76 ± 2.1 mg TE/g extract). Similarly, in the present study, the antioxidant activity of the hydroalcoholic extract of *C. monogyna* flower-bearing branches (319.29 ± 12.60 mg TE/g) was found to be higher than that of the *C. monogyna* fruit extract (132.89 ± 20.32 mg TE/g). Additionally, the antioxidant effect of the *C. monogyna* flower-bearing branches was 3.5 times higher than that of the previous study, whereas the antioxidant effect of the *C. monogyna* fruit extract was 8 times higher than that of the previous study. Rocchetti et al. (180) investigated the DPPH radical scavenging activity of three extracts methanolic, decoction and infusion) obtained from the leaves of the *C. orientalis.* According to their results, the methanolic extract exhibited the highest antioxidant activity (96.71 ± 0.09 mg TE/g extract). In this study, the hydroalcoholic extract of *C. orientalis* leaves showed three times more antioxidant effect compared to the methanolic extract in the previous study (313.59 ± 13.11 mg TE/g). Martín-Garcia et al. (181) investigated the DPPH radical scavenging activity of 15 different extracts prepared by varying the acetone –water ratio of extract from *C. monogyna* leaves. The values they found ranged between 37.68 mg TE/g extract and 101.3 mg TE/g extract. In the current study, the DPPH radical scavenging activity of the hydroalcoholic extract of *C. monogyna* leaves exceeded these values (243.63 ± 8.18 mg TE/g). This difference could be attributed to variations in the plant's growing locations and differences in the extraction method (181).

According to the results of the ABTS radical scavenging activity assay, the hydroalcoholic extract of flower-bearing branches belonging to *C. monogyna* showed the highest antioxidant activity (457.27 ± 38.03 mg TE/g) statistically among all the extracts, which may be attributed to the high content of chlorogenic acid and epicatechin compounds in the flower-bearing branches of the plant. Among the flower-bearing branches, leaves and fruits and of *C. turcicus*, the hydroalcoholic extract of flower-bearing branches (380.780 ± 20.11 mg TE/g) exhibited the highest antioxidant activity. The hydroalcoholic extract of *C. turcicus* leaves (365.47 ± 25.53 mg TE/g) was found to have statistically higher antioxidant activity compared to the hydroalcoholic extracts of *C. orientalis* leaves (334.43 ± 15.68 mg TE/g) and *C. monogyna* leaves (289.05 ± 10.26 mg TE/g). Furthermore, the fruit extract of *C. turcicus* (130.78 ± 4.63 mg TE/g) as found to have a higher antioxidant activity than the fruit extract of *C. orientalis* (119.45 ± 11.95 mg TE/g).

Froehlicher et al. (165) conducted a study on ABTS radical scavenging activity of the fruit and flower extracts of the *C. monogyna.* According to the results they obtained, the flower extract (26.03 ± 0.3 mg TE/g extract) showed highest antioxidant activity compared to the fruit extract (12.5 ± 0.7 mg TE/g extract). Similarly, in the current study, the hydroalcoholic extract of the *C. monogyna* flower-beaing branches (457.27 ± 38.03 mg TE/g) exhibited a higher antioxidant effect than the fruit extract (215.97 ± 24.11 mg TE/g) based on the results of the ABTS radical scavenging activity assay. In a study conducted by Rocchetti et al. (180), it was determined that the methanolic extract obtained from the leaves of the *C. orientalis* using three different extraction methods has the highest antioxidant effect according to the results of the ABTS radical scavenging activity assay (130.77 ± 0.25 mg TE/g extract). In the current study, the hydroalcoholic extract of the *C. orientalis* leaves exhibited an antioxidant effect 2.5 times higher than the previous study based on the results of ABTS radical scavenging activity assay (334.43 ± 15.68 mg TE/g). Martín-Garcia et al. (181) conducted a study in which they examined ABTS radical scavenging activity of 15 extracts derived from *C. monogyna* leaves, with the acetone-water ratio being varied. The observed values fell within the range of 37.61 mg TE/g extract to 102.43 mg TE/g extract. However, in the present study, the ABTS radical scavenging activity of hydroalcoholic extract of *C. monogyna* leaves surpassed these values (289.05 ± 10.26 mg TE/g). This variation could be attributed to variances in the plant's geographical origins and discrepancies in the extraction process (181). Özyürek et al. (169) performed ABTS radical scavenging activity study using the methanolic extracts of flowers and leaves from *C. monogyna, C. orientalis* and *C. pentagyna*. According to their findings, the ABTS radical scavenging activity values were determined to be between 16.51 ± 10.010 mg TE/g extract and 97.61 ± 0.08 mg TE/g extract for *C. monogyna* flower extracts, between 8.760 ± 0.004 mg TE/g extract and 82.59 ± 0.052 mg TE/g extract for *C. monogyna* leaves extracts, between 14.26 ± 0.016 mg TE/g extract and 31.03 ± 0.016 mg TE/g extract for *C. orientalis* flower extracts, between 17.27 ± 0.004 mg TE/g extract and 23.02 ± 0.020 mg TE/g extract for *C. orientalis* leaves extracts, between 15.51 ± 0.007 mg TE/g extract and 33.78 ± 0.013 mg TE/g extract for *C. pentagyna* flower extracts, between 41.54 ± 0.0024 mg TE/g extract and 105.87 ± 0.112 mg TE/g extract for *C. pentagyna* leaves extracts. In the current study, the ABTS radical scavenging activity values for hydroalcoholic extracts were found to be 457.27 ± 38.03 mg TE/g for *C. monogyna* flower-bearing branches, 289.05 ± 10.26 mg TE/g for *C. monogyna* leaves, 400.34 ± 14.59 mg TE/g for *C. orientalis* flower-bearing branches,

334.43 ± 15.68 mg TE/g for *C. orientalis* leaves, 442.72 ± 32.10 mg TE/g for *C. pentagyna* flower-bearing branches and 397.07 ± 29.02 mg TE/g for *C. pentagyna* leaves.

According to the results of the FRAP assay, the hydroalcoholic extract of flower-bearing branches belonging to *C. monogyna* showed the highest antioxidant activity (371.52 ± 9.04 mg TE/g). Among the hydroalcoholic extracts of leaves, *C. turcicus* (226.50 ± 8.66 mg TE/g) showed the highest antioxidant activity, and similarly, among the hydroalcoholic extracts of flower-bearing branches, *C. turcicus* (304.48 ± 6.89 mg TE/g) again displayed the highest antioxidant activity. It was determined that the fruit extract of *C. turcicus* (105.51 ± 6.3 mg TE/g) had a statistically higher antioxidant effect than the fruit extract of *C. orientalis* (66.52 ± 3.28 mg TE/g).

Martín-Garcia et al. (181) conducted research where they investigated the FRAP metal-reducing acitivty of 15 extracts obtained from *C. monogyna* leaves, altering the acetone-water ratio. The measured values were found to range from 49.62 mg TE/g extract to 134.68 mg TE/g extract. However, in the current study, the FRAP metal-reducing activity of the hydroalcoholic extract of *C. monogyna* leaves exceeded these values (162.66 ± 3.33 mg TE/g). Bardakçı et al. (89) investigated the FRAP metal-reducing activity of the fruit extracts from *C. turcicus*, *C. monogyna* and *C. orientalis*. In their findings, the *C. monogyna* (2.83 ± 0.04 Mm $FeSO_4$ equivalents/g extract) exhibited the highest antioxidant activity, followed by *C. turcicus* (0.86 ± 0.16 Mm $FeSO_4$ equivalents/g extract) and *C. orientalis* (0.73 ± 0.01 Mm $FeSO_4$ equivalents/g extract), respectively. In this study, similar to the previous research, when examining the FRAP metal-reducing activity of *C. turcicus, C. monogyna* and *C. orientalis* fruit extracts, the highest antioxidant effect was observed in the *C. monogyna* (128.81 ± 11.67 mg TE/g), followed by *C. turcicus* (105.51 ± 6.3 mg TE/g) and then *C. orientalis* (66.52 ± 3.28 mg TE/g). Özyürek et al. (169) carried out FRAP metal-reducing activity study, using the methanolic extracts obtained from flowers and leaves from *C. monogyna, C. orientalis* and *C. pentagyna*. Based on their findings, the FRAP metal reducing activity values were determined to range from 4.00 ± 0.001 mg TE/g extract to 37.29 ± 0.003 mg TE/g extract for *C. monogyna* flower extracts, from 16.51 ± 0.00 mg TE/g extract to 35.29 ± 0.011 mg TE/g extract for extracts of *C. monogyna* leaves, from 7.00 ± 19.77 mg TE/g extract to 19.77 ± 0.012 mg TE/g extract for *C. orientalis* flower extracts, from 9.51 ± 0.001 mg TE/g extract to 10.51 ± 0.001 mg TE/g extract for extracts of *C. orientalis* leaves, from 9.51 ± 0.002 mg TE/g extract to 20.52 ± 0.001 mg TE/g extract for *C. pentagyna* flower

extracts, from 23.27±0.003 mg TE/g extract to 33.03 ± 0.001 mg TE/g extract for extract of *C. pentagyna* leaves. In the present study, FRAP metal reducing activity values of hydroalcoholic extracts were found to be 371.52 ± 9.04 mg TE/g extract for *C. monogyna* flower-bearing branches, 162.66 ± 3.33 mg TE/g for *C. monogyna* leaves, 271.72±5.84 mg TE/g for *C. orientalis* flower-bearing branches, 198.72 ± 7.68 mg TE/g for *C. orientalis* leaves, 272.860 ± 9.44 mg TE/g for *C. pentagyna* flower-bearing branches and 217.48 ± 11.91 mg TE/g for *C. pentagyna* leaves.

In the antioxidant activity experiments, looking at the results of the CUPRAC assay, similar to the ABTS and FRAP assays, the extract of *C. monogyna* flower-bearing branches showed the highest antioxidant activity statistically (852.09 ± 21.16 mg TE/g). Among the extracts of leaves, it was determined *C. turcicus* exhibited highest antioxidant activity (692.83 ± 39.22 mg TE/g). Additionally, it was found that the fruit extract of *C. turcicus* (277.03 ± 20.28 mg TE/g) has a higher antioxidant activity compared to the fruit extracts of *C. orientalis* (222.13 ± 11.82 mg TE/g) and *C. pentagyna* (180.87 ± 0.61 mg TE/g).

Bardakçı et al. (89) performed the CUPRAC assay on the fruit extracts of *C. turcicus, C. monogyna* and *C. orientalis*. In their findings, *C. monogyna* (560.08 ± 0.10 mg ascorbic acid equivalents/g extract) demonstrated the highest antioxidant activity followed by *C. turcicus* (254.72 ± 3.43 mg ascorbic acid equivalents/g extract) and *C. orientalis* (190.37 ± 8.99 mg ascorbic acid equivalents/g extract), respectively. According to the CUPRAC assay results of the current study, consistent with the previous research, *C. monogyna* fruits exhibited the highest antioxidant activity (359.33 ± 11.57 mg TE/g), followed by *C. turcicus* fruits (277.03 ± 20.28 mg TE/g) and *C. orientalis* fruits (222.13 ± 11.82 mg TE/g), respectively. In their research, Özyürek et al. (169) conducted a study on the CUPRAC assay by utilizing methanolic extracts derived from flowers and leaves from *C. monogyna, C. orientalis* and *C. pentagyna*. Their findings revealed that CUPRAC assay results varied across different extracts. *C. monogyna* flower extracts exhibited values ranging from 10.01 ± 0.005 mg TE/g extract to 93.6 ± 0.004 mg TE/g extract, extracts of *C. monogyna* leaves showed 31.78 ± 0.011 mg TE/g extract and 91.85 ± 0.004 mg TE/g extract, *C. orientalis* flower extracts displayed values between 19.77 ± 0.009 mg TE/g extract and 50.80±0.065 mg TE/g extract, extracts of *C. orientalis* leaves ranged from 15.26 ± 0.002 mg TE/g extract to 18.27 ± 0.07 mg TE/g extract, *C. pentagyna* flower extracts displayed values between 25.77 ± 0.002 mg TE/g extract and 55.56 ± 0.007 mg

TE/g extract and extracts of *C. pentagyna* leaves demonstrated values between 47.80 ± 0.007 mg TE/g extract and 94.60 ± 0.004 mg TE/g extract. In the current study, CUPRAC assay results of hydroalcoholic extracts were similarly observed to be 852.09 ± 21.16 mg TE/g for *C. monogyna* flower-bearing branches, 568.10 ± 10.41 mg TE/g for *C. monogyna* leaves, 779.56 ± 41.05 mg TE/g for *C. orientalis* flower-bearing branches, 596.73 ± 32.51 mg TE/g for *C. orientalis* leaves, 847.94 ± 30.557 mg TE/g for *C. pentagyna* flower-bearing branches and 553.50 ± 28.91 mg TE/g for *C. pentagyna* leaves.

In this study, the anti-inflammatory effect of *Crataegus* species was determined by examining the levels of nitric oxide (NO). According to the results obtained, all the samples exhibited anti-inflammatory effects. Among all the plant extracts studied, the hydroalcoholic extract of *C. pentagyna* leaves showed significantly higher inhibition of NO in LPS-stimulated RAW 264.7 cells compared to the other extracts. This effect may be attributed to the high content of orientin, as detected by the HPLC analysis, in the *C. pentagyna* leaves. Overall, when examining the flower-bearing branches, leaves and fruits, it was determined that the hydroalcoholic extracts of leaves and flower-bearing branches has higher anti-inflammatory effects compared to the fruit extracts. The hydroalcoholic extract of leaves belonging to *C. turcicus* was found to have a higher anti-inflammatory effect compared to the hydroalcoholic extract of *C. pentagyna* leaves. Additionally, it was found that the flower-bearing branches of *C. turcicus* exhibited higher anti-inflammatory effect compared to the flower-bearing branches of *C.monogyna* and *C. pentagyna*. Furthermore, the fruit extract of *C. turcicus* showed higher anti-inflammatory effect compared to the fruit extracts of *C. pentagyna* and *C. orientalis.* In a study, the anti-inflammatory effect of the *C. orientalis* was examined. According to the results of the study, it was observed that *C. orientalis* ethanol extract has a dose-dependent anti-inflammatory activity on carrageenan-induced paw edema (182). In another study conducted by Šavikin et al. (61), the anti-inflammatory activity of leaves and fruit hydroalcoholic extracts of *C. orientalis* was examined. According to the results, both extracts exhibited anti-inflammatory activity. Furthermore, it was found that the extract obtained from leaves had a higher anti-inflammatory activity compared to the extract obtained from fruits.

To evaluate the analgesic activity, the inhibition level of prostaglandin E_2 (PGE_2) released from LPS-stimulated RAW 264.7 cells was determined for the extracts. According to the results, fruit extracts were found to cause greater decrease in PGE_2 levels

compared to extracts obtained from leaves and flower-bearing branches. Among all samples, the fruit extract *C. turcicus* showed the highest reduction in PGE_2 levels. Additionally, the leaves extract of *C. turcicus* exhibited a higher inhibition of PGE_2 compared to the leaves extracts of *C. monogyna* and *C. pentagyna.* According to the results of the IL-6 releasing inhibition assay, which examined the anti-inflammatory activity, the fruit extract of *C. turcicus* showed a higher inhibition of IL-6 releasing compared to the fruit extracts of *C. orientalis* and *C. pentagyna.* Additionally, it was found that *C. turcicus* exhibited higher IL-6 releasing inhibition among the hydroalcoholic extracts of leaves as well. Lastly, the decrease in TNF-α levels were analyzed and it was observed that all extracts decreased TNF-α levels. Additionally, the hydroalcoholic extract of *C. turcicus* leaves showed a significant anti-inflammatory activity by inhibiting TNF-α release by 93%.

6. CONCLUSION

C. turcicus was comparatively evaluated with other *Crataegus* species (*C. monogyna, C. orientalis, C. pentagyna*) in terms of chemical composition and bioactivity profiles. As a result, the phenolic profile, antioxidant capacity and anti-inflammatory activity of *C. turcicus* did not exhibit notable discrepancies compared to the other investigated *Crataegus* species, yielding similar outcomes. The study revealed that each extract exhibited anti-inflammatory effects, inhibiting the secretion of IL-6, TNF-α and NO. However, the analgesic activity effect of the extracts was found to be less potent than indomethacin, as observed in the PGE_2 assay. In this study, all *Cratageus* species, including *C. turcicus, C. monogyna, C. orientalis* and *C. pentagyna*, demonstrated antioxidant effects in their extracts of flower-bearing branches, leaves and fruits. Additionally, it is noteworthy that the extracts of flower-bearing branches and leaves belonging to these *Cratageus* species had higher phenolic content and stronger antioxidant activity compared to their fruit extracts. However, such a difference was not observed in the anti-inflammatory experiments. Interestingly, the fruit extracts exhibited higher analgesic effects than the extracts of flower-bearing branches and leaves and showed almost similar analgesic effect to that of indomethacin.

Moreover, this research provides valuable insights into the potential therapeutic applications of *C. turcicus* and other investigated *Crataegus* species. The comparable phenolic profiles and bioactivities among these *Cratageus* species suggest that they could be utilized interchangeably in traditional medicine and formulations. Furthermore, the antioxidant and anti-inflammatory properties observed in the flower-bearing branches, leaves and fruits highlight their potential in combating oxidative stress-related diseases and inflammatory conditions. The findings of this research contribute to the growing body of knowledge on the chemical composition and biological activities of *C. turcicus, C. monogyna, C. orientalis* and *C. pentagyna*, emphasizing their significance as a natural source of bioactive compounds. Future studies could delve deeper into the underlying mechanisms of action and explore the synergistic effects of combining different *Crataegus* species to maximize their therapeutic potential.

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8. CURRICULUM VITAE

Kişisel Bilgiler

Adı	Tansu	**Soyadı**	Turnalar Ülger
Doğum Yeri	İstanbul	**Doğum Tarihi**	[redacted]
Uyruğu		**TC Kimlik No**	
E-mail		**Tel**	

Öğrenim Durumu

Derece	Alan	Mezun Olduğu Kurumun Adı	Mezuniyet Yılı
Doktora	Farmakognozi	**Yeditepe Üniversitesi**	2023
Lisans	Eczacılık Fakültesi	**Yeditepe Üniversitesi**	2015
Lise	-	**İstek Özel Uluğbey Fen Lisesi**	2010

Bildiği Yabancı Dilleri	Yabancı Dil Sınav Notu (#)
İngilizce	YDS:

Başarılmış birden fazla sınav varsa(KPDS, ÜDS, TOEFL; EELTS vs), tüm sonuçlar yazılmalıdır

İş Deneyimi (Sondan geçmişe doğru sıralayın)

Görevi	Kurum	Süre (Yıl - Yıl)
Eczacı	Nuray Eczanesi	2015-

Bilgisayar Bilgisi

Program	Kullanma becerisi
Microssoft Office	Çok iyi
GraphPad Prism	Çok iyi
Minitab	Çok iyi
EndNote	Çok iyi
ChemDraw	Çok iyi

*Çok iyi, iyi, orta, zayıf olarak değerlendirin

Bilimsel Çalışmaları

SCI, SSCI, AHCI indekslerine giren dergilerde yayınlanan makaleler

Turnalar Ülger T, Oçkun MA, Güzelmeriç E et al. Comprehensive Analysis of the Chemical and Bioactivity Profiles of Endemic *Crataegus turcicus* Dönmez in Comparison with Other *Crataegus* Species. *Molecules.* 2023, 28, 6520.

Diğer dergilerde yayınlanan makaleler

Oçkun MA, Turnalar Ülger T, Güzelmeriç E, Kan Y, Yeşilada E. Crataegus turcicus (Türk alıcı) meyvelerinin Avrupa Farmakopesi'ne göre uygunluğunun değerlendirilmesi. *Türk Farmakope Dergisi*. 2020, 5(2), 14-24.
Turnalar T, Terlemez H, Us Yılmaz D, Sipahi H, Kadıoglu B, Deniz I et al. Synthesis and Pharmacological Activities of Some Novel N,N′-Disubstituted Urea Derivatives. *International Journal of Research In Pharmacy and Chemistry.* 2016, 6(2), 363-371.

Uluslararası bilimsel toplantılarda sunulan ve bildiri kitabında (*Proceedings*) basılan bildiriler

Hakemli konferans/sempozyumların bildiri kitaplarında yer alan yayınlar

Diğer (Görev Aldığı Projeler/Sertifikaları/Ödülleri)
