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NEAR EAST UNIVERSITY
HEALTH SCIENCES INSTITUTE

INVESTIGATION ON THE POSSIBILITY OF BECOMING A HERBAL MEDICINE OF *CRATAEGUS PALLASII* GRISEB. FROM LIBYA AND *CRATAEGUS AZAROLUS* L. FROM NORTHERN CYPRUS

NAJAT ABUBAKER A. AGIEL

DOCTORAL THESIS

FACULTY OF PHARMACY
DEPARTMENT OF PHARMACOGNOSY

SUPERVISOR
PROF. DR. FİLİZ MERİÇLİ

CO-ADVISOR
PROF. DR.ALİ HİKMET MERİÇLİ

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NICOSIA

DEDICATION

To my mother, sister and family

STATUTORY DECLARATION

Hereby I declare that this thesis study is my own study, I had no unethical behaviour in all stages from planning of the thesis until writing thereof, I obtained all the information in this thesis in academic and ethical rules, I provided reference to all of the information and comments which could not be obtained by this thesis study and took these references into the reference list and had no behaviour of against patent rights and copyright infringement during the study and writing of this thesis.

Najat Abubaker A. Agiel

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ABBREVIATIONS

ACN: Acetonitrile

ANOVA: Analysis of variance

CaC: *Crataegus azarolus* from Cyprus

CaCd: *Crataegus azarolus* dry sample from Cyprus

CaCf: *Crataegus azarolus* fresh sample from Cyprus

CNS: Central Nervous System

CpE: *Crataegus pallasii* from El-Merj in Libya

CpT: *Crataegus pallasii* from Tripoli (Attar) in Libya

CpEd: *Crataegus pallasii* dry sample from El-Merj in Libya

CRC: Colorectal cancer

DLLME: Disperative Liquid Liquid Micro Extraction

EtOAc: Ethyl acetate

FBS: Fetal Bovine Serum

GC-FID: Gas Chromatography - Flame Ionization Detector

GC-MS: Gas Chromatography–Mass Spectrometry

HCT-116: Human colorectal carcinoma cell line

HeLa: Human cervix carcinoma cell line

Hep-2: Human epithelial type 2 (human laryngeal carcinoma)

HEP-G2: Hepatocellular carcinoma cells

Hep3B: Hepatoblastoma cell line

IDLLME: Indirect Dispersive Liquid-Liquid Microextraction

IC_{50}: The half maximal inhibitory concentration

IMR-32: Neuroblastoma cell line

LRI: Linear Retention Index

MCF-7: Michigan Cancer Foundation-7 (Breast cancer cells)

MeOH: Methanol

MTT: (3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyl-tetrazolium bromide)

NCI-H460: Human non small cell lung carcinoma

OPC: Oligomer Procyandines

RP-HPLC-DAD: Reverse Phase-High Performance Liquid Chromatography-Diode Array Detector

RPM: Revolutions per minute

RPMI: Roswell Park Memorial Institute Medium

SF-295: Human glioblastoma multiforme cells

SKOV-3: Human ovary cancer cell line

SV: Saphenous Vein

SW480: Human colon adenocarcinoma cell line

SUMMARY

Name of the student: NAJAT ABUBALKER A. AGIEL

Mentor: PROF. DR. FİLİZ MERİÇLİ
Co-advisor: PROF. DR.ALİ HİKMET MERİÇLİ
Department: PHARMACOGNOSY

Aim: Phytochemical screening of *Crataegus pallasii* and *Crataegus azarolus* for important bioactive metabolites and their evaluation as raw material for the production of herbal drugs.

Material and Method: Phytochemical screening tests were carried out to determine important groups of metabolites. Evaluation of the volatile oil components was carried out by GC-MS and GC-FID. RP-HPLC-DAD analysis was performed for identification, separation, and quantitation of bioactive flavonoids in the ethyl acetate extract and the results were compared with Crataegutt® Trophen. The relaxant effect of total extracts was tested on isolated segments of human saphenous vein (SV). Anticancer activity was carried out against Breast cancer cell line, MCF-7.

Results and Discussion: This research displayed for the first time the phytochemical constituents and the biological activity of two un-investigated species of *Crataegus* genus; *C.pallasii* and *C.azarolus*. The preliminary phytochemical screening tests revealed the presence of flavonoids, saponins, and tannins. The essential oil investigations revealed fifty-four compounds major groups of which were the alkane series mainly tricosane, pentacosane, heptacosane, tetracosane, docosane, and eicosane. Carvacryl acetate and carvone are reported for the first time from *Crataegus* species in *C. pallasii* essential oil. HPLC-DAD analysis of the EtOAc showed that the percentage concentration of vitexin 2''-*O*-rhamnoside, rutin, vitexin, and hyperoside were in accordance with that stated by the pharmacopeias. A novel approach indirect-dispersive liquid-liquid microextraction (IDLLME) technique was carried out to reduce the matrix effect prior to HPLC analysis. The total ethanolic extracts showed a negative relaxant effect on the human saphenous vein. The cell viability test on breast cancer cell line, MCF-7 showed that *C. pallasii,* extract was only slightly effective.

Keywords: *Crataegus pallasii*, *Crataegus azarolus,* Crataegutt®Trophen, phytochemical screening, herbal drug

ÖZET

Öğrenci adı: NAJAT ABUBALKER A. AGIEL
Danışman: PROF. DR. FİLİZ MERİÇLİ
Eş Danışman: PROF. DR.ALİ HİKMET MERİÇLİ
Bölüm: FARMAKOGNOZİ

Amaç : Bu çalışmada üzerinde herhangi bir araştırma bulunmayan Libya'da yetişen *Crataegus pallasii* ile Kuzey Kıbrıs'da yetişen *Crataegus azarolus* bitkilerinin çiçek yaprak örneklerinin önemli biyoaktif fitokimyasallarının belirlenmesi ve önemli bir fitofarmasötik ile karşılaştırılarak bitkisel ilaçların üretiminde hammadde olarak kullanılabilirliğinin tespiti amaçlanmıştır.

Gereç ve Yöntem : Önemli metabolit gruplarını belirlemek için fitokimyasal tarama testleri yapılmıştır. Uçucu yağ bileşenleri GC-MS ve GC-FID yöntemleri kullanılarak belirlenmiştir. Etil asetat ekstraktındaki biyoaktif flavonoidlerin tanımlanması, ayrılması ve kantitasyonu RP-HPLC-DAD yöntemi ile analiz edilerek, sonuçları önemli bir bitkisel ilaç olan Crataegutt® Trophen ile karşılaştırılmıştır. Toplam extrelerin gevşetici etkisi, insan safen veninin (SV) izole edilmiş bölümleri üzerinde test edilmiştir. Antikanser aktivitesi ise MCF-7 meme kanseri hücre hattlarında incelenmiştir.

Sonuç ve Tartışma: Bu çalışma Libya'da yetişen *Crataegus pallasii* ile Kuzey Kıbrıs'da yetişen Crataegus *azarolus* bitkilerinin çiçek yaprak örneklerinin önemli biyoaktif fitokimyasalları ve biyolojik aktiviteleri ile ilgili ilk araştırmadır. Ön fitokimyasal tarama testleri ile flavonoidler, saponinler ve tanenlerin varlığı belirlenmiştir. Uçucu yağın bileşiminde başlıca alkan serileri olan elli dört bileşik saptanmıştır. C. pallasii uçucu yağında varlığı saptanan Carvacryl asetat ve carvone *Crataegus* türlerinde ilk kez tespit edilmiştir. EtOAc ekstresinin HPLC analizinde, vitexin 2 "- O-ramnoside, rutin, vitexin ve hyperosid konsantrasyonunun farmakopelerde belirtilenlere uygun olduğu saptanmıştır. HPLC-DAD analizinden önce matris etkisini azaltmak için dolaylı-dispersif sıvı-sıvı mikroekstraksiyon (IDLLME) tekniği uygulanarak geliştirilmiştir. Toplam etanol ekstresinin, insan safen damarı üzerinde kayde değer bir gevşetici etki görülmemiştir. MCF-7 meme kanseri hücre hattı üzerindeki hücre canlılığı testi de, *C. pallasii* ekstresinin biraz daha etkili olduğunu göstermiştir.

Anahtar Kelimeler: *Crataegus pallasii, Crataegus azarolus*, Crataegutt®Trophen, fitokimyasal tarama, bitkisel ilaç.

CHAPTER 1

1. INTRODUCTION

Nowadays, cardiovascular diseases are considered a serious health problem with a high mortality rate reaching up to 17.1 million deaths yearly recorded worldwide according to World Health Organization (WHO). The most serious and prevalent of such diseases is arterial hypertension which is also associated with other conditions such as myocardial infarction and stroke. *Crataegus* (hawthorn) is one of the most outstanding medicinal plant in phytotherapy and has been recently a topic of concern in treatment of diseases related to the cardiovascular system. However, ethnobotanical knowledge and scientific research support the effectiveness of *Crataegus* species in the treatment of different ailments, most importantly being in the cure of circulatory problems. Several pharmacologically active metabolites have been reported to be present in *Crataegus* extracts of which the most important are flavonoids and oligomeric procyanidins (OPC) which are responsible for its pharmacological activity.

Since the interest in herbal medicine has been growing worldwide which has raised the international trade of herbal medicine and attracted pharmaceutical companies in commercializing herbal drugs. Some countries are producing drugs from *Crataegus* species growing on their land. Today, a number of herbal medicinal preparations of *Crataegus* are manufactured by world-leading companies in the field of herbal medicines including teas, tablets, syrups, tinctures, and oral drops and may be available as authorised prescription drugs, over-the-counter (OTC) medications, authorized herbal medicinal products, dietary supplements, or unregulated herbal remedies which are sold in pharmacies. Whereas, some *Crataegus* species are mentioned in the Pharmacopoeias and licensed for the production of herbal drugs, many others are under investigation.

1.1. The Genus *Crataegus*

Crataegus species Rosaceace, more commonly known as Hawthorn worldwide, 'alıç', 'yemişen' or 'haziran' in Cyprus and as 'Zaarour' in the middle east countries, is a diverse genus of flowering, fruit-bearing shrubs or small trees that grow mostly in temperate zones, including countries of North Africa, European and the Mediterranean, Western Asia, India, China and North America (Kumar et al.,2012). The genus *Crataegus* is estimated to include from 150 to 1200 species (Yanar et al., 2011;Phipps et al., 2003). In Libya, *Crataegus genus* is represented in 3 species *C. mexicana, C. laevigata, C. pallasii* (Jafri and EL-Gadi,1977), and 2 species in Cyprus *C. azarolus* and *C. monogyna* (Viney, 1994).

1.1.1. General botanical description

Crataegus is usually multi-branched ranging from shrubs to small trees, usually armed with thorns that are gray dark, and sharp which grows along the branches. Normal size trees can reach a height of up to 10 m. However, average hawthorn trees have a height between 2-5 m. The Hawthorn trees prefer the forest margins of lower and warmer areas (Yanar et al., 2011; Edwards et al., 2012).

Leaves of the most species of *Crataegus* have two leafy bracts, their stalk meets the twig. Leaves are bright to dark green color, 15 mm-5 cm long, glabrous, broad-ovate, or obovate, and have to tooth margins with three to seven lobes. The flowers grow in clusters of 5-12 with color ranges from white to pink, pink to red. They contain both male and female sexes and are mostly fertilized by insects. Fruits of berry type are greenish when first appear, gradually turning bright yellow or red then deep red, containing one or two stony seeds (Davis, 1972).

1.1.2. Ethnobotanical uses of *Crataegus* species

Crataegus species (Hawthorn) have been used traditionally since ancient times. Preparations from leaves and flowers of certain species of *Crataegus* have been mentioned in medical literature since the 1st century A.D. The first report of patients treated with *C. oxycantha* who were suffering from various heart illness was in 1896

(Holubarsch et al., 2017). Jütte and his colleagues in 2017 (Jütte et al., 2017) described that *Crataegus* traditional use was traced back to Dioscorides (c. 40-90 CE) with a continuous usage through the middle ages and until early modern times for compliments of the heart in addition to other diseases such as gastrointestinal disorders. In North America, its use to treat heart problems dates back to 1800. Until dated, *Crataegus* is accepted to have active ingredients and its extracts to be biologically active (Byeon et al., 2018). As *Crataegus* is widely distributed throughout the planet, numerous reports record the use of hawthorn in different cultures (Table 1.1) in European, American, African and Asian societies, especially in traditional Chinese medicine (TCM) (Kumar et al., 2012).

Hawthorn is also known for relieving stagnated food, by its action on the stomach, and has a strengthening effect on the spleen, and act on the liver channel and blood for promoting blood circulation and removing blood stasis. Fruits are an excellent source of antioxidants and are used for their astringent properties in heavy menstrual bleeding and diarrhea. Both the flowers and the berries act as diuretics and are used to treat kidney problems (Qader et al., 2017).

Hawthorn medicinal extract has long been a favorite herbal remedy in Europe (Dahmer and scott, 2010). In the Arabian traditional medicine system a decoction of leaves and unripe fruits from *Crataegus aronia* is used to treat cardiovascular diseases, cancer, diabetes, and sexual weakness (Kumar et al., 2012). According to the review done by Jie et al., 2013 and his colleagues *C. monogyna* and *C. laevigata* are the major hawthorn species in middle Europe, *C. pentagyna*, *C. nigra*, and *C. azarolus* in Southern Eastern Europe, and *C. pinnatifida* and *C. scabrifolia* in China.

Table 1.1: Summarizes some of the traditional use of *Crataegus* in different parts of the world.

Continent	Species & origin/ part used	usage	Reference
Africa	In the Middle East *C. oxyacantha* and *C. aronica.* In Egypt *C. sinaica* In Algeria and Tunisia *C.azarolus* and *C.azarolus* var. *eu-azarolus, C. azarolus* var. *aronia, C. oxyacanthus* ssp. *monogyna* In Morocco *C. laciniata.*	Hypotensive, cough, and insomnia. Hypertension Cardiovascular ailments, diabetes, renal problems, and laxative	(Nerman et al., 2009; Cherifa and Zidi, 2011; Amel, 2015) (El Hilaly et al., 2003; Bahri et al., 2009)

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America	*C.monogyna*, *C.leaviqata*, *C.douqlasii* and *C. okanaqanenis* (North American hawthorn) Extracts of *C. phaenopyrum* and *C. pubescens* (Mexican hawthorn)	For cardiovascular diseases and diabetes (early stages)	(Kumar et al., 2012; Lund et al., 2017)
Asia	Fruits of *C. pinnatifida* and *C. oxyacantha* (Chinese, Taiwan hawthorn) and *C. cuneata* (Japanese hawthorn)	For cardiovascular diseases, to lower plasma lipids, and for digestive problems, dyspnea, insomnia, asthma, and diabetes.	(Caliskan et al., 2016;Wang et al., 2013; Bahmani et al., 2014)
	C. azarolus subsp *aronia* and *C.azarolus* (Lebanese *Crataegus*) *C. pontica* (Persian Hawthorn) *C. ambigua* (Russian hawthorn)	Hypertension, heart tonic, antihypertensive, and headache	(Kumar et al., 2012; Ghasemi et al., 2013; Preedy and Watson, 2014; Kallassy et al., 2017; Reza et al., 2017)
Europe	Fruits, leaves, flowers of *C. laeviqata*, *C. monogyna*, *C.oxyacantha*, *C. pentagyna*, *C.azarolus*, *C.orientalis*, and *C.rhipidophylla*	Treatment of heart problems; cardiotonic, hypotensive, and antiatherosclerotic effects.	(Caliskan et al., 2012; Caliskan et al., 2016; Rastogi et al., 2016)

1.1.3. Phytochemical studies on *Crataegus* species

Different classes of active compounds are found in *Crataegus* species which have been used in therapy as cardioactive drugs (Bahri-Sahloul et al., 2009). A diverse chemical profile of chemical constituents in the leaves, unripe fruits, and flowers in *Crataegus* include flavonoids, sugars, sugar alcohols, organic and phenolic acids, terpenes, essential oils (including mixtures of monoterpenes, sesquiterpenes, and alkenes), and phenylpropanoids (including hydroxycinnamic acids, and lignans) (Lund et al., 2017).

1.1.3.1. Flavonoid compounds in *Crataegus* species

Bioflavonoid-like complexes that appear to be primarily responsible for the cardiac actions of the plant include oligomeric procyanidins (OPC), and flavonoids as vitexin, quercetin, and hyperoside. The action of these compounds on the cardiovascular system has led to the development of leaf and flower extracts, which are widely used in Europe as herbal pharmaceutical products. Other chemical constituents include vitamin C, saponins, tannins, cardiotonic amines (phenylethylamine, tyramine, isobutylamine, *O*-methoxy phenylethylamine, choline, and acetylcholine), purine derivatives (adenosine, adenine, guanine, caffeic acid, and amygdalin), triterpene acids, ursolic acid were also determined. These compounds

have been thoroughly investigated for their pharmacological activity (Wang et al., 2013;Kumar et al., 2012). In Table 1.2 below mentions a number of flavonoids that have been isolated from *Crataegus* species.

Table 1.2: Major bioactive flavonoids from *Crataegus* species

Species	Compound name	Reference
C. armena Pojark	Arbutin, hyperoside, kaempferol, quercitrin, apigenin.	(Manukyan et al., 2019)
C. aronia (L.) DC	Vitexin-2''-*O*-rhamnoside, hyperoside, quercetin, chlorogenic acid, rutin, spiraeoside, isoquercetin (-)-epicatechin.	(Orhan et al., 2007)
C. atrosaguinea Pojark	Rutin, chlorogenic and caffeic acids, hyperoside, vitexin.	(Amanzadeh et al., 2007)
C. brettschneideri C.K. Schneid.	Catechin / epicatechin, isoquercetin, hyperoside, ideain.	(Liu et al., 2011; Liu et al., 2009; Liu, 2019)
C. cuneata Siebold. & Zucc	Vitexin, vitexin-2''-*O*-rhamnoside, vitexin-4''-*O*-glycoside, quercetin, hyperoside, rutin.	(Liu et al., 2005; Gao et al.,1995; Ma et al., 2007)
C. curvisepala Lindm.	Cratenacin, rutin, hyperoside, and vitexin.	(Amanzadeh et al.,2007)
C. davisii Browicz.	Hyperoside, vitexin -2-*O*-rhamnoside, vitexin-4'-*O*-rhamnoside, rutin, quercetin.	(Sözer et al., 2006; Preedy and Watson 2014)
C. gracilior J.B.Phipps	Rutin, quercetin, kaempferol and (+)-catechin.	(Hernández-Pérez et al., 2014)
C. germanica (L.) Kuntze	Verbascoside, quercetin.	(Gao et al., 1995)
C. grayana Eggl.	Hyperoside, (-)-epicatechin, luteolin-C-hexoside, methyl luteolin-C- hexoside, methoxykaempferol-methypentosylhexoside, methoxykaempferol-pentoside, quercetin hexoside acetate.	(Liu et al., 2011)
C.hupehensis Sarg.	Vitexin, hyperoside, rutin.	(Gao et al., 1995)
C.kansuensis E.H. Wilson	Vitexin, hyperoside, rutin, (-)-epicatechin.	(Gao et al.,1995;Yang and Liu, 2012)
C. laevigata (Poir.) DC	Epicatechin-(4 β→8)-epicatechin-(4 β→6)-epicatchin, pentamer of (-)-epicatchin uniteslinked through C-4 β/C-8 bonds, vitexin, vitexin-2''-O-rhamnoside, acetylvitexin-2-O-rhamnoside, isoquercetin, hyperoside, saponaretin, rutin,	(Svedström et al., 2002; Liu et al., 2011)

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	spiraeoside, (R)-and (S)-eriodictyol-7-O-β-D-glucuronide, luteolin-7-O-β-D-glucuronide.	
C. macrocarpa Hegetschw	Eriodictyol-7-gluuronide, luteolin-7-*O*-Glucuronide,vitexin, vitexin-2''-O-rhamnoside, isovitexin, rutin, hyperoside, isoquercitrin, (R)-and (S)-eriodictyol-7-O- β-D-glucuromide, luteolin-7-O- β-D-glucuronide, saponaretin.	(Ringl et al., 2007)
C. maximowiczii C.K.Schneid.	8-methoxykaemferol, vitexin, hyperoside, quercetin, rutin.	(Gao et al.,1995)
C. meyeri Pojark	vitexin, hyperoside, chlorogenic and caffeic acids, rutin.	(Amanzadeh et al., 2007)
C. microphylla C. Koch	Hesperetin, apigenin, vitexin, vitexin-4''-*O*-rhamnoside, quercetin, hyperoside, eriodictoyl, luteolin, rutin.	(Amanzadeh et al., 2007; Melikoglu et al., 2004)
C.monogyna Jacq.	Vitexin, vitexin-2-*O*-rhamnoside, acetylvitexin-2''-O-rhamnoside, vitexin-4''-O-glucoside, hyperoside, rutin, chlorogenic acid, epicatechin, apigenin-6,8-di-*C*-glycosides, saponaretin, saponaretin rhamnoside, quercetin, isoquercetin, spiraeoside,8-methoxykaempferol-3-O-glucoside, orientin, orientin-2''-O-rhamnoside, iso-orientin, iso-orientin-2''-O-rhamnoside, catechin, apigenin-7-O-glucoside.	(Orhan et al., 2007; Bahorun et al., 2003; Prinz et al., 2007; Ringl et al., 2007; Bahri-Sahloul, 2009; Froehlicher et al., 2009; Bernatoniene et al., 2008; Martino et al., 2008; Liu et al., 2005; Bardakci et al., 2019; Alirezalu et al., 2018)
C. orientalis Pall. ex Bieb	Quercetin and vitexin.	(Bardakci et al., 2019)
C. oxyacatha Linn.	Quercetin, hyperoside, rutin, vitexin-4'-rhamnoside, epicatechol, quercetin-3-*O*-β-glucoside, naringenin and epicatechin.	(Verma et al., 2007; Aneta and Oszmianski, 2007; Benabderrahmane et al., 2019)
C. pentaegyna Waldst. & Kit.	Vitexin, vitexin-2''-*O*-rhamnoside, acetylvitexin-2-*O*-rhamnoside, saponaretin, saponaretin rhamnoside, isoquercetin, rutin, 8-methoxykaempferol-3-*O*-glucoside, orientin, orientin-2''-*O*-rhamnoside, iso-orientin, iso-orientin-2''-*O*-rhamnoside, hyperoside.	(Amanzadeh et al., 2007; Ebrahimzadeh and Bahramian, 2009; Prinz et al., 2007)
C. pinnatifida Bunge.	Pinnatifin C, pinnatifin D, oleanolic acid, urosolic acid, pinnatifinoside A, pinnatifinoside B, Pinnatifinoside C, pinnatifinoside D, shanyenoside A(5,4-dimthoxy-biphenyl-4-ol-3-*O*-β-D-glycoside), vitexin, vitexin-2''-*O*-rhamnoside, vitexin-2''-*O*-	(Zhang et al., 2001; Ning et al., 2009; Liu et al., 2011; Gao et al.,1995; Chen et al., 2007; Jurikova et al., 2012; Venskutonis, 2018; Zhang and Xu, 2003; Chu et al., 2019)

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	glucoside, β-glucopyanoside and n-triacontanol, quercetin, isoquercetin, hyperoside, rutin, epicatechin.	
C. pontica C. Koch	Quercetin	(Bardakci et al., 2019)
C. pseudoheterophylla Pojark	Vitexin, vitexin-2-*O*-rhamnoside, hyperoside, rutin.	(Amanzadeh et al., 2007; Orhan et al., 2007; Bernatoniene et al., 2008)
C. rhipidophylla Gand.	(R)-and (S)-eriodictyol-7-*O*-β-D-glucuronide, luteolin-7-*O*-β-D-glucuronide, vitexin, vitexin-2-'-*O*-rhamnoside, hyperoside, qurcetin.	(Prinz et al., 2007; Bardakci et al., 2019)
C. sanguinea Pall.	Vitexin, vitexin 2″-*O*-rhamnoside.hyperoside, rutin, citric, ascorbic, taratric and malic acid, ergosterol 3-*O*-β-D-glucopyranoside, p-coumaric acid 4-*O*-β-D-glucopyranoside, trifolin, quercitrin, sanguineoside (5,7,3′,5′-tetrahydroxyflavanone 7-*O*-β-D-glucopyranoside), oleanolic and caffeic acid.	(Gao et al., 1995; Vladimir et al., 2019)
C. scbrifolia (Franch.)Rehd.	Vitexin, vitexin 2''-*O*-rhamnoside, vitexin-4''-*O*-glucoside, quercetin, epicatechinrutin, isoquercetin, hyperoside.	(Liu et al., 2005; Gao et al.,1995; Liu et al., 2011)
C. sinaica Boiss.	Hyperoside, quercetin, vitexin-2''-*O*-rhamnoside, epicatechin.	(Refaat et al., 2010)
C. tanacetifolia (Poir.)Pers.	Hyperoside.	(Koçyıldız et al., 2006)
C. turcicus Dönmez	Isoorientin, vitexin, and quercetin.	(Bardakci et al., 2019)
C.xmacrocarpa Hegetschw.	Isoquercetin, hyperoside, rutin, (R)-and (S)-eriodictyol-7-O-β-D-glucuronide, luteolin-7-O-β-D-glucuronide.	(Ringl et al., 2007; Yang and Liu, 2012)
C.wilsonii Sarg.	Hyperoside, rutin, chlorogenic acid.	(Yang and Liu, 2012)

1.1.3.2. Volatile oil constituents of *Crataegus* Species

While intensive work has been done on the major bioactive compounds in the chemical profile of *Crataegus* species which is pointed out in Table 1.2 above, little attention has been given to the volatile constituents (Edwards et al., 2012). This could be due to the low concentrations of these metabolites but they could contribute to the synergic effect of the plant and explain some therapeutic effects such as the sedative

effect (Wang et al., 2013). The volatile oils extracted from some *Crataegus* species are mentioned in Table 1.3 below.

From another point of view, the volatile compounds from Hawthorn fruit (*Crataegus* spp.) were also found to act as behavioral attractants for hawthorn-infesting files *Rhagoletis pomonella*. The reported volatiles were; ethyl acetate, 3-methylbutan1-ol, isoamyl acetate, 4,8-dimethyl-1,3(E),7-nonatriene, butyl hexanoate, and dihydro-β-ionone (Nojima et al., 2003).

It is worth to mention here that the seed oil of *Crataegus* species was also investigated to reveal the presence of linoleic acid, oleic acid, oxalic acid, bis(trimethylsilyl) ester, palmitic acid, tetracosamethylcyclododecasiloxane, benzaldehyde, 3-pyridine carboxaldehyde, 4-methoxybenzaldehyde, 4-methoxybenzoic acid methyl ester (Bechkri et al., 2017; Zder et al., 2016)

Table 1.3: Volatile oil from different *Crataegus* species.

Species	Essential oils (%/dry weight)	Name of volatile oil	Reference
C. aestivalis (Walter) Torr. & A. Gray		Hexanal, butyl acetate, (E)-2-hexenal, butyl butyrate, linalool, butyl hexanoate, methyl octanoate, pentyl hexanoate, hexyl hexanoate.	(Horvat et al., 2007)
C. armena Pojark	0.04 ± 0.001	Butyraldehyde hexanol, benzaldehyde, capronaldehyde, (β-myrcene, β-caryophyllene.	(Manukyan et al., 2019)
C. monogyna Jacq.	0.05 - 0.20	Heneicosane, linalool n-hexadecanoic acid, nonadecane, (E,E)-α-farnesene, caryophyllene oxide, and methyl eugenol.	(Kowalski et al., 2018; Zder et al., 2016; Robertson et al., 1993)
C. opaca Hook & Arn.		Hexanal, butyl acetate, (E)-2-hexenal, butyl butyrate, linalool, butyl hexanoate, methyl octanoate, pentyl hexanoate, hexyl hexanoate.	(Horvat et al., 2007)
C. orientalis subsp. *orientalis*		2-Hexenal, 3-hexenol, capronaldehyde, benzaldehyde, butyraldehyde.	(Özderin et al., 2015); Zder et al., 2016)
C. orientalis subsp. *Szovitsii* (Pojarkova) K.I.Chr.		Propyl methyl ketone, butyraldehyde, 2-Hexenal.	(Özderin et al., 2015;Zder et al., 2016)
C. oxyacantha L.	0.45	Eugenol, Longifolenaldehyde, β-Selinene.	(Meddah, 2018)

Continued……..

C. pentagyna subsp. *Pentagyna* Waldst. & Kit. ex Willd		Benzaldehyde, butyraldehyde, (E)2-hexenal.	(Zder et al., 2016)
C. robesoniana Sarg.	0.03	Tricosane, squalene, phthalate, nonadecane, pentacosane, nonacosane.	(Kovaleva et al., 2009)
C. *rufula* Sarg.		Hexanal, butyl acetate, (E)-2-hexenal, butyl butyrate, linalool, butyl hexanoate, methyl octanoate, pentyl hexanoate, hexyl hexanoate.	(Horvat et al., 2007)
C. tanacetifolia Pers.		Benzaldehyde, butyraldehyde, (E)2-hexenal.	(Zder et al., 2016)
C. flabellata (Bosc ex Spach) K. Koch.	0.04	Phthalate, heneicosane, tricosane, pentacosane, nonacosane.	(Kovaleva et al., 2009)
C. jackii Sarg.	0.08	Phthalate, heneicosene-1, tricosane, pentacosane, nonacosane.	(Kovaleva et al., 2009)

1.1.4. Analytical techniques

In the last decade, important progress was registered regarding the extraction, identification, and quantification of bioactive compounds needed for adequate quality control of plant material used as herbal medicines. Thus, a fingerprinting approach has become obligatory for identification and direct analysis process. However, different features could constitute to the overall fingerprint as genetic, (Beccaro et al., 2012) quality (Donno et al., 2010), sensory (Canterino et al., 2010), and morphological (Mellano et al., 2012) features which are used to create a complete fingerprint. Whereas, the power of combining separation techniques with spectroscopic techniques for quantitative and qualitative analysis of compounds in plant extracts and fractions has been demonstrated. Thus, to obtain structural information about the compounds under investigation high-performance liquid chromatography (HPLC) and gas chromatography (GC) are linked to spectroscopic techniques UV-Vis absorbance and mass spectrometry (MS) (Satyajit and Latif 2006).

1.1.4.1. GC-MS and GC-FID analysis

All-natural products namely, volatile oils are highly complex. Therefore, hyphenated methodologies have to be invented for their analysis. The analytical

technique GC which is used for the advancement, development, and quality control of many industries especially drug manufacturing is particularly useful for this purpose. With the requirement of trace compound analysis and development of the capillary column in GC, sensitive detectors were investigated. Flame based detectors such as flame ionization detector (FID) were regarded as more sensitive for carbon-containing compounds. However, FID detectors do not provide structural information about the analyzed molecules and relay primarily on retention indices for peak assignment. The hyphenated technique of coupling GC and MS was to be used. The mass spectra offer structural information based on the interpretation of fragmentations. The fragment ions with relative abundance can be compared with that of the library data or could be integrated with on-line MS databases for reference compounds. However, compounds that are volatile and stable at high temperatures can be easily analyzed by GC (Bartle and Myers, 2002).

Investigations by GC on plant volatiles first started when professor Liberti in 1950 analysed the *Citrus* volatile oil. Then A.T. James and A.I.P. Martin in 1952 first described the gas-liquid chromatography for volatile compounds analysis. In addidtion to *Citrus* volatile oils, rose oil, chamomile, rosemary, many other essential oils have been studied extensively for their constituents (Baser and Buchbauer, 2010). *Crataegus* species, which is a famous medicinal plant due to the presence of active flavonoid compounds has also been investigated for its volatile oil constituents by many researchers as mentioned in section 1.1.3.2. above. Although according to studies the percentage concentration of the volatile constituents in *Crataegus* species is low, the scope of synergistic effect is relatively possible.

1.1.4.2. HPLC analysis

The advanced technique for phytochemical fingerprinting used today is the HPLC coupled to UV-visible diode array detector (DAD) or mass spectrometry (MS) is one of the most reported hyphenated technique for the analysis of medicinal plant extracts. Reverse Phase HPLC (RP-HPLC) using C18 column with gradient elution of mobile phase composition is the method of choice for the identification and quantification of phenolic compounds from plant material (Stalikas, 2007; Kong et al., 2009; Albe Slabi

et al., 2019; Ebrahimi-Najafabadi et al., 2019). HPLC is a powerful analytical technique used for the qualitative analysis of compounds such as phenolics, terpenoids, and alkaloids. The results of the qualitative analysis are interpreted based on the consistency in the retention time of reference standards and the compounds in the analysed sample. While quantitative estimation is based on the calibration graphs generated after reference standards are injected at different concentration levels (Kumar, 2017).

However, sample preparation prior to HPLC analysis an important step to avoid matrix interference and preconcentration of analytes present at low concentrations. dispersive liquid-liquid microextraction (DLLME) is a novel microextraction technique preceded for the first time by Rezaee et al., 2006 is primarily used for this purpose (Maryam et al., 2019; Lee et al., 2019b). In conventional DLLME, the analytes are extracted into a micro-volume of a water-immiscible organic solvent, where they are preconcentrated. The analyte-rich extraction phase is separated from the mixture, evaporated to dryness and the analytes are reconstituted into a water-miscible solvent. The latter is then injected into HPLC for analysis.

The detection of bioactive compounds in hawthorn species with HPLC diode array detection (DAD) has been carried out by many researchers (Pan et al., 2014;Weon et al., 2016; Donno et al., 2017; Khokhlova et al., 2018; Vladimir et al., 2019) using different chromatographic conditions and solvent systems (methanol, ethanol) in the extraction of bioactive phenolic compounds (Benzie and Strain, 1999).

1.1.5. Biological overview of *Crataegus* species

Crataegus species are well known for their medicinal properties and possess immense pharmacological activities. Most important and common is its cardiovascular effects and antitumor activities which are discussed in some detail below. However, accumulating data regarding other biological effects of *Crataegus* is continuously updated

1.1.5.1. Cardiovascular activity

Among the cardioactive medicinal plants that do not contain typical cardiac glycosides, hawthorn has taken a special position for centuries (Refaat et al., 2010). The use of fruits for the treatment of heart ailments dates back to the late 1800s (Donno et al., 2017). Currently, the evidence is accumulating from various *invivo* and *invitro* studies shows that hawthorn extracts exert a wide range of cardiovascular pharmacological properties, including, positive inotropic, antiplatelet aggregation, vasodilation, endothelial protective, protective effect against ischemia/reperfusion injury, antiarrhythmic, lipid-lowering and decrease of arterial blood pressure effects. On the other hand, reviews of placebo-controlled trials have reported both subjective and objective improvements in patients with mild forms of heart failure (NYHA I-III)(Wang et al., 2013). Due to the above mentioned effects, *Crataegus* species has also become a popular herbal medicine in phytotherapy (Caliskan et al., 2016).

Crataegus extracts are reported to stimulate the activation of heart muscle cells, thereby regulating the blood flow in the coronary arteries (Badalica-Petrescu et al., 2014). Studies revealed that oral administration of standardized *Crataegus* extracts induces a significant decrease in mortality after ischemia reperfusion in animals (Veveris et al., 2004). The aqueous extract of the leaves of *C. tanacetifolia* (Koçyıldız et al., 2006) and *C. oxyacantha* (Abdul-Ghani et al., 1987) have shown a significant hypotensive effect. *C. aronia* has been demonstrated to have an anticoagulant effect and to prolong the bleeding time via the inhibition of thromboxane B2 synthesis (Amel, 2015).

Hawthorn (*C. oxyacantha*) extract was also added to drinking water and feed intake of broiler chickens to aid in the prevention of physiological cardiac disorders and pulmonary hypertension which usually leads to mortality and slow growth of the chickens (Ahmadipour et al., 2017;2019).

C. pinnatifida was recommended by Dong and his colleagues in the prevention of atherosclerosis were they demonstrated that flavonoids of *C.pinnatifida* can attenuate the development of atherosclerosis (Dong et al., 2017).

C. gracilior is used in traditional Mexican medicine as a hypotensive agent, has been documented to produce a relaxant effect on the isolated rat aortic rings. This

effect could be claimed to the presence of compounds as euscapic and corosolic acids (Torres-Ortiz et al., 2019).

However, many *Crataegus* species possess immense medicinal applications other than the cardiovascular therapy (section 1.1.5.3. below), but limited evidence is provided on other traditional uses. On the other hand, only a few species have been screened for their biological activities (Dahmer and Scott, 2010).

1.1.5.2. Antitumor activity.

Recently plant-derived products including bioactive compounds have attracted the researchers for their potential antitumor activity. *Several Crataegus* species have been investigated by researchers for its activity against various cancer cells (Table 1.4). The main bioactive compound in *Crataegus* that was suggested to be responsible is vitexin (apigenin-8-C-D-glucopyranosid), a flavonoid that has been isolated from some hawthorn species and has shown cytotoxic activity against a number of human cancers (Yang et al., 2013).

Table 1.4: Anticancer activities of some *Crataegus* species.

Species name	Type of tumor	Intensity of effect	Reference
C. armena Pojark.	HepG2 cells	High cytotoxicity effect	(Manukyan et al., 2019)
C. cuneata Sieb. et. Zucc	Human oral squamous carcinoma and salivary gland tumor cell lines	Slightly reduced at lower concentrations, stimulated the cytotoxic action at higher concentrations	(Satoh et al., 1998)
C. monogyna Jacq.	HEp-2 cells, MCF-7, NCI-H460, HeLa, HepG2.	Stronger in vitro activity	(T Sáenz et al., 1997; Rodrigues et al., 2012)
C. pinnatifida Bunge	Human hepatocellular carcinoma: HepG2 and Hep3B cells, MCF-7, SKOV-3.	Moderate and significant inhibition of growth	(Guo et al., 2019; Wu et al., 2017)
C. sinaica Boiss	HEP-G2, HCT-116, MCF-7.	Significant potency	(Atef et al., 2017)
C. songarica K. Koch	MCF-7, HeLa, HepG2, SF-295, SW480, IMR-32.	Potent anticancer activity	(Ganie et al., 2016)

1.1.5.3. Other important biological activities of *Crataegus* species

In addition to the above mentioned pharmacological effects, *Crataegus* shows a high antioxidant and immunostimulating activity (Li et al., 2009). *Crataegus* has also shown effects related to CNS such as anxiety, mild depression (Hanus et al., 2004) and in the treatment of Alzheimer's disease (Lee et al., 2019a). The anticataract effect of the leaves extracts eye drops of *C. pinnatifida* was evaluated to report a significant effect (Wang et al., 2011). Antimicrobial, anti-inflammatory, and gastroprotective effects were also demonstrated in *C.leavigata*, *C.monogyna*, and *C.oxycantha* (Tadic et al., 2008). Improvement in the mobility of sperms *in vitro* of asthenospermia patients was proved by the root extract of *C.cuneata* (Hu and Xiong, 2006). Lowering of the blood glucose levels was observed in streptozotocin-induced diabetic rats treated with a decoction of the leaves and unripe fruits of *C.aronia* (Ljubuncic et al., 2006). In addition to increasing the coronary artery dilation effect caused by theophylline, caffeine, papaverine, sodium nitrate, adenosine, and epinephrine, hawthorn procyanidins have also been reported to increase barbiturate-induced sleeping times (Upton, 1999). In addition to the above mentioned, *Crataegus* species displays several other biological activities which was reported by Kumar et al., 2012.

1.1.5.4. Biological evaluation methods

Plants are a good source of inspiration for pharmaceutical drugs. The biological activities of phytochemicals are investigated with both total extract and enriched fractions or pure substances isolated from the plant. Recently, to discover new sources and their pharmacological activity, attention has been paid to the biological activities of the plants. For this reason, pharmacological proof of plant extracts is essential (Tlili et al., 2019).

1.1.5.4.1. Effect of plant extracts on Saphenous Vein (SV)

Cardiovascular diseases are the leading cause of death. Most prevalent is arterial hypertension which is also associated with other conditions as myocardial infarction and stroke. Ethnobotany has documented various plant species used to treat this drastic

disease. Researchers studied the vasodilation effect of plant extracts and compounds isolated from them. The findings suggest that compounds derived from plants may have great therapeutic potential as they involve multiple mechanisms of action in their vascular relaxant activity. Related studies have been conducted *invivo* assay as well as *invitro* on isolated tissues. Many research studies for this purpose used rat thoracic aorta rings or isolated segments of human saphenous vein (SV) tissues (Luna-Vázquez et al., 2013). However, since saphenous vein provides multiple graft segments and is the most common bypass graft used and is readily available and obtainable, it is preferably used (Daci et al., 2017; Marinko et al., 2018).

The relaxant effect of *Crataegus* extract and the isolated compounds were studied on albino rat's thoracic aorta (Al-habib et al., 2015; Al-habib and Shekha, 2010; Hernández-Pérez et al., 2014) and on human mammalian coronary bypass artery (Brixius et al., 2006) and isolated guinea pig aorta rings (Vierling et al., 2003). More information on the vascular and cardiac activity properties of *Crataegus* is discussed in more detail in section 1.1.5.1.

1.1.5.4.2. Antitumor activity; MTT assay

Several *invitro* methods have been established to measure pure compounds isolated from plant extracts or total plant extracts against their antitumor activity (Ganot et al., 2013). MTT is the most commonly used and popular, for antitumor evaluation of plant extracts. It is a simple, accurate, and highly reliable colorimetric based assay that could be performed on a wide range of cell lines (Chanda and Nagani, 2013). It is based on the enzymatic reduction of MTT molecule to formazan when exposed to viable cells, the outcome is the color change of the molecule. Measuring the absorbance relative to control determine the percentage of remaining viable cells after treatment with varying concentrations of test compound and translated to the compound anticancer activity and its IC50 values. Traditional knowledge and scientific evidence document the anticancer activity of *Crataegus* species as pointed out in sections 1.1.2 and 1.1.5.2. Based on this, the antitumor activity of some *Crataegus* has been reported using MTT method (Kmail et al., 2015; Quadros et al., 2017).

1.1.6. Species of *Crataegus* selected as herbal drug

More than twenty species of hawthorn are used as herbal drugs or drug materials in the world. In the U.S market hawthorn is sold as a popular herbal supplement (Dahmer and Scott, 2010). Hawthorn products are currently marketed as an alternative treatment for hypertension, angina, arrhythmia, and congestive heart failure (Furey and Tassell, 2008; Edwards et al., 2012). Some of them are officially listed in the pharmacopeias of many countries as illustrated in Table 1.5 below. A good example from Germany is the Rote Liste which lists more than 40 preparations of *Crataegus*; 5 of them are liquid as elixir and drops and the others are solid dosage forms which include film-coated tablets and capsules (Rote Liste Service Gmbh, 2009).

However, *C. laevigata* (native to Europe and North America) and *C.monogyna* (native to Europe, Asia, and North Africa) are the two hawthorn species that are often studied and used to produce phytopharmaceticals and are the major hawthorn species utilized in the British and European Pharmacopoeias (Kirakosyan et al., 2004). *C.pinnatifida*, has been extensively used for foodstuffs and traditional medicine in Europe, Asia, and North America (Qader et al., 2017).

The available hawthorn products include tinctures, tablets, teas, and aqueous extracts. Extracts may be prepared using hydroalcoholic (ethanol) or water-based extraction and are derived from various plant parts including, most commonly, berries or leaves and flowers (Wang et al., 2013; Jennifer E. Edwards et al., 2012;Chang et al., 2002) .

Table 1.5: World wide acceptance of *Crataegus* species

Pharmacopoeias	Species accepted	Part used
British Herbal Pharmacopoeia 1996	*C. oxyacantha* L., *C.monogyna* Jacq (Lindn., *C. laevigata* (poir) D.C.	Fruits, leaves, and flowers
European Pharmacopoeia 6.0 2008	*C. oxyacantha* L., *C. monogyna* Jacq (Lindn.), *C. laevigata* (poiret) D.C, *C. oxyacanthoides* (Thuill), *C. pentagyna* Waldst et Kit ex Wild, *C.nigra*Waldst. et Kit, *C. azarolus* L.	Fruits and flowers
USP Official Monographs 2009	*C. monogyna* Jacq (Lindn.), *C. laevigata* (poiret)D.C.	
Chinese Pharmacopoeia 1997	*C. pinnatifida* Bge., *C. pinnatifida* Bge. Var. *major* NE Br.	Fruits

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Pharmacopée Française 1998	*C. oxyacantha* L., C.*monogyna* Jacq.	Fruits
German Pharmacopeia.Deutsches Arzneibuch (DAB1997) and Pharmacopoea Helvetica 1997	*C. oxyacantha* L., *C.monogyna* Jacq., *C.pentagyna* Waldst., *C. nigra* Waldst, *C.azarolus* L.	Leaves and flowers
Brazilian Pharmacopeia 1977	*C.oxycantha*	Fruit and flowers
The State Pharmacopoeia of the USSR1990	*C. altaica* (Loud.) Lang, *C. sanquinea*Pall,*C. laevigata* (poiret) D.C, *C.korolkowii L.*Henry, *C. monogyna* Jacq (Lindn.), *C. dahurica* Koehne ex.C.K. Schneid,*C. chlorosarca* Maxim., *C. wattiana* Hemsl.&Lace	Fruits and flowers
Upton 1999 (A H P)	*C. monogyna* Jacq (Lindn.), *C. laevigata* (poiret)D.C	Fruits

1.1.6.1. Herbal teas of *Crataegus* species

As mentioned above, there are many dosage forms of *Crataegus* products but herbal medicinal teas are mostly consumed because it is easy to use and its increased consumption lead to market expansion (Başgel and Erdemoğlu, 2006; Mathivha et al., 2020). However, herbal teas and other natural medicinal products should be prepared with licensing procedures that incorporate the standards for quality, safety, and efficacy. As contaminants can originate in conditions related to cultivation and post-harvest treatment of herbal materials as finished manufacturing stages (Kosalec et al., 2009). Therefore, there are Good Agricultural and Collection Practices in the European and WHO guidelines for raw herbal materials which ensure the quality and safety of herbal medicines (World Health Organization (WHO), 2005; World Health Organization (WHO), 2007). Herbal teas of *Crataegus* species are given in Table 1.6 below.

Table1.6: Herbal teas of *Crataegus* species

Trade name	Dosage form	Usage	*Crataegus* species	Medicinal type	Reference
1.Coarsely cut packing teas					
Aurica® hawthorn tea		Vasodilator contraction-enhancing blood circulation	hawthorn leaves with flowers	mono	https://www.shop-apotheke.com/arzneimittel/4453916/aurica-weissdorn-tee.htm
HERBOTRADE Hawthorne fruit tea		For circulation	Hawthorn berries of *Crataegus monogyna*	mono	https://www.amazon.com/Hawthorn-berries-Crataegus-monogyna-fruits/dp/B071ZJ7PYQ
Rosemary and Hawthorn Herbal Tea		Helps improve circulation	*Rosmarinus officinalis* and *C.oxycantha*	Mixture	https://www.woodlandherbs.co.uk/acatalog/Rosemary_Hawthorn_loose_herbal_tea.html
Sidroga hawthorn		Alternative therapy for feelings of oppression in the area of the heart and deteriorating performance of the heart: with a vasodilating properties	Hawthorn leaves and flowers	mono	https://www.sidroga.de/tees/finder/lose-arzneitees/weissdornblaetter-mit-blueten-loser-arzneitee/
2. Filter bag teas					
Alvita Hawthorn berry		Used to support cardiovascular health	*C. leavigata*	mono	https://www.alvita.com/herbal-teas/crataegus-laevigata.html#.XhhWG_4zbIU
L'Angelica Hawthorn Functional Herbal Tea		Helps regulate blood pressure	*C. monogyna* (flowers and leaves) *+lavandula officinalis* (flowers)	mixture	https://www.amazon.it/LAngelica-Tisana-Funzionale-Biancospino-35/dp/B01EAC8E3I

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Sidroga hawthorn		For heart problems such as palpitations, pressure, and feeling of tightness or stinging in the area of the heart, rapid pulse, or dizziness.	Hawthorn leaves with flowers	mono	https://www.sidroga.ch/tees/finder/gesundheitstees/weissdorn/
Organic Hawthorne with *Hibiscus* herbal tea		Promotes heart health	Hawthorne berries and *Hibiscus* flowers	mixture	https://www.amazon.in/Traditional-Medicinals-Hawthorn-Hibiscus-formerly/dp/B007W6AU9Q
HERBARIA Hawthorne flowers and leaves		Helps to normalize blood pressure by regulating heart activity	*C.oxycantha*	mono	https://herbarianorthamerica.com/our-teas/hawthorn-flowers-leaves/
Kneipp weissdorn tee		Improve cardiovascular function	Hawthorn leaves	mono	https://diskontshop.eu/AP-02473292/
Bombastus hawthorn leaves with flowers		For declined cardiac performance	Hawthorn leaves and flowers	mono	https://www.shop-apotheke.com/arzneimittel/5467346/bombastus-weissdornblaetter-mit-blueten.htm
Bad Heilburnner Weissdorn tee		Support cardiovascular function	Hawthorn leaves and flowers	mono	https://internet-apotheke-freiburg.de/shop/bad-heilbrunner-2296140

1.1.6.2. Liquid and solid preparations of *Crataegus* species.

As pointed out earlier, finished *Crataegus* products must meet the specifications set out within the official monographs concerning phytochemical content i.e., the minimum content of active components (flavonoids /or proanthocyanidins). Thus the European Pharmacopeia 2008, states that the quantified liquid extract produced from Hawthorn leaf with flower should contain 0.8 - 3.0 % flavonoids, expressed as hyperoside of the specified *Crataegus* species. The United States Pharmacopoeia 2009 states that the minimum required is 0.6% of *C*-glycosylated flavones expressed as vitexin and not less than 0.45% of *O*-glycosylated flavones expressed as hyperoside

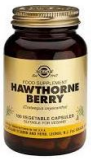
calculated on dry basis for leaves and flowers of (*C. monogyna* Jacq (Lindm), or *C. laevigata* (Poiret) D.C. According to the Pharmacopoeia Commission of the People's Republic of China, 2005 *C. pinnatifida* and *C. pinnatifida* var *major*, leaves should contain not less than 7% flavonoids calculated as anhydrous rutin. Phytopharmaceuticals produced using *Crataegus* extracts are given below in Table 1.7. Randomized, controlled trials in patients with heart failure demonstrated that *Crataegus* herbal medicinal products increase functional capacity, alleviates disabling symptoms, and improves health-related quality of life all of which have become important targets of heart failure therapy according to current diseases management guidelines.

Table 1.7: Phytopharmaceuticals produced using *Crataegus* extracts

Trade name	Extract type	Dosage form	uses	Phytomedicine	Reference
1.Liquid preparations					
Hawthorn® Florida Herbal Pharmacy	Leaf and flower of *C. leavıgata*	tıncture	Promotes cardıovascular health		https://www.amazon.com/Florida-Herbal-Pharmacy-Crataegus-oxyacantha/dp/B011FF068O
Crataegutt Tropfen® (Elsadig and Kuhnert 2015)	*C. monogyna* and *C. laevigata*, leaves, fruits	oral drops	Hyperlipidemia, hypertension, arrhythmia, angina, and New York Heart Association (NYHA) class I-III heart failure		https://www.pharmasana.co.uk/crataegutt-herz-kreislauf-tropfen-100-ml-11885622
A.Vogel *Crataegus* Oxy®	*C. Oxyacantha* berries	tincture and drops	Heart tonic		https://peoplespharmacy.co.za/product/a-vogel-crataegus-oxy-50ml/
Madaus Diacard Gold Drops	mixture	drops	Deals with palpitations, anxiety, heart pain, regulate a pulse, heart tonic		https://www.healthmug.com/product/madaus-diacard-gold-drops-25ml/1055090453

Continued……..

2.Solid preparations

Bomacorin 450mg	Hawthorn leaves with flowers	tablet	Strengthen and maintain heart function		http://www.hevert.com/market-de/de/arzneimittel/arzneimittel_von_a-z/produkt/bomacorin-450-mg-weissdorntabletten-n
Craegium novo 450mg	Hawthorn leaf and flowers dry extract	tablet	For weak heart		https://www.homoempatia.eu/product/craegium-novo-450-mg-film-coated-tablets.77803.html?language_code=en
Crataegus 450mg AL	Hawthorn leaf and flower dry extract	tablet	Strengthen weak heart		https://www.arzneiprivat.de/product/crataegus-al-450-mg-filmtabletten.748.html?language_code=en
CRATAEGUTT 450 mg Herz-Kreislauf-Tabletten	Hawthorn leaf and flower dry extract	tablet	Support cardiovascular function		https://www.pharmasana.co.uk/crataegutt-450-mg-herz-kreislauf-tabletten-200-st-14064541
Dineh Cardioton®	*C. monogyna* dry extract	tablet	Cardiotonic, antihypertensive, antiarrhythmic		https://www.alibaba.com/product-detail/Dineh-Cardioton-Tablet_109288690.html
Hawthorn® Berries	*C. laevigata*	capsule	Help maintain a healthy cardiovascular system.		https://www.naturessunshine.com.au/products/hawthorn-berries
Nature's Way Premium Herbal Hawthorn Berries	Hawthorn berries	capsule	Support cardiovascular health		https://www.amazon.com/Natures-Way-Hawthorn-Berries-Veg-capsules/dp/B0002PU5N0
Solgar Hawthorne Berry	*C. Oxyacantha* berries	capsules	Tonic effects on the heart, circulation, and blood vessels and is also a protective antioxidant.		https://www.tecanada.com/solgar-hawthorne-berry-p-517.html

Currently, the most studied hawthorn extracts is WS 1442 and LII32 (Dahmer and Scott, 2010). Scientific evidence shows that WS1442 is safe and has a beneficial effect in patients with heart failure corresponding to **New York Heart Association (NYHA)**

classes II or III. The benefit-risk assessment for WS 1442 is therefore positive (Holubarsch et al., 2017). However, a comparative review on the safety of hawthorn preparations founded that it is well tolerated by patients (Daniele et al., 2006) with some side effects as dizziness, vertigo, headache, gastrointestinal problems, and palpitation (Chang et al., 2005; Dahmer and Scott, 2010).

It must be taken into consideration that preparations made from hawthorn enhance the effects of cardiac glycoside which has been used with such drugs especially in German clinical medicine to reduce the toxic effects. In addition, hawthorn has been used intermittently with digitalis which resulted in modest changes in digoxin pharmacokinetics. But the difference did not achieve statistical significance which suggests that both drugs can be given together with studied doses (Tankanow et al., 2003).

Homeopathic medicines of herbal or natural origin have been anchored in the German law since 1978, they are very popular and meet widespread need (Schwabe, 2000). It must be pointed out here that there are *Crataegus* homeopathic medications, mostly the well-known is Diacard (Madaus) and *Crataegus* oxy. Researches were based on homeopathic preparations of *Crataegus* to treat patients with cardiac malfunctions especially mild cardiac insufficiency, (NYHA) class II. In comparison with ACE inhibitor/diuretics, the homeopathic preparations revealed similar results (Schroder et al., 2003).

1.2. *Crataegus* Species in Libya and Cyprus

Libya and Cyprus are not *Crataegus*-rich countries. In Libya, the only *Crataegus* species that still exist is *C.pallasii* which is used medicinally by the natives. While in Cyprus *C.azarolus* is the only widespread *Crataegus* species, its fruits are edible by the Cypriot people, while other species of *Crataegus* are rare.

1.2.1. *Crataegus pallasii* Griseb.

C.pallasii which is native to Krym (Crimea), South European Russia, Transcaucasus (Kurtto et al., 2013). In Libya, *C. pallasii* is introduced and reported from Bata, an area that lies north of El Merj city northeast of Libya on the edge of the fields. It lies in an upland valley separated from the Mediterranean Sea by a range of hills, which are part of the Jebel Akhdar Mountains, Eastern to Bengazi the second largest city of Libya.

In Libya, medicinal plants have faced the danger of extinction due to climatic changes, collection of medicinal and woody plants for local use, overgrazing, and hazards which occur frequently. The monitoring center announced that about 58 plant species are threatened in Libya (Louhaichi et al., 2011). As due three *Crataegus* species are mentioned in the Libyan flora *C. pallasii*, *C. laevigata,* and *C. mexicana.* Only *C. pallasii* has been recorded until dated that has survived diverse environmental conditions and is traditionally used by the native people to treat cardiac problems where other *Crataegus* species have been extinct (Jafri and EL- Gadi, 1977).

1.2.1.1. Botanical description of *Crataegus pallasii*

Shrubs with, reddish glabrescent twigs. Leaves 30-35mm long as well as broad, with 5-7 deep lobes, light green beneath Corymb villous. Flowers c.1.5 cm in diameter; style (1-) 2. Fruit 10-12 mm, ellipsoid-globose, first yellow, later red, blackish when ripe, crowned by deflexed sepals; pyrenes (1-)2 (Jafri and EL- Gadi, 1977).

Classification:

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida – Dicotyledons

Subclass	Rosidae
Order	Rosales
Family	Rosaceae
Genus	*Crataegus*
Species	*Crataegus pallasii* Grisb.

a

b

c

Figure 1.1: *Crataegus pallasii*,

a: tree, Photograph was taken by Dr. Fathi Al-Rateb in 2012 in Bata region.

b: flowers, Photograph was taken by Fatma El-Theera in 2018 in Bata region.

c: fruits, Photograph was taken by Dr. Fathi Al-Rateb in 2012 in Bata region.

1.2.1.2. Traditional uses

In Libya, *C.pallasii* is used for the treatment of cardiovascular problems and sold in Attar in the Libyan markets for this purpose.

1.2.2. *Crataegus azarolus* L.

C. azarolus L. also known as the Mediterranean "medlar fruit" or "Azarole" hawthorn (syn. *Crataegus azarolus* var.*aronia* L.; *Crataegus azarolus* var *azarolus*) cultivated for centuries in Mediterranean areas, is a species of hawthorn that thrives in semiarid conditions and area with no shade, in light, medium and heavy soils. Requiring moist or wet soil and tolerate drought. Thus it is found in woods and hedges on dry hills sides and mountains. Besides its use for medicinal purposes which will be mention later in more detail the fruits of *C.azarolus* are used as jam and syrups (Donno et al., 2017; Esmaeili et al., 2013; Hashempour et al., 2010).

Classification:

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida – Dicotyledons
Subclass	Rosidae
Order	Rosales
Family	Rosaceace
Genus	*Crataegus*
Species	*Crataegus azarolus* L.

1.2.2.1. Botanical description of *Crataegus azarolus*

Deciduous tree to 6 m with deep-fissured bark, branches, spiny, young twigs densely hairy, wedge-shaped, bluntly lobed and toothed at the broad end, smooth above and downy below; fls 10-20 in a flat-topped terminal spray sticky-scented, with 5 round white petals c. 8 mm across; frt. roundish, 1.5 cm, or more in diameter. Yellowish or latter Reddish-tinged, sweetly acid, containing 2-3 stones. Fl. Mar-Apr.

Rocky mountainsides, particularly above Kyrenia and Lepta; at lower levels it has often been planted e.g around Tepebasi. Medit. Region east to Iraq, and Iran. Fruits are very variable in size and color with weight ranges from 2-8 g and usually up to 25 mm in diameter, and color from yellow through bright red to black. Each fruit contains 1-3 large seeds The fruit is excellent, preserved, or made into jam and the timber is prized for carving (Viney, 1994).

a b c

Figure 1.2: *Crataegus azarolus*,

a: tree, Photograph was taken by Najat Agiel in Near East University campus in 2018 in Lefkosa,

b: flowers, Photograph was taken by Najat Agiel in Near East University, Pharmacognosy Lab in 2018 in Lefkosa

c: fruits, Photograph was taken by Najat Agiel Near East University campus in 2018 in Lefkosa

1.2.2.2. Traditional uses of *Crataegus azarolus*

In traditional Arab medicine *C.azarolus* is used to treat cardiovascular diseases especially those associated with high blood pressure as well as cancer, diabetes and sexual weakness as a decoction and infusion of the seed, flower and the fruits. Especially the fruits and the flowers that are considered the medicinal part of the plant (Abu-Darwish and Efferth 2018; Baydoun et al., 2015; Ameen et al., 2013; Afifi et al.,

2011) has a strong hypotensive effect and are used as a mild heart tonic. In most European countries, especially Germany, it is used to treat irregular heartbeat (Esmaeili et al., 2013). However, fruits has attracted increasing attention in the field of functional foods, nutraceuticals and medicine because of its widely reported health benefits (Donno et al., 2017). In Algeria, the leaves of *C. azarolus* are recommended as a hypotensive agent (Bouaziz et al., 2014). Another report of traditional use of *C. azarolus* was from Kurdistan in Iraq for bladder inflammation (Ahmed, 2016). Whereas, an ethnobotanical study that focused on wild edible fruits in Cyprus, stated that *C. azarolus* is among the fruits that are consumed as dessert in the fresh form (Della et al., 2006).

1.2.2.3. Phytochemicals of *Crataegus azarolus*

C. azarolus is considered as a valuable variety from the nutritional and antioxidant point of view due to the presence of high contents of flavonoids, condensed and hydrolysable tannins, and sugars (Yahyaoui et al., 2019). Several studies reported the antioxidant and measured the level of bioactive compounds as phenolics and organic acids (citric acid, malic acid, oxalic acid, and tartaric acid), flavonols (quercetin) tannins (castalagin and vescalagin) catechins (catechin and epicatechin), monoterpenes (limonene, phellandrene, sabinene, g-terpinene, and terpinolene), cinnamic acids (caffeic acid, chlorogenic acid, coumaric acid, and ferulic acid) and vitamin C (expressed as the sum of ascorbic acid and dehydroascorbic acid), and other bioactive compounds include sabinene and citric acid in the fruits, flower buds and opened flowers of *C. azarolus* (Bahri et al., 2009; Hashempour et al., 2010; Ameen et al., 2013; Bouaziz et al., 2014; Lakache et al., 2016; Donno et al., 2017).

Fruits of *C.azarolus* were studied by Donno and his colleagues 2017, the most important class of bioactive compounds in the fruits of *C. azarolus* was organic acids (55.0 %) followed by the polyphenols (25.1 %) monoterpenes (15.4 %), and vitamins (4.5 %). In the polyphenol group, the most important class were the catechins (11.6 %) and tannins (9.2 %) followed by flavonols, benzoic acids, cinnamic acids and anthocyanins were all refer to the total content of bioactive compounds.

Seeds of *C.azarolus* revealed on analysis that it is a good source of siloxanes, (Bechkri et al., 2017) : palmitic acid (28.37 %), oleic acid (26.57 %) linoleic acid (25.37 %) (Radhia et al., 2014). The summary of the phytochemical constituents in *C.azarolus* is presented in Table 1.8 below.

Table 1.8: Bioactive compounds isolated from *Crataegus azarolus* L

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Chemical group	Part used	Compound name	Reference
Polyphenols	Fruit	Chlorogenic acid, quercetin, spiraeoside, epicatechin, apigenin, luteolin, vitexin-rhamnoside, kaempferol, neoategolic,succinic, malic, citric, quinic and ursolic acids, nonacosan, procyanidin B2, hyperoside, rutin, isoquercitrin, quercetin, 3β-O-acetyl ursolic acid, ellagic acid, quercetin 3-O-β methyl ether, apigenin 7-O-rutinoside.	(Bignami et al., 2004; Bahri et al., 2009; Hashempour et al., 2010; Hamahameen and Jamal 2013; Bahri-Sahloul et al., 2014; Preedy and Watson 2014; Donno et al., 2017; Abu-Gharbieh and Shehab, 2017).
	Fruit and leaves	Gallic acid, catechin, vescalagin, citric acid, dehydroascorbic acid.	(Donno et al., 2017; Amel, 2015)
	Leaves	Quercetin 3-O-galactoside.	(Amel. 2015)
Sugars	fruits	Xylose, sucrose, fructose, myo-inositol	(Bignami et al. 2004)
Organic acids, Flavanols, Procyanidins	Flowers, callus cultures	Chlorogenic acid, hyperoside, rutin, spiraeoside, quercetin, (-)-Epicatechin, vitexin, vitexin-2''-O-rhamnoside, 4''-O-acetylvitexin-2''-O-rhamnoside, Procyanidins B2, euscaphic acid, azarolic acid, arjunic acid, 2-oxopomolic acid, dihydroxy-3-oxo-urs-12-en-28-oic acid.	(Bahri et al,. 2009;Bahri-Sahloul et al., 2014; Mahmud ,2016)
Fixed oils	Seed oil	Tetradecamethylcycloheptasiloxane, 3,4-dihydroxytetramethylsilyl mandelic acid, dodecamethylcyclohexasiloxane decamethylcyclopentasiloxane and 3-isopropoxy-1,1,1,7,7,7-hexamethyl3,5,5 tris(trimethylsiloxy)tetrasiloxane.	(Bechkri et al., 2017)
Volatile oils	Young branches with flowers and leaves	2,4-bis (1,1-dimethyl ethyl)-phenol, tridecanoic acid 12-methyl-methyl ester, pentadecanoic acid 14-methyl-methyl ester, 8-octadecanoic acid methyl ester, and isobutyl nonyl phthalate.	(Amina et al., 2018)
	fruits	Phellandrene, sabinene, γ-terpinene, terpinolene and limonene	(Donno et al. ,2017)
	Leaves and fruit	Hexadecanoic acid, p-xylene, thymol and thymolethanoate	(Hamedi et al., 2017)

Continued……..

	Leaves and flowers	Benzaldehyde, butyraldehyde, 2-hexenal n-hexadecanoic acid, tricosane and α-farnesene	(Zder et al. ,2016; Lakache et al., 2014; Dönmez, 2015)
	jam	2-Furaldehyde, Cyclohexane-2-methyl-1-propenyl, 1H-3α,7-Methanoazulene,2,3,4,7,8,8α-hexahydro-3,6,8,8-tetramethyl, δ-Selinene, 3-Cyclohexene-1-methanol,α,α,4-trimethyl	(Hadjimitsi and Zabetakis 2005)

1.2.2.4. Pharmacology of *Crataegus azarolus*

As with other *Crataegus* species, *C.azarolus* is used primarily for the treatment of cardiovascular diseases and the latest reports have also documented its use in cancer therapy which will be explained in more detail later. Researchers have also reported the effect of *C.azarolus* to treat other malfunctions as diabetes (Abu-Gharbieh and Shehab, 2017). It was also evaluated to serve as a promising source for anti-inflammatory (Mustapha et al., 2016) and antimicrobial drugs (Moradi et al., 2018; Abu-Gharbieh and Shehab, 2017; Belkhir et al., 2013). The methanolic extract of the stems of *C.azarolus* representing the highest content of phenolic compounds was selected to be the most potent against the arginase enzyme, the increase of which is a marker of many pathological diseases and its inhibition is a promising treatment (Attia et al., 2019).

The antioxidant activity of *C.azarolus* was also evaluated by a number of studies (Mustapha et al., 2016; Egea et al., 2010; Mraihi et al., 2013; Bahri et al., 2009; Bahri-Sahloul et al., 2014; Belkhir et al., 2016) which suggests that this species of *Crataegus* should be explored as a novel potential immunomodulatory drug.

1.2.2.4.1. Cardiovascular activity

Reports that the methanol extract and the ethyl acetate of *C.azarolus* when administered intravenously decreased the mean arterial blood pressure, systolic and diastolic blood pressure in anesthetized rats dose-dependently was documented (Bouaziz et al., 2014). Euscaphic acid isolated from *C. azarolus var aronia* showed anticontraction effects on the isolated rats' aortic smooth muscles which justifies the use of this *Crataegus* species in Cardiovascular diseases (Al-habib and Mahmud, 2015).

1.2.2.4.2. Antitumor activity

The ethyl acetate extract of *C.azarolus* and the respective compounds isolated from it, as vitexin-2''-*O*-rhamnoside exhibited significant anti-proliferative activity against B16F10 melanoma cells after incubation for 48 hours (Mustapha et al., 2015). The antiproliferative and pro-apoptotic activity of the ethyl acetate extract of *C. azarolus* towards HCT-116 and HT-29 human colorectal cancer cell (CRC) lines were also evaluated (Mustapha et al., 2016).

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Aim of the study

The present work aims to investigate the possibility of two *Crataegus* species; which have not been investigated prior to this work; *Crataegus palllasii* Griseb. from Libya and *Crataegus azarolus* L. from Northern Cyprus for the production of *Crataegus* derived herbal drugs upon achieving the following specific aims.

1- Phytochemical screening including volatile oil evaluation.
2- Extraction of phenolic compounds using percolation and Soxhlet extraction methods.
3- Identification and quantification of major phenolic compounds using RP-HPLC-DAD and comparing the results with the well-known herbal pharmaceutical drug Crataegutt® Tropfen.
4- Investigate of the relaxant and cytotoxic effect of total ethanolic extracts.

CHAPTER 2

2. MATERIALS AND METHODS

2.1. Plant Material

Crataegus pallasii Griseb. samples from Libya were collected from El-merj city (CpE) from Bata region on 23.3.2018 and purchased from attar from the local market in Tripoli (CpT) on 25.8.2017. Both samples were authenticated by Dr. Mohammed Nuri Abuhadra and deposited in the Herbarium of the Faculty of Science, Botany Department, the University of Tripoli in Libya, voucher number: D6831131.

Crataegus azarolus L. samples were collected from two regions in Northern Cyprus; Cengizköy / Lefke (CaC) on 1.3.2018 and from Near East University Campus (CaCf) in Nicosia on March 26.3.2018. Samples were deposited at the Herbarium of the Near East University with voucher numbers NEUN 6899 and NEUN 6900, respectively.

The *Crataegus*-derived phytopharmaceutical drops Crataegutt® Tropfen purchased from Marien Apotheke, Saarlouis, Germany in 2018.

2.2. Phytochemical Screening

2.2.1. Chemicals and apparatus.

Sodium hydroxide, dichloromethane, conc. Sulphuric acid and Hydrochloric acid from Sigma Aldrich Germany. Ethanol from Merck KGaA and glacial acetic acid from Riedel-de Haen Germany. 3, 5-dinitrobenzoic acid from Aldrich China. Acetic anhydride, ferric chloride, magnesium powder and Mayer's reagent. Apparatus used mainly ranks, test tubes, flasks, and pipettes.

2.2.2. Methods

To detect the presence groups of secondary metabolites, 10 g of powdered flower and leaf of *C. pallasii*, and *C. azarolus* were macerated in water, dichloromethane, and ethanol overnight. Phytochemical screening tests were performed according to (Trease and Evans, 1989; Satyajit and Latif, 2006) with slight modification.

Test for Alkaloids.

Mayer's reagent test: To 1 ml of extract, 2 ml of Mayer's reagent was added. The appearance of a dull white precipitate indicates the presence of alkaloids.

Cardiac glycosides.

Keller-Killani test: 5ml of the extract was treated with 2 ml of glacial acetic acid and 1 drop of 0.1% ferric chloride then add slowly by side of the test tube with 1 ml conc. H_2SO_4. A brown ring at the interface indicates the presence of deoxysugars.

Kedde Test: The addition of 3, 5 dinitrobenzoic acid and NaOH to the alcoholic extract solution causes an intense violet coloring.

Test for Flavonoids.

Shibata test: To 1 ml of extract magnesium powder was added and a few drops of conc. HCL, an orange-pink color indicate the presence of flavones or flavonols type of flavonoids

Test for Saponins.

The frothing test: 1 ml of the extract was shaken with 2 ml distilled water vigorously for about 5 mins. A froth that stands for 15 minutes is an indicator of the presence of saponins.

Test for Sterols.

Libermann- Burchard's test: To 2 ml of the extract, 2 ml acetic anhydride, and 1ml of conc. H_2SO_4 was added. A blue ring indicates the presence of sterols.

Tannins.

To 1 ml of extract, 1 ml of neutral ferric chloride was added. The formation of dark blue color which changes to green on the addition of more ferric chloride has confirmed the presence of tannins.

2.3. Determination of Volatile Oils

2.3.1. Obtaining of volatile oils

150 g of fresh inflorescences and unripe fruits of *C. azarolus* (CaCf) was distilled in water for 3 h using the Clevenger-type apparatus by hydrodistillation. The volatile oil obtained was given the code number (KHCB020). An inflorescence was weighed fresh and weighted after drying to evaluate the moister content.

150 g of dry inflorescences and unripe fruits of *C. azarolus* (CaCd) were distilled with water for 3 h using the Clevenger-type apparatus by hydrodistillation. The volatile oil obtained was given the code number (KHCB022).

85 g of dry inflorescences and unripe fruits of *C. pallasii* (CpE) were distilled with water for 3 h using the Clevenger-type apparatus by hydrodistillation. The volatile oil obtained was given the code number (KHCB024). The resulting oils were trapped with hexane and stored at 4℃ until used.

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2.3.2. Analysis of volatile oils

2.3.2.1. GC-MS Analysis

The GC-MS analysis was carried out with an Agilent 5977B GC-MSD system. Innowax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) from USA was used with Helium as carrier gas (0.8 mL/min). GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4°C/min, and kept constant at 220°C for 10 min and then programmed to 240 °C at a rate of 1 °C/min. The split ratio was adjusted at 40:1. The injector temperature was set at 250 °C. Mass spectra were recorded at 70 eV. The mass range was from m/z 35 to 450. The sample was dissolved in 10 % n-hexane and 1 µL was injected.

2.3.2.2. GC-FID Analysis

The GC analysis was carried out using an Agilent 7890B GC system. FID detector temperature was 300°C. To obtain the same elution order as with GC-MS, a

simultaneous duplicate auto-injection was performed with the same column and operational conditions. The relative percentage of the separated components were calculated from FID chromatograms.

2.4. Extraction Procedures

2.4.1. Chemicals and apparatus

For extraction of plant material; Leaves and flowers were ground using commercial blender WARING® CB15V from USA. Heating mantle MTOPS E102 from Bor-Kim in Turkey. Rotatory evaporator was purchased from Buchi Labortechnik AG Switzerland. Freeze drying was carried out with Martin Christ Gefriertrocknungsanlagen GmbH. Other glassware materials include Soxhlet apparatus, round flasks, and conical flasks.

All chemicals and reagents used in the study were purchased from Fluka, Merck, and Sigma Chemical Co. Ethanol which was purchased from Merck-Chemicals in Germany. Ethyl acetate, petroleum ether, and toluene were obtained from Sigma-Aldrich GmbH.

2.4.2. Preparation of total extract

2.4.2.1. Percolation

The extraction method was carried according to (Reza et al., 2017), 100 g of powdered dried leaves and flowers of *C. pallasii* (CpE), *C.pallasii* (CpT), and *C. azarolus* (CaC) were weighed and extracted separately by maceration in 500 ml of 96 % ethanol for 48 hrs, then extracted with 500 ml alcoholic water 60:40 (v/v) for 24 hrs. A third-time extraction was carried out with 300 ml of alcoholic water 60:40 (v/v) for 24 hrs. The extract was then collected and filtered for drying using the rotary evaporator and the extraction yield was recorded.

2.4.2.2. Soxhlet extraction

Extraction procedures were carried out according to (Sözer et al., 2006) with some modifications. 100 g of powdered dried leaves and flowers of *C. pallasii* (CpE), *C. pallasii* (CpT), and *C. azarolus* (CaC) were weighed and extracted separately in the soxhlet apparatus using 96 % ethanol. After extraction, the extract was cooled, then filtered for evaporation of the excess solvent in the rotary evaporator. The extraction yield was recorded respectively for each sample.

2.4.2.3. Fractionation

Fractionation of total ethanolic extract was carried out according to the liquid-liquid extraction technique. Firstly total ethanolic extract was washed in a separatory funnel with petroleum ether to remove fatty compounds, then washed with toluene to remove chlorophyll (Fig. 2.1). To obtain an extract with a high amount of phenolic compounds, a third washing was carried out with ethyl acetate. The ethyl acetate fraction obtained was evaporated to dryness in rotary evaporator. After freeze-drying, the ethyl acetate extracts were stored at a temperature of 4℃.

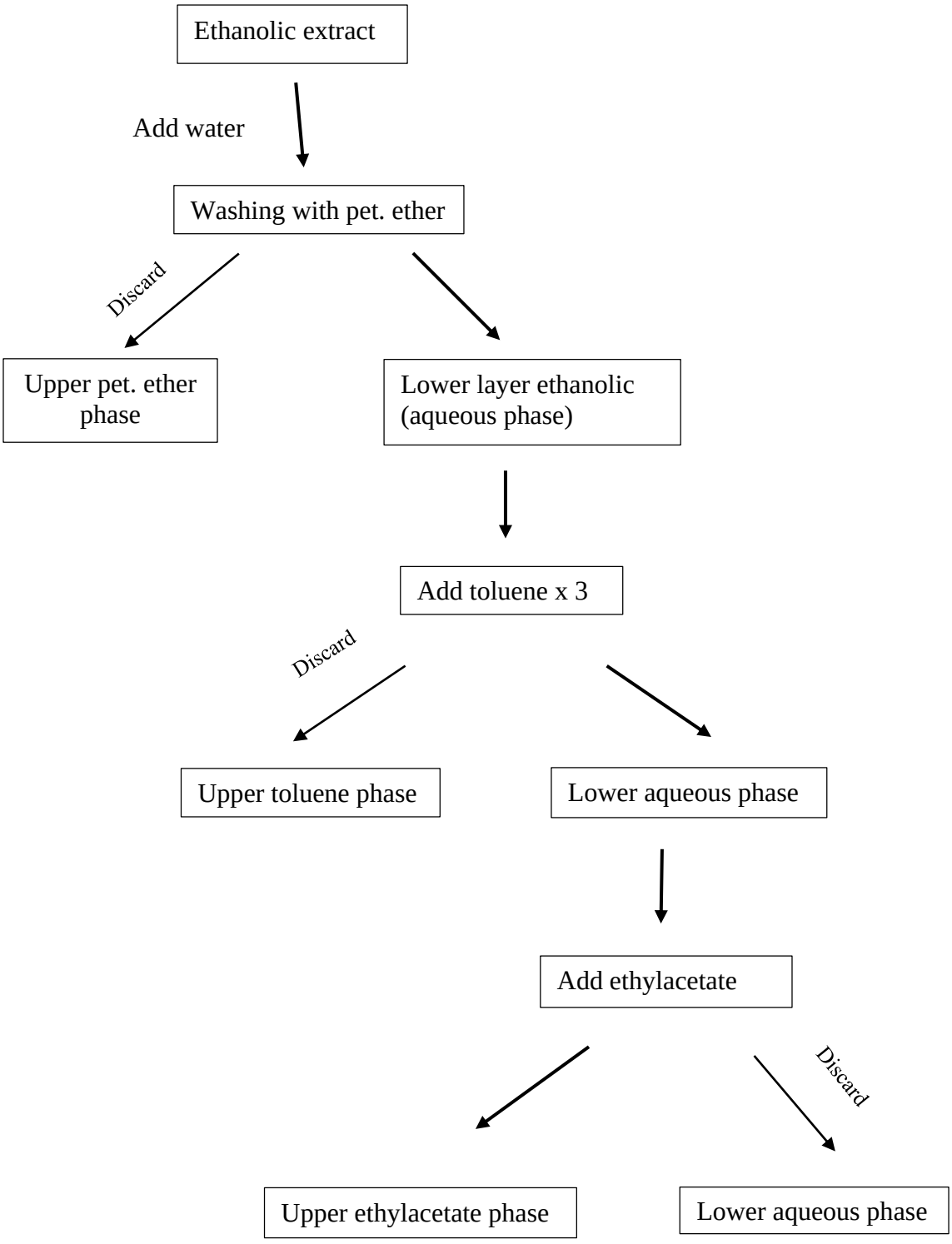

Figure 2.1: Scheme of liquid-liquid extraction of ethylacetate extract.

2.4.2.4. Dispersive Liquid-Liquid Microextraction (DLLME)

Sample preparation prior to HPLC analysis is a vital step to suppress matrix interference and preconcentration of analytes at low concentrations in the sample. Dispersive liquid-liquid microextraction (DLLME) is a microextraction technique preceded for the first time by Rezaee (Rezaee et al., 2006) is primarily used for this purpose (Ghalebi et al., 2019). Since its introduction in 2006, DLLME has found wide prominence among scientists as an efficient sample preparation method prior to analysis (Lee et al., 2019b). In conventional DLLME, the analytes are extracted into a micro-volume of a water-immiscible organic solvent, where they are preconcentrated. The analyte-rich extraction phase is separated from the mixture, evaporated to dryness and the analytes are reconstituted into a water-miscible solvent and then injected into HPLC for analysis. In order to achieve an efficient sample clean-up of hydrophobic matrix components which might pose a potential interference on analyte peaks, and due to the nature of the compounds under investigation Indirect DLLME (IDLLME) was thought to be possible (section 2.5.3).

2.5. Quantitation and Identification of Phenolic Compounds by HPLC Analysis

2.5.1. Chemicals and apparatus

All reagents for analysis were HPLC grade. Absolute ethanol 99.9 % purchased from TEKKIM KIMYA SAN Turkey. Phosphoric acid from Milipor, USA. Acetonitrile and chloroform from BDH PROLABO® VWR European Economic Community. Ultra-pure water (deionized water and degassed by ultra-sonication). The Standard compounds hyperoside (Purity ≥ 98 %) and vitexin (purity 96.4 %) were obtained from HWI Pharma Services GmbH. Rutin (purity ≥ 94 %) was ordered from Sigma Life Science, Germany. The authentic standard vitexin 2"-*O*-rhamnoside (purity ≥ 98.0 %) was purchased from Sigma –Aldrich France.

Bandelin Sonrex digital ultrasonic bath was used for ultra-sonication and centrifugation was performed with Hettich Eba 20 centrifuge and vortexing was performed on a Heidolph Reax top Vortex all were purchased from Germany.

Eppendorf micropipette from Sigma-Aldrich, USA. Tips were used for sample collection and transfer. Nylon membrane filters 0.2µm, diameter 47mm Whatman, GE Healthcare, Life Sciences, USA used for filtering ultra-pure water for HPLC analysis. ChromFil Nylon Filter 0.22 µm used for sample filtration.

2.5.2. Instrumentation and chromatographic conditions

Chromatographic separation was performed using high-performance liquid chromatography Agilent Technologies 1200 series (USA) equipped with quaternary pump solvent degasser G1322A, automatic injector ALS G1329A, column oven TTC G1316A and diode array detector DAD G1315B. An ACES-C8 column (4.6 mm ID x 25 cm, 5 µm) at column temperature 20 °C was used for separation. The mobile phase consisted of water (A) Acetonitrile (B) using a gradient elution system of 20 % B at 0 min to 60 % B at 12 min. The flow rate of 0.8 ml/min. The ultraviolet (UV) wavelength was selected and monitored at 264 nm for hyperoside and 342 nm for the other compounds which correspond to their wavelength of maximum absorption, and the injection volume was 0.8 ml/min.

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2.5.3. Sample preparation

In a step to obtain a phenolic rich extract the total ethanolic extract was washed with hexane and toluene the ethyl acetate fraction was evaporated to dryness, freeze-dried, and stored at a temperature of 4℃ until used as indicated in section 2.4.2.3. To prepare the sample solution, 25 mg of ethyl acetate extract was weighed, and to reduce the matrix effect IDLLME procedure was carried out by adding 4.5 ml of DI water to the extract and sonicating the mixture for 5 min. Next, 500 µl ACN (as a disperser solvent), 100 µl of chloroform (as an extractant for interferences), and 100 µl of phosphoric acid were added. The solution was vortexed for one min and centrifuged (1 min, 6000 rpm). The organic phase containing the interferences was discarded and the aqueous phase was directly injected into HPLC for analysis.

2.5.5. Preparation of standard stock solution for standard compounds

To prepare the appropriate concentrations for obtaining the calibration curves, 1.0 (±0.01) mg of each standard was weighed and dissolved in ethanol in an HPLC vial to obtain 1000 mg/L stock solution of individual standards, which were stored at 4 °C until use. Intermediate stock solutions containing 100 mg/L were then freshly prepared in ethanol.

2.5.6. Preparation of sample solution for Crataegutt® Tropfen

1 mL of the crude syrup was diluted to 10 mL with the aid of vortex to make the syrup 10% v/v with ethanol. The solution was filtered through a 0.22 µm syringe filter paper as a sample solution. 100 µL of the sample solution was diluted to 1000 µL before injection into HPLC. Subsequently, the spiked concentration of the standards was added and injected into HPLC to plot a standard addition calibration curve.

2.6. Biological Activity Studies

In this work, the biological studies were carried out with the total ethanolic extracts of *C.pallasii* and *C.azarolus* to determine the relaxant effect on isolated segments of human Saphenous Vein (SV) and evaluate the cytotoxic effects on human breast cancer MCF-7 cells in *invitro* conditions.

2.6.1. The relaxant effect of total extracts on isolated segments of human saphenous vein (SV)

2.6.1.1. Isolated vascular preparations

The study was performed on isolated segments of the human saphenous vein (SV), with intact endothelium obtained from patients (3 males, aged 53±3) who had undergone coronary artery bypass surgery.

2.6.1.2. Chemicals and apparatus

Organ baths containing Kreb's solution (concentration mM): NaCl 139.2, KCl2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, $NaHCO_3$11.9, NaH_2PO_4 0.4, glucose 5.5, gassed with 5% CO_2 and 95% O_2 at 37˚C and pH 7.4, Phenylephrine (1–10 µM), distilled water, acetylcholine (0.01-100 µM).

2.6.1.3. Methodology of relaxant effect of total extracts on isolated segments of human saphenous vein (SV)

The study was performed on isolated segments of the human saphenous vein (SV), according to the work done by (Ozen et al., 2013). The isolated segments were obtained from patients (3 males, aged 53±3) who had undergone coronary artery bypass surgery, SV preparations (cut as rings) were set up in 10mL organ baths containing Kreb's solution (concentration mM): NaCl 139.2, KCl2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, $NaHCO_3$11.9, NaH_2PO_4 0.4, glucose 5.5, gassed with 5% CO_2 and 95% O_2 at 37˚C and pH 7.4. Each ring was initially stretched to an optimal load (1.5-2.0 g). Changes in force were recorded by an isometric force-displacement transducer. Rings were equilibrated for 90 min with bath fluid changes taking place every 10 min. After the equilibration period, the viability (contractility) of the vessel specimens was checked with potassium chloride (KCl, 40 mM) stimulation and the preparations were washed until the initial resting tone was re-established. The vessels were pre-contracted with Phenylephrine (1–10 µM) and then increasing concentrations of *C.pallasii* or *C.azarolus* (0.2-2.0 mg/mL) were established cumulatively. The extract is dissolved in distilled water (0.1 mg extract /µl distilled water). To investigate the endothelium capacity of vessels, after pre-contraction with Phenylephrine (1–10 µM), acetylcholine is applied as a concentration-dependent manner (0.01-100 µM). The relaxations induced by *Crataegus* extract or acetylcholine are calculated as a percentage of pre-contraction levels.

2.7.2. Antitumor cell viability study

The breast cancer cell line, MCF-7, was used in this study (ATCC: HTB-22). MCF-7 cells were maintained in media containing RPMI-1640 medium (Biochrom, FG 1215), 10 % heat-inactivated fetal bovine serum (FBS) (Capricorn Scientific, FBS-11B), 1 % penicillin-streptomycin (Biochrom, A2213) and 1 % glutamine (EMD Millipore, K0282). Cells were cultured in a humidified atmosphere at 37 °C in 5 % CO_2. As the cultured cells reached confluency state, they were sub-cultured using 0.25 % trypsin-EDTA solution (Biochrom, L 2143).

2.7.2.1. Assay of cell viability and growth

The cytotoxicities were measured using a MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Biotium, #30006). MTT is based on colorimetric measurement of the reduction of 3-(4, 5-dimethylthialzol-2-yl)-2, 5-diphenyl tetrazolium bromide which is reduced by living cells to yield purple formazan product. Stock *C. pallasii* and *C. azarolus,* extracts were prepared in dimethylsulfoxide (DMSO, Sigma-Aldrich), 100 mg/mL and diluted in culture medium with five different concentrations (5 µg/mL, 10 µg/mL, 20 µg/mL, 50 µg/mL and 100 µg/mL). MCF-7 cells were collected, suspended in medium, and seeded in 96-well culture dishes at a density of 5 x 10^3/mL cells in each well with 100 µL medium. The negative control row neither contained any cells nor extracts and the positive control row only had cells seeded in. Extract dilutions were triplicated and both cell lines were incubated for 24 and 48 h. After incubation MTT solution was heated to 37 °C and then 10 µL was added to each well. After 4 h incubation at 37 °C in 5 % CO_2, 200 µL DMSO was added to dissolve the formazan salts. The absorbance was measured at 570 nm with a spectrophotometer (Versa Max, Molecular Device, Sunnyvale, USA). All experiments were performed in triplicate for each extract.

CHAPTER 3

3. RESULTS AND DISCUSSION

According to the procedures that have been displayed in chapter two on the total extracts, ethyl acetate extract, and volatile oil of *C.pallasii* and *C.azarolus* to investigate their major metabolites and biological activity the results were recorded and discussed in this chapter.

3.1 Results

In the following sections the results are interpreted individually.

3.1.1 Preliminary phytochemical screening

Preliminary phytochemical screening provides a quick scan of important group of compounds with simple tests. The water, dichloromethane, and ethanol extracts of *C.pallasii* and *C.azarolus* leaves and flowers were evaluated to detect important secondary metabolites; alkaloids, cardiac glycoside, flavonoids, saponins, sterols, and tannins. This phytochemical study was qualitatively expressed as positive (+) or negative (-) as shown in Table 3.1 below. The results of the phytochemical screening tests of *C.pallasii* and *C.azarolus* leaf and flower extracts revealed that the aqueous and ethanol extracts revealed the highest concentration of flavonoids. While dichloromethane extract showed a low concentration of flavonoids. A moderate concentration of tannins was also detected in the aqueous and ethanolic extracts and saponins were detected in the aqueous extract only. However, all extracts showed the absence of cardiac glycoside, alkaloids, and sterols.

Table 3.1: Phytochemical screening of extracts

Metabolites	Extracts								
	CaCw	CaCd	CaCe	CpEw	CpEd	CpEe	CpTw	CpTd	CpTe
Alkaloids	-	-	-	-	-	-	-	-	-
Cardiac glycoside									
Keller Killani test	-	-	-	-	-	-	-	-	-
Kedde test	-	-	-	-	-	-	-	-	-
Flavonoids									
Shibata test	+++	+	+++	+++	+	+++	+++	+	+++
Saponins	++	-	-	++	-	-	++	-	-
Sterols									
Liberman test	-	-	-	-	-	-	-	-	-
Tannins	++	-	++	++	-	++	++	-	++

+ = Present, ++ = present in medium concentration, +++ = present in high concentration. - = Absent. CaC: *C.azarolus* from Cyprus; CaCw: water extract, CaCd: dichloromethane extract, CaCe: ethanol extract, CpE: *C.pallasii* from El-merj; CpEw: water extract, CpEd: dichloromethane extract, CpEe: ethanol extract, CpT: *C.pallasii* from Attar in Tripoli; CpTw: water extract, CpTd: dichloromethane extract, CpTe:ethanol extract.

3.1.2. Volatile oil analysis results

Identification of the components in the essential oils that were obtained from the unripe fruits and inflorescence of the dried *Crataegus pallasii* (CpE) and fresh *Crataegus azarolus* (CaCf) and dried *Crataegus azarolus* (CaCd) were accomplished by comparison of their relative retention times with authentic samples or their linear retention index (LRI) to series of n-alkanes. Computer matching was also performed against commercial (Wiley GC/MS Library and NIST Chemistry WebBook) McLafferty and Stauffe, 1989; Linstrom and Mallard, 2011) and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data was used for identification (Joulain and Koenig, 1998; ESO 2000, 1999).

Fifty-four compounds in the essential oil of *C.pallasii* and fresh and dry samples of *C.azarolus* were identified by GC-MS and GC-FID. The chemical profiles, the percentage contents, and linear retention indices of the components are shown in Table 3.2 given below. Thirty-one compounds comprising 97.6% of the total oil composition of *C.pallasii* (CpE) were identified. Fresh *C.azarolus* (CaCf) revealed thirty-three compounds forming 91.1% of the total oil composition and twenty-seven were

identified from the dried samples of *C.azarolus* (CaCd) forming 85.8% of the total oil composition. The oil yield was less than 0.01% on a dry weight basis of all samples. Moisture content for fresh *C. azarolus* was calculated as 65.6%. However, the major outstanding group of compounds was the volatile alkanes with a concentration in CaCf was 81.8% (Fig.3.1), in CaCd was 72% (Fig.32) and in CpE was 85.8% (Fig.3.3).

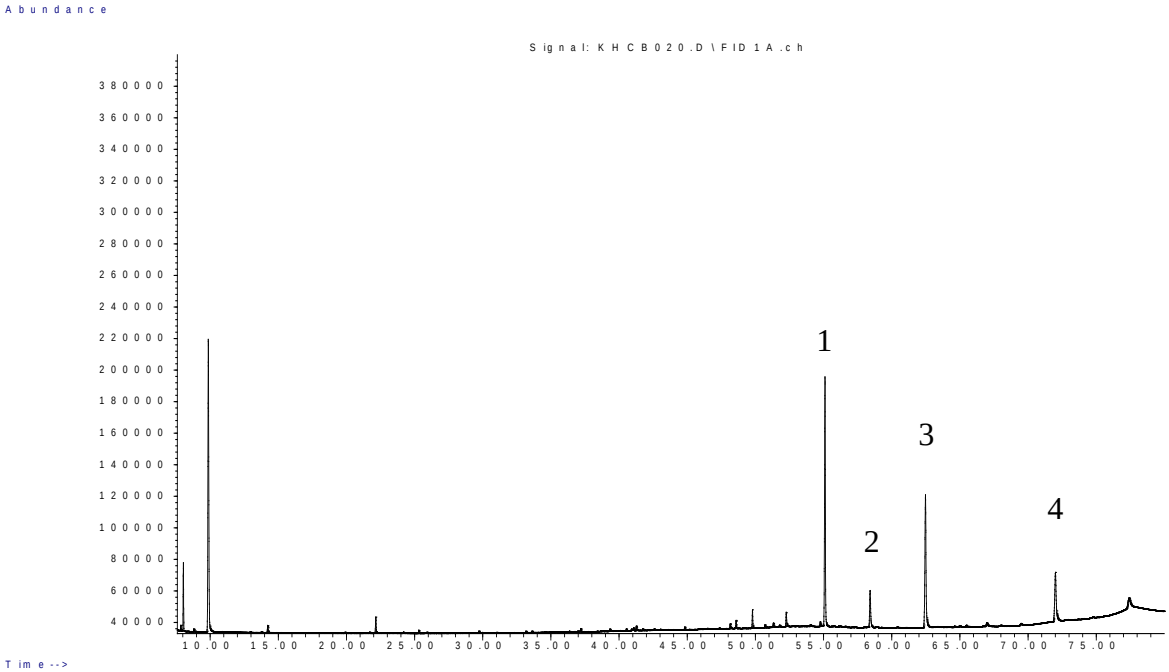

Figure 3.1: Chromatogram of main compounds of *Crataegus azarolus* (A) fresh inflorescences and immature fruits- NEU Campus (CaCf) / Sample A (1: Tricosane, 2: Tetracosane, 3: Pentacosane, 4: Heptacosane)

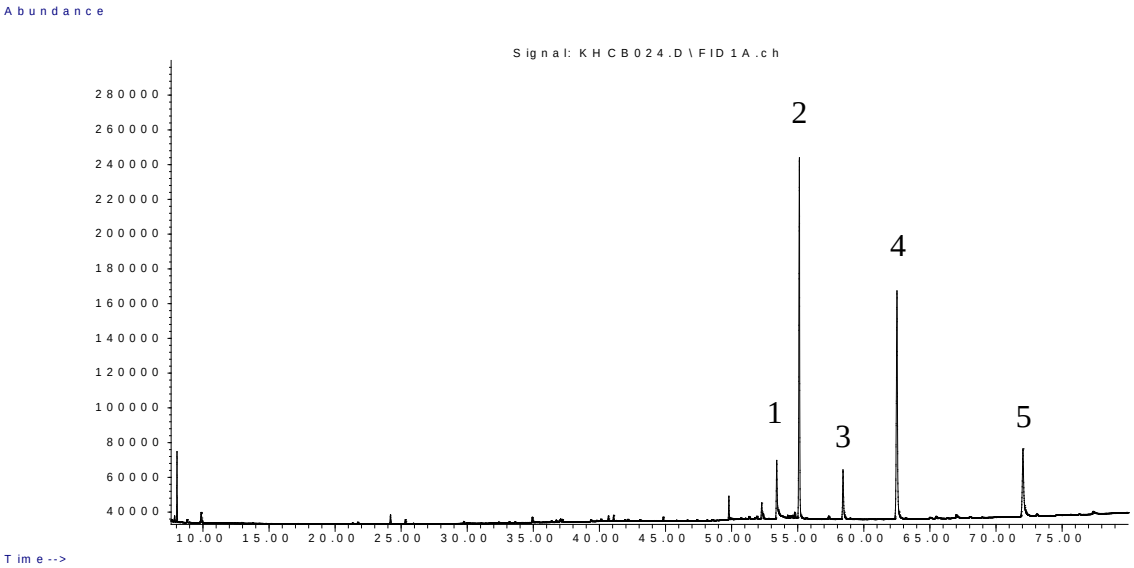

Figure 3.2: Chromatogram of main compounds of *Crataegus azarolus* (B) dry inflorescences and immature fruits- Cengizköy-Lefke(CaCd) / Sample B (1: Eicosane, 2: Tricosane, 3: Tetracosane, 4: Pentacosane, 5: Heptacosane)

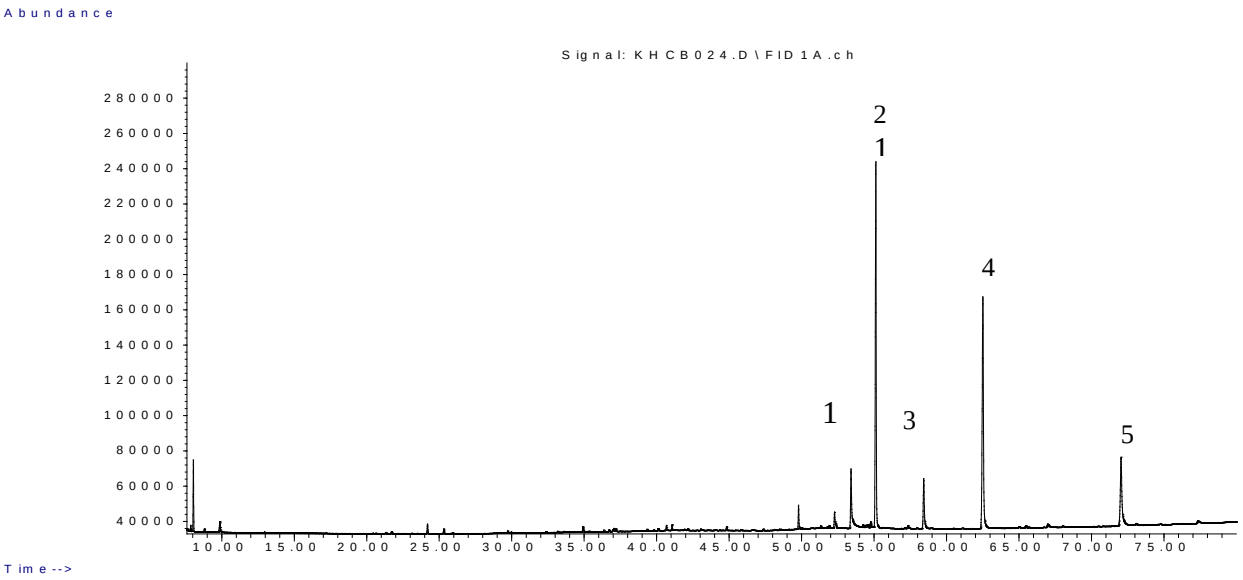

Figure 3.3: Chromatogram of main compounds of *Crataegus pallasii* (C) dry inflorescences and immature fruits- Bata Region-Libya (CpE)/ Sample C (1: Carvacrol, 2: Tricosane, 3: Tetracosane, 4: Pentacosane, 5: Heptacosane

Table 3.2: Volatile oil composition of infloresence and immature fruits of *Crataegus pallasii* and *Crataegus azarolus*

LRI $_{EXP}$	LRI $_{LIT}$*	Compound Name	A%	B%	C%
1020	1025[b]	α-pinene	0.9	0.3	-
1190	1177[b]	α-terpinene	-	-	0.1
1213	1212[c]	Limonene	1.9	0.5	-
1259	1245[b]	γ-terpinene	-	-	0.7
1288	1282[c]	*p*-cymene	0.4	-	0.3
1408	1391[b]	Nonanal	0.2	0.2	0.1
1514	1491[b]	α-copaene	0.2	-	-
1555	1543[b]	Linalool	-	-	0.4
1613	1591[b]	β-elemene	0.1	0.2	0.2
1620	1617[a]	Terpinen-4-ol	-	-	0.3
1622	1617[a]	Undecanal	0.1	0.3	0.2
1624	1623[a]	β-caryophyllene	0.3	0.2	0.2
1728	1719[a]	Borneol	-	-	0.2
1744	1726[a]	Germacrene D	0.2	-	-
1748	1741[a]	β-bisabolene	-	-	0.3
1758	1742[d]	β-selinene	0.2	-	-
1761	1744[a]	α-selinene	0.1	-	-
1763	1760[a]	(*E*,*E*)-α-farnesene	0.3	0.4	0.4
1770	1740[a]	Valencene	0.5	-	-
1770	1751[a]	Carvone	-	-	tr
1786	1755[b]	δ-cadinene	0.2	-	-
1817	1797[a]	Selina-3,7(11)-diene	0.2	-	-
1835	1830[a]	Tridecanal	-	0.2	0.1
1854	1838[a]	(*E*)-β-damascenone	-	0.1	-
1875	1854[b]	(*Z*)-geranyl acetone	-	0.1	-
1893	1880[e]	2,2,4-trimethyl-3-carboxyisopropyl pentanoic acid isobutyl ester	-	0.1	-
1901	1890[a]	Carvacryl acetate	-	-	0.3
1941	1931[a]	Phenylethyl alcohol	-	-	0.1
1973	1954[a]	(*E*)-β-Ionone	-	-	0.1
2033	2008[a]	Caryophyllene oxide	0.5	-	-
2052	2053[a]	(*E*)-nerolidol	0.8	1.0	-
2075	2055[a]	Anisaldehyde	-	-	0.1
2102	2100[a]	Eicosane	1.9	3.8	1.6
2110	2095[a]	Hexyl benzoate	0.1	0.3	0.1
2138	2118[a]	Hexahydrofarnesyl acetone	0.4	0.2	-
2159	2144[a]	Spathulenol	0.2	-	-
2163	2148[a]	(*Z*)-3-hexenyl benzoate	0.5	0.6	0.2
2182	2170[f]	(*E*)-2-hexenyl benzoate	0.1	0.2	0.1
2197	2191[a]	3,4-dimethyl-5-pentylidine-2(5H)-furanone	-	tr	-
2202	2200[d]	Docosane	1.7	2.0	1.3
2210	2198[a]	Thymol	-	-	0.1
2228	2223[a]	Methyl hexadecanoate	-	0.3	-
2230	2233[a]	δ-cadinol	0.2	-	-
2242	2239[a]	Carvacrol	-	-	6.8
2265	2278[d]	Torilenol	0.3	-	-
2303	2300[d]	Tricosane	33.8	29.3	34.0
2332	2351[a]	Eudesma-4(15),7-dien-1-β-ol	0.2	-	-
2370	2369[a]	(2*E*,6*E*)-farnesol	-	6.3	0.4
2402	2400[d]	Tetracosane	6.0	5.6	5.7
2416	2376 [a]	Manoyl oxide	0.2	0.7	-
2503	2500[d]	Pentacosane	24.6	21.1	30.8
2602	2600[d]	Hexacosane	1.3	-	0.7
2623	2613[a]	Phytol	-	1.6	-
2703	2700[d]	Heptacosane	12.5	10.2	11.7

Continued…..

Total%	99.1	85.8	97.6
Grouped compounds %			
Alkanes	81.8	72	85.8
Monoterpene hydrocarbones	3.2	0.8	1.1
Oxygenated monoterpenes	-	0.1	7.8
Sesquiterpenes hydrocarbones	2.3	0.8	1.1
Oxygenated sesquiterpenes	2.2	7.3	0.4
Esters	0.7	1.5	0.7
Others	0.9	3.3	0.7

A: *Crataegus azarolus* fresh inflorescences and immature fruits (CaCf), NEU Campus,TRNC;
B: *Crataegus azarolus* dried inflorescences and immature fruits (CaCd), Cengizköy / Lefke, TRNC;
C: *Crataegus pallasii* dried inflorescences and immature fruits (CpE) , Bata region, Libya
LRIEXP: Linear retention indices calculated against n-alkanes by using FID data. LRI LIT*: LRI from literatures: [a](Linstrom and Mallard, 2011) , [b](Babushok et al., 2011), [c](http://www.pherobase.com/database/kovats/kovats-%20detailsulcatone.php),[d](Demirci et al,. 2009), [e](Tekin et al., 2018), [f] (Baser et al., 2006), t: trace (<0.1 percent).

3.1.3. Extraction procedures and extraction yield

To obtain a high extraction yield two extraction methods were evaluated. The results revealed that the Soxhlet extraction method presented a good extraction yield with a lower quantity of solvent (ethanol) as compared to the percolation method as illustrated by Table 3.3 below. So, it was decided that it is the method of extraction that will be undertaken throughout the research.

Table 3.3: Extraction yield by percolation and Soxhlet extraction methods.

Method of extraction	Extraction yield %		
	CaC	CpE	CpT
Percolation	5.27%	5.05 %	4.092%
Soxhlet	20.90%	15.00%	12.84%

CaC: *Crataegus azarolus* from Cyprus, CpE: *Crataegus pallasii* from El-Merj-Libya, CpT: *Crataegus pallasii* from Tripoli –Libya.

3.1.4. Flavonoids in *C.azarolus* and *C.pallasii* in relation to Crataegutt Tropfen®

3.1.4.1. Optimum chromatographic conditions

For quantification of the flavonoids in *C.pallasii* and *C.azarolus* and comparison with that of Pharmaceutical *Crataegus* product Crataegutt® Tropfen, Optimisation of chromatographic conditions was carried out using a one-factor-at-a-time approach

(OFAT) (Sahu et al., 2018). ACN was selected as the optimum mobile phase (Fig.3.4). A column with short-chain aliphatic stationary phases C8 and mobile phase starting from 20 % ACN to a final composition of 60 % ACN in water (Fig. 3.5) in an optimum gradient time of 12 min (Fig. 3.6) at a temperature of 20 °C (Fig. 3.7) with a flow rate of 0.8 mL/min (Fig. 3.8) was chosen for all analytical peaks. Optimum chromatographic conditions are summarized in Table 3.4.

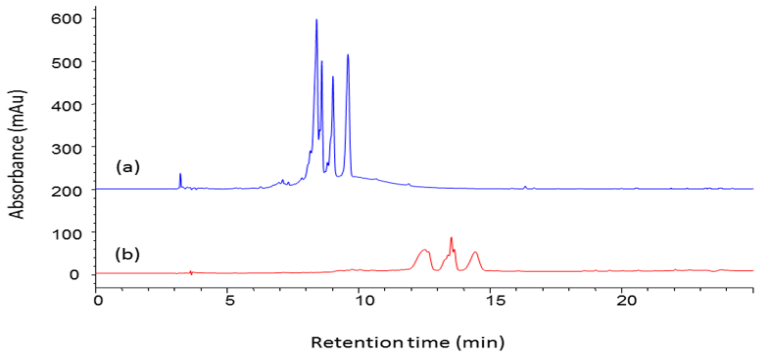

Figure 3.4: Effect of type of organic solvent type in the mobile phase on separation: (a) ACN and (b) methanol.

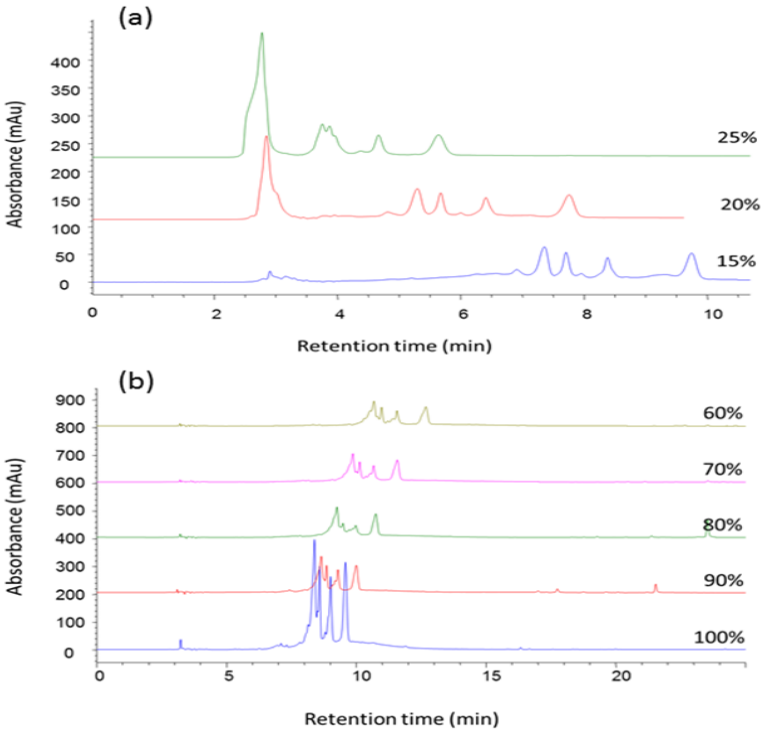

Figure 3.5: Effect of (a) initial and (b) final concentration of ACN in the mobile phase on separation.

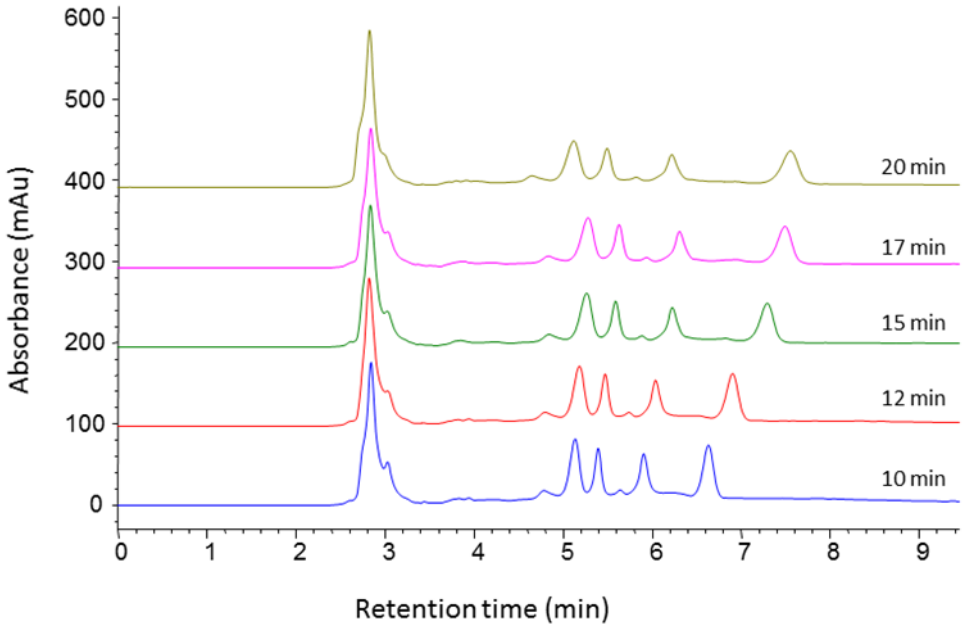

Figure 3.6: Effect of gradient time on separation.

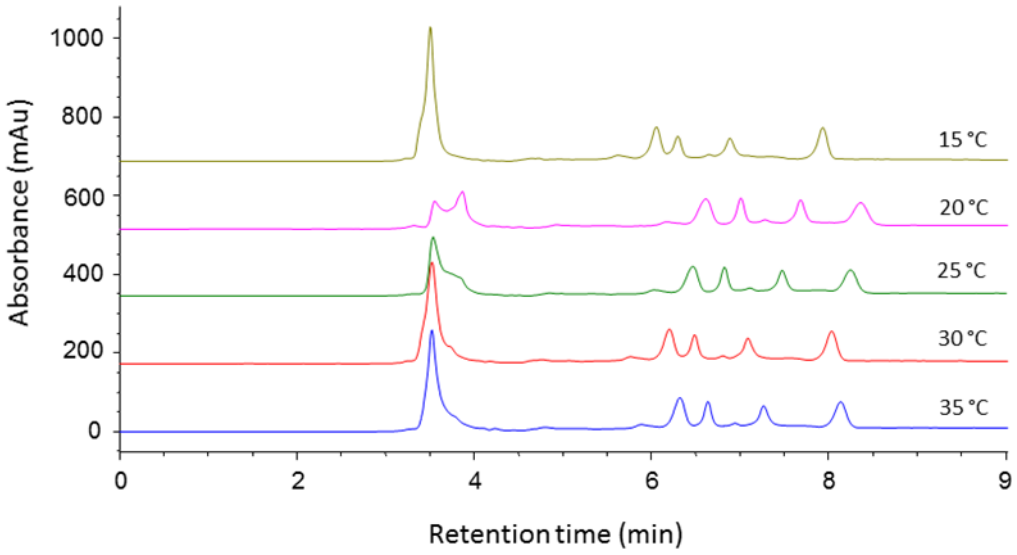

Figure 3.7: Effect of column temperature on separation.

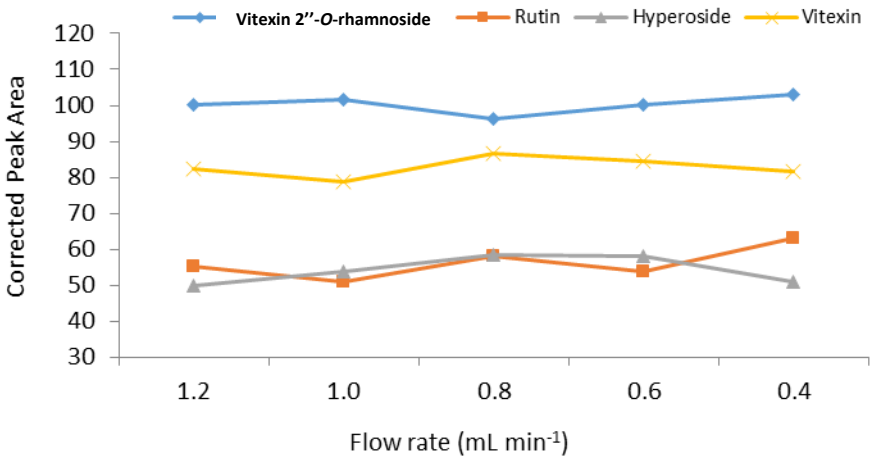

Figure 3.8: Effect of flow rate on corrected peak area.

Table 3.4: Optimum chromatographic conditions.

Column	ACE5-C8, 4.6 mm ID x 25 cm (5 µm)
Detector/wavelength	DAD, 264 nm for hyperoside and 342 nm for the other compounds, bandwidth: 16 nm.
Injection volume (µL)	20
Mobile phase	(A) H_2O: (B) ACN, 20% B at 0 min to 60% B at 12 min
Temperature (°C)	20
Flow Rate (mL min^{-1})	0.8

3.1.4.2. Effect of IDLLME

In this method potential matrix constituents, were extracted into the organic extraction phase, i.e., chloroform, using indirect-dispersive liquid-liquid microextraction (IDLLME), and compounds remained in the aqueous phase. The baselines obtained in both cases were compared (Fig.3.9). It was found that IDLLME was an efficient sample clean-up method, which significantly reduced the matrix effect.

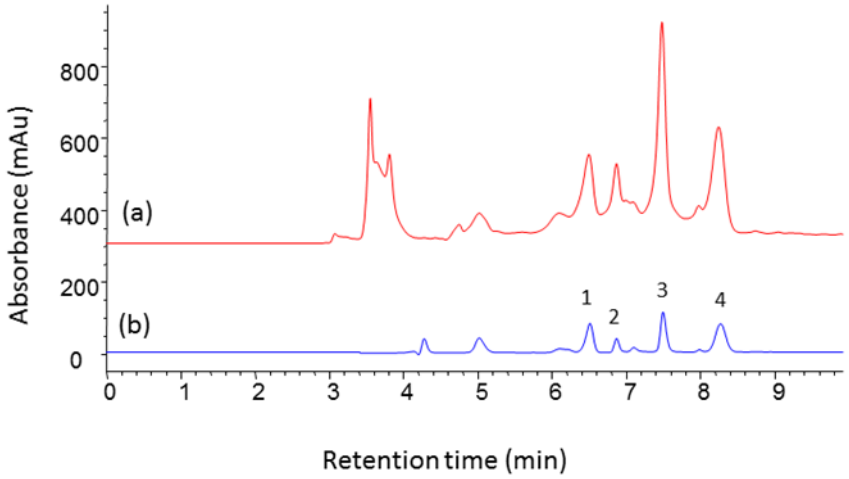

Figure 3.9: Effect of IDLLME on the baseline, (a) before (b) after IDLLME, Peaks: 1, vitexin 2"-*O*-rhamnoside, 2, rutin, 3, vitexin, and 4, hyperoside.

3.1.4.3. Analytical Performance

Standard-addition calibration graphs were plotted by spiking known concentrations of the standards into the sample and plotting the peak area versus concentrations of standard solutions within the range of 0 - 25.0 mg/L, with each measurement repeated three times (n=3). Analytical figures of merit of the method were found as follows: Limits of detection (LOD), (calculated based on $3S_b/m$) and LOQ limits for quantitation (calculated based on $10S_b/m$), where S_b is the standard deviation of the intercept and m is the slope of the regression equation, ranged from 0.4 to 3.4 mg/g and from 1.3 to 11.3 mg/g, respectively. Linear regression equations were presented as $Y = aX + b$ (where, Y is the peak area, a is the slope of the calibration graph, X is the analyte concentration in mg g^{-1} and b is the intercept). The response was found to be linear with coefficients of determination (R^2) higher than 0.9950. The results showed good precision, which was expressed as percent relative standard deviation (%RSD) at all concentrations of the analytes with intraday and interday precisions ranging from 1.0 to 2.8 and from 1.5 to 4.3, respectively which indicate good precision of the method. The method showed high accuracy based on the relative recovery of the analytes shown to be more than 98 % by spiking known concentrations of the standards (5.0, 10.0, and 15.0 mg/L into a fixed amount of samples of *C. pallasii*, *C. azarolus*, and Crataegutt® Tropfen. Method validation

parameters are indicated in Table 3.5. From the chromatograms (Fig. 3.9 and 3.10), four peaks were identified as vitexin 2"-*O*-rhamnoside, rutin, vitexin, and hyperoside by comparing their retention times and the UV spectra with those obtained with the standard compounds. Using single variable analysis of variance ANOVA, the P values of the concentration of each analyte between pairs of selected samples of *C. pallasii, C. azarolus*, and Crataegutt® Tropfen were compared. In general, $P<0.05$ for all comparisons indicates that there is a significant difference in the concentration of each analyte between the samples except for vitexin 2"-*O*-rhamnoside where $P> 0.05$ which indicates no significant difference between the pair of *Crataegus* species (CaC and CpE). Percentage mass concentration (%, w/w) of the four analytes found in, *C. pallasii*, *C. azarolus*, and Crataegutt® Tropfen are given in Table 3.6 and Fig. 3.11.

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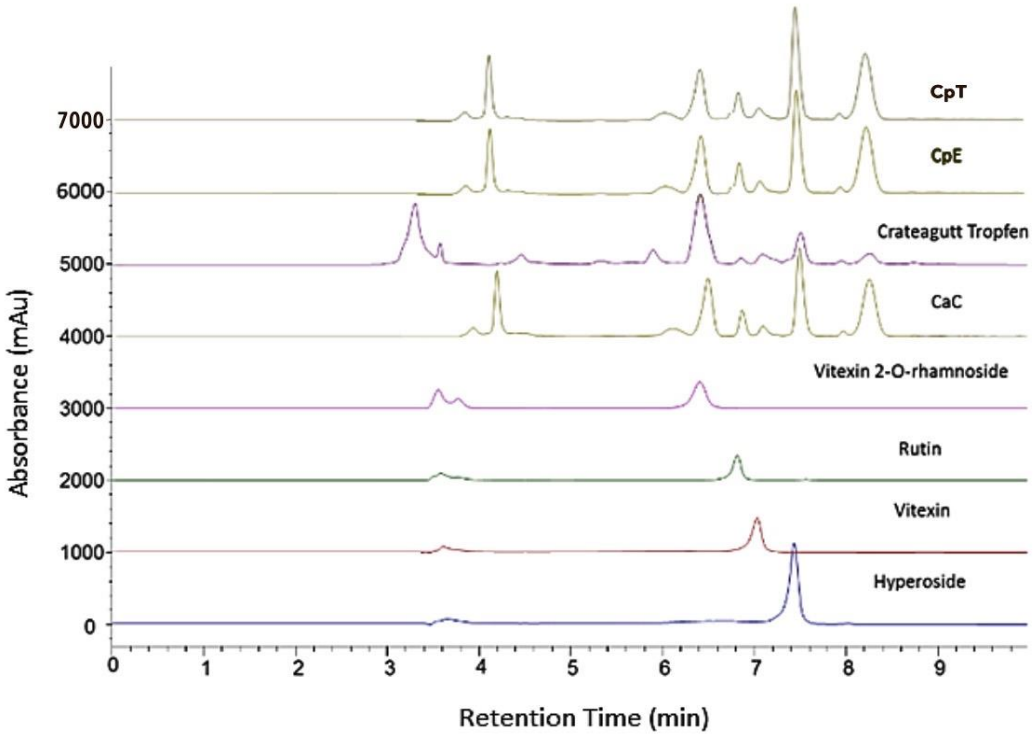

Figure 3.10: Peak characterisation in the crude extracts and standard compounds standards: *Crataegus pallasii*; (CpT), (CpE), Crataegutt®Trophen, *Crataegus azarolus* (CaC).

Table 3.5. : Validation parameters of RF-HPLC-DAD method for standard compounds determination

Parameter	Analytes			
	Vitexin 2"-*O*-rhamnoside	Rutin	Vitexin	Hyperoside
Regression[a] equation	y = 49.5(±0.66) x+611.21(±9.93)	y = 26.9(±0.13) x+636.26(±2.0)	y = 26.5(±0.24) x+552.8(±3.59)	y= 43.04(±0.41) x +476.3(±6.20)
R^2	0.9970	0.9970	0.9990	0.9980
LOD[b]	1.0	0.4	2.2	3.4
LOQ[c]	3.3	1.3	7.3	11.3
%RSD[d](intraday)	2.8	1.0	2.5	1.9
%RSD[d](interday)	3.2	1.5	4.3	3.0
Relative Recovery %RR				
Added (mg/l)				
5	100.73±1.02	100.09±0.13	98.95±1.50	99.40±0.85
10	98.21±2.55	99.53±0.67	98.91±1.55	99.95±0.07
15	98.67±1.90	99.75±0.35	99.48±0.74	101.62±2.28

[a] $Peak\ area\ =\ Slope(\pm SD)\ \times\ [Analyte\ concentration\ (mg/l)] +\ Intercept(\pm SD)$
[b]Limit of detection (mg/g)
[c]Limit of quantitation (mg/g)
[d]Percentage relative standard deviation, n= 3

Table 3.6: Concentration of standard compounds in the real samples.

Sample	Concentration (%, w/w)			
	Vitexin-2"-*O*-rhamnoside	Rutin	Vitexin	Hyperoside
CaC	4.4±0.08*	2.9±0.03	1.7±0.07	4.4±0.23
CpE	4.4±0.08*	2.6±0.05	1.4±0.02	4.8±0.12
CpT	$3.8\pm5.44x10^{-16}$	2.4±0.04	1.4±0.02	8.2±0.59
Crataegutt®Tropfen	1.6±0.05	1.0±0.07	0.6±0.02	0.4±0.02

CaC: *C. azarolus* from Cyprus; CpE: *C. pallasii* from El-Merj; CpT: *C. pallasii* from Tripoli.
*P>0.05

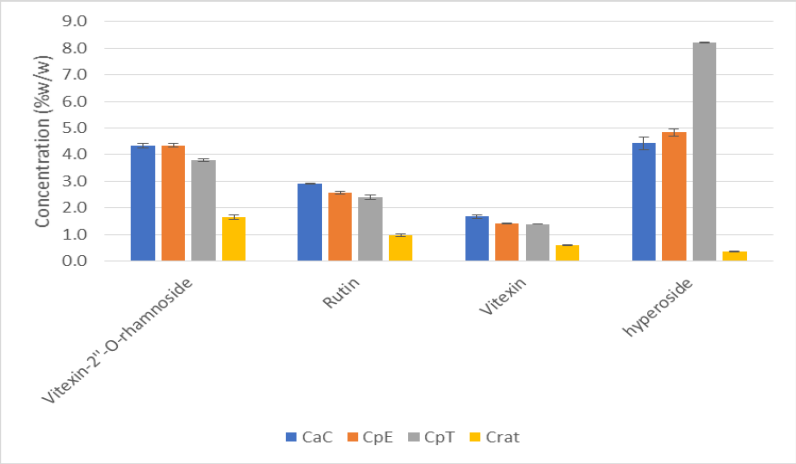

Figure 3.11: Percentage content of standard compounds. CaC: *Crataegus azarolus*, CpT: *Crataegus pallasii* from Tripoli, CpE: *Crataegus pallasii* from El-merj city, Crat: Crataegutt® Tropfen.

3.1.5. Effect of *Crataegus* extracts on isolated segments of human saphenous vein (SV)

The total ethanolic extracts of Libyan *C. pallasii* and Cypriot *C. azarolus* were tested for the first time on the human saphenous vein segments with intact endothelium obtained from patients (3 males, aged 53±3) who had undergone coronary artery bypass surgery. Pre-contraction levels obtained with Phenylephrine (1–10 µM) is 3.22±1.03 g. The maximal relaxation (E_{max}) induced by *C.pallasii* or *C.azarolus* is 9.5±4.4 %. The endothelium capacity of SV preparations is 4.2±2.5%. Thus, the results showed no relaxant effect on the phenylephrine contracted human saphenous vein segments. This result could be proposed to factors as environmental species variability (Fig. 3.12, Table 3.7).

Table 3.7. The relaxant effect of *Crataegus* extract (*C.pallasii* and *C.azarolus*) and Acetylcholine on saphenous vein preparation pre-contracted by phenylephrine

	E_{max}	n
Crataegus extract	9.5±4.4 %	3
Acetylcholine	4.2±2.5 %	3

E_{max} indicates maximal relaxation expressed as a percentage of precontraction induced by Phenylephrine. Values are means ± s.e.mean derived from (n) different patients.

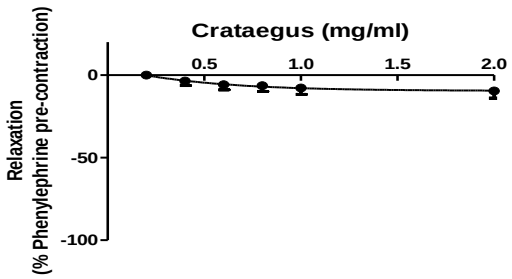

Figure 3.12: The relaxation induced by *C.azarolus* or *C.pallasii* (0.2-2.0 mg/mL) in pre-contracted saphenous vein (SV) preparations. Values are means ± s.e.mean derived from (n=3) different patients.

3.1.6. Cell viability and cytotoxicity of *Crataegus* Extracts.

MCF-7 cells were treated with different concentrations of (5-100 µg/ml) *C. pallasii* and *C. azarolus*, extracts for 24 and 48 hours. The cell viability was determined as described above in (section 2.7) by MTT assay. Our results showed that the total ethanolic extract of *C. pallasii* (CpE) was only slightly effective in terms of reduction of cell viability at 100 µg/mL doses, incubated for 48 h (Fig. 3.13). On the other hand, Cypriot *C. azarolus* (CaC) and *C. pallasii* (CpT) extracts didn't show cell viability reduction effects on MCF-7 cells at any concentration, incubated for 24h and 48 h (Fig. 3.14 and 3.15). Therefore, overall results suggest that the *C. pallasii*, *C. azarolus*, extracts are ineffective for reduction of cell viability and show the absence of cytotoxic effects in breast cancer MCF-7 cells *invitro* conditions.

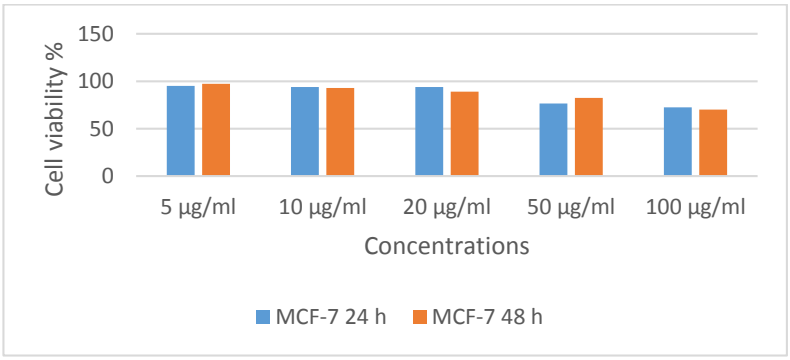

Figure 3.13: Effect of different concentrations of *C. pallasii* (CpE) extract on cell viability of MCF-7 cells at two incubation periods

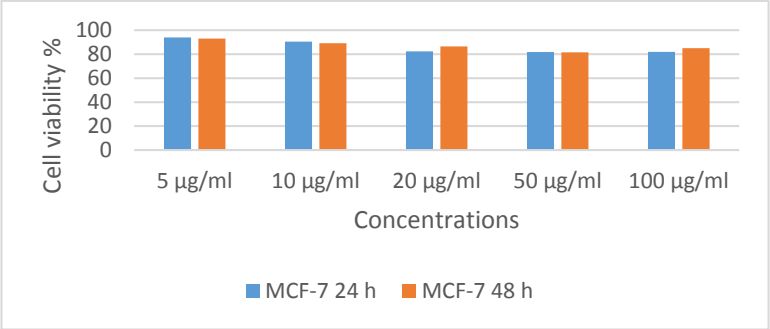

Figure 3.14: Effect of different concentrations of *C. azarolus* (CaC) extract on cell viability of MCF-7 cells at two incubation periods

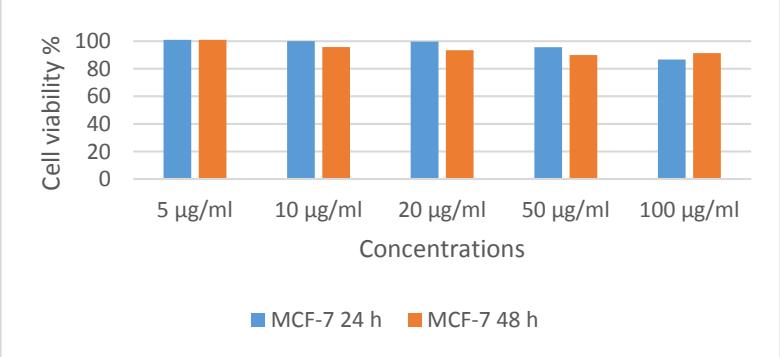

Figure 3.15: Effect of different concentrations of *C. pallasii* (CpT) extract on cell viability of MCF-7 cells at two incubation periods

3.2 Discussion.

Preliminary phytochemical screening is a vital step for the detection of important metabolites and discovers the bioactive profile of plants of therapeutic importance in which may alternatively lead to drug discovery (Karande et al., 2016). The results of the phytochemical screening tests of *C.pallasii* and *C.azarolus* leaf and flower extracts revealed the highest concentration of flavonoids, in water and ethanolic extracts and moderate concentrations of saponins and tannins respectively. While a very low concentration of flavonoids was detected in the dichloromethane extract (Table 3.1). The researchers Lakache et al., 2016 also reported the results of the phytochemical screening of the leaves and flowers of *C.azarolus* from Algeria, indicating the presence of a high concentration of flavonoids in addition to tannins, steroids, and triterpenes.

Since our research focuses on the use of *C.pallasii* and *C.azarolus* as raw plant material for the production of *Crataegus* derived phytomedicines and the literature points to the fact that flavonoids are the group of compounds responsible for the bioactivity of *Crataegus* species. It was obvious to follow extraction with ethanol.

While many researches were directed to the bioactive compounds in various *Crataegus* species, little attention has been awarded to the volatile oil constituents (Edwards et al., 2012). Although in low concentrations the volatile oil constituents could contribute to the synergic effect of the plant and explain some therapeutic effects like those related to the CNS such as the sedative effect (Wang et al. 2013). Therefore, another step was taken to evaluate the volatile constituents of *C.pallasii* and *C.azarolus* essential oil which was obtained by the hydrodistillation method of the inflorescence and unripe fruits of fresh *C.azarolus* and dry samples of *C.azarolus* and *C.pallasii* (Table 3.2). The results indicate that fourteen compounds were common to all samples of *Crataegus* species tested; fresh *C.azarolus* (CaCf), dry *C.azarolus* (CaCd), and dry *C.pallasii* (CpE). The major outstanding essential oils were the alkanes namely; tricosane (33.8 %, 29.3 % and 34.0 %), pentacosane (24.6 %, 21.1 % and 30.8 %), heptacosane (12.5 %, 10.2 % and 11.7 %) and tetracosane (6.0 %, 5.6 %, and 5.7 %), respectively. Whereas, docosane (1.7 %, 2.0 %, and 1.3 %), showed lower concentrations in all samples. In addition to the above mentioned compounds nonanal (0.2, 0.2, 0.1 %), β-elemene (0.1, 0.2, 0.2%), undecanal (0.1, 0.3, 0.2 %), β-caryophyllene (0.3, 0.2, 0.2 %), farnesene (0.3, 0.4, 0.4 %) , eicosane (1.9, 3.8, 1.6 %), hexyl benzoate (0.1, 0.3, 0.1 %), (z)-3-hexenyl benzoate (0.5, 0.6, 0.2 %), and (E)-2-hexenyl benzoate (0.1, 0.2, 0.1 %) were also detected in all samples at lower concentrations.

According to the results thirteen compounds were detected in CpE uncommon to CaCf *and* CaCd samples; α-terpinene (0.1 %), γ-terpinene(0.7 %), linalool(0.4 %), terpinen-4-ol (0.3 %), borneol (0.2 %), β-bisabolene (0.3 %), carvone (tr), carvacryl acetate (0.3 %), phenylethyl alcohol (0.1 %), (*E*)-β-ionone (0.1 %), anisaldehyde (0.1 %), thymol (0.1 %), carvacrol (6.8 %). It is worth mentioning here that the essential oils, carvacryl acetate and carvone are determined for the first time from *Crataegus* species in *C. pallasii* essential oil. Uncommon to CaCf and CpE six essential oils were determined only in CaCd samples; (E)-β-damascenone (0.1 %), (Z)-geranyl acetone

(0.1 %), 2,2,4-trimethyl-3-carboxyisopropyl pentanoic acid isobutyl ester (0.1 %), 3,4-dimethyl-5-pentylidine-2(5H)-furanone (tr), methyl hexadecanoate (0.3 %), phytol (1.6 %). In CaCf samples twelve compounds were determined which were not common to the other two samples; α-copaene (0.2 %), germacrene D (0.2 %), β-selinene (0.2 %), α-selinene (0.1 %), valencene (0.5 %), δ-cadinene (0.2 %), selina-3,7(11)-diene (0.2 %), caryophyllene oxide (0.5 %), spathulenol (0.2 %), δ-cadinol (0.2 %), torilenol (0.3 %), eudesma-4(15),7-dien-1-β-ol (0.2 %). Whereas, only five essential oils compounds were common to both samples of *C.azarolus* (CaCf and CaCd) but with varying concentrations: α-pinene(0.9, 0.3 %), Limonene (1.9, 0.5 %), (*E*)-nerolidol (0.8, 1.0 %), Hexahydrofarnesyl acetone (0.4, 0.2 %), Manoyl oxide (0.2, 0.7 %) respectivly. In general, this variation is related to fact that samples has been extracted in different conditions (dry and fresh) and from different localities in Cyprus.

While this is the first report on the essential oil of Libyan *C. pallasii* and *C.azarolus* growing in Cyprus, the essential oil of *C. azarolus* species in other parts of the world has been studied by other researchers. Some results were per our results whereas, others were completely different. As many Cypriot homes are famous for the jam made from *Crataegus* a study was conducted on the volatile compounds of commercial jam made from *C. azarolus* in Cyprus by the simultaneous distillation–extraction method which revealed the presence of 44 components that were identified by GC-MS. The major constituent was 2-furaldehyde (21.4 %). Two compounds γ –Terpinene (0.2 %) and Limonene (1.9 %) were also reported by our study (Hadjimitsi and Zabetaki, 2005).

Lakache and his colleagues reported sixty-one compounds from the essential oil of leaves and flowers of *C. azarolus* growing in Algeria by two extraction methods; hydrodistillation assisted by microwave heating (HD-MW) and hydrodistillation (HD). The outstanding groups of essential oils were acids and esters (12 compounds) 27.5 % for HD-MW, 50.7 % for HD respectively. The alkanes and alkenes (17 components) comprised 40.8 % for HD-MW, and 29.5 % for HD. Corresponding with our study, a high concentration of the alkane group, is reported in addition to seventeen other volatile compounds that were in accordance with our study; Hexyl benzoate (2.7, 8.5 %), (*E*)-2-hexenyl benzoate (1.2, 2.0 %), tetracosane (1.7, 1.5%), pentacosane (0.6, 0.5 %), hexacosane (0.2, 0.1 %), heptacosane (4.9, 3.7 %), tricosane (16.7, 12.3 %),

β-elemene (0.1, 0.1 %), β-damascenone (tr., 0.3 %), α-farnesene (16.9, 7.2 %), caryophyllene oxide (0.2%, tr.), hexahydrofarnesyl acetone (0.1, 0.4 %), (*E*)-nerolidol (0.3, 0.4 %), undecanal (0.3, 0.6 %), nonanal (0.1, 0.3 %), Phytol (2.4, 0.3 %), and limonene (tr.,tr.) (Lakache et al., 2014). The Algerian *C. azarolus* which was also investigated by Amina et al., 2018. The fraction of the silica gel column of the $CHCl_3$ extract of the young branches, leaves, and flowers revealed five volatile compounds; 2,4-bis (1,1-dimethyl ethyl)-phenol, tridecanoic acid 12-methyl-methyl ester, pentadecanoic acid 14-methyl-methyl ester, 8-octadecanoic acid methyl ester, and isobutyl nonyl phthalate.

Another study that was conducted on the chemical profile of *C. azarolus* fruits in Northern Italy, five monoterpenes were documented; phellandrene, sabinene, γ-terpinene, terpinolene, and limonene constituting a total of 15.4 % of the total oil composition. Only one compound, limonene was common with our results (Donno et al.,2017).

In Persian nutrition culture and folk medicine, *C. azarolus* leaf and fruits are used to make hydrosol beverages for the treatment of cardiovascular diseases. When analysed by GC–MS, reveal the presence of hexadecanoic acid (7.7 %), p-xylene (20.1 %), thymol (28.7 %), and thymolethanoate (Hamediet al., 2017).

Thus, studies on the essential oil of *C.azarolus* have recorded considerable variation. This explains that the formation of essential oil in plants is extremely effected by climatic conditions, genetic background, and other factors as part of the plant distilled, stage of development, and method of extraction (Baser and Buchbauer, 2010). The essential oil of *C.azarolus* and other *Crataegus* species was discussed in more detail in (Sections 1.1.3.2, 1.2.2.3. and Table 1.8). Scientific studies also document that compounds as tricosane, pentacosane, hexacosane which were the major compounds in both *C.pallasii* and *C.azarolus* are considered important in pest management programs (Sharma et al., 2019). Therefore, the alkanes concentration of *C. pallasii* and *C. azarolus* could be evaluated for these purposes. Our results also reveal that *C.pallasii* has a high concentration of Oxygenated monoterpenes (7.8 %) of the total oil concentration. The literature documents that Oxygen-containing monoterpenes exhibit certain pharmacological actions as; antispasmodic, sedative, tranquilizing and is beneficial to metabolic processes in the human body (Gurib-

Fakim, 2006) which explains some pharmacological activities of *Crataegus* species (Kumar et al., 2012) and the possible use *C. pallasii* to treat these ailments. The antimicrobial compound (2*E*,6*E*)-farnesol (Jabrsa et al., 2006) with a concentration of 6.3 % in dry *C.azarolus* (CaCd) could explain the antimicrobial activity *C.azarolus* that has been documented by the researchers (Section 1.2.2.4.) and enhance the use of this *Crataegus* species as antimicrobial agent.

Since extraction forms are a vital step in medicinal plant research, as the preparation of crude extracts is the starting point in the isolation of chemical constituents from plant material. However, the varying and insufficiently defined composition of extracts investigated as a result of different raw materials and extraction methods, makes comparison of studies very difficult. However, the efficiency of soxhlet extraction has been demonstrated by many researches. In the fact that it is an automated and simple extraction method which requires less time and less solvent, with a higher recovery yield of total extract as compared with percolation. Moreover, it is regarded as an efficient method for extraction of phenolic compounds from plant material (Seidel, 2012; Zhang et al., 2018). On the other hand, as extracting solvent ethanol, revealed a high extraction yield of phenolic content (Abu-Gharbieh and Shehab, 2017). Accordingly, and in confirmation with our results (Table 3.3), Soxhlet apparatus extraction using ethanol as the extracting solvent has been undertaken throughout our research study.

In order to determine the availability of *C.pallasii* and *C.azarolus* for production of phytopharmaceutical *Crataegus* derived products a RP-HPLC-DAD analysis was carried out. For this purpose the bioactive flavanoids in the well-known *Crataegus* drug Crataegutt® Trophen were evaluated in relation to the bioactive flavonoids in the ethyl acetate extracts of *C. pallasii* and *C. azarolus*. However, many studies has investigated *Crataegus* based preparations (Elsadig and Kuhnert, 2017; Schroder et al., 2003) but this is the first comparative report between *C.pallasii*, *C.azarolus* and Crataegutt® Trophen. Preliminary HPLC chromatograms of the ethanolic extract revealed separation of overlapping peaks was not possible due to impurities. Nonetheless, previous studies have mentioned that the ethyl acetate fraction of the total ethanolic extract is highly enriched with flavonoids (Lakache et al., 2016). Thus, as a

further step, fractionation of total ethanolic extract was carried out to obtain the ethyl acateate fraction with low impurities and enriched with flavonoids (Fig. 2.1).

Optimisation of the RP-HPLC-DAD method was carried out using a one-variable-at-a-time approach (Sahu et al., 2018). Short-chain aliphatic stationary phases (i.e., C4 and C8) were considered for preliminary experiments. A gradient scan using both columns with a mobile phase consisting of acetonitrile (ACN) and water over the range of 0 to 100 % (v/v) ACN within 20 min revealed that C8 was more suitable for separation. Compared with methanol, ACN showed a better resolution and separation efficiency and hence, the latter was selected as the optimum mobile phase (Fig.3.4). Preliminary experiments also revealed that isocratic elution was not possible. Optimum baseline resolution was obtained using a gradient composition starting from 20 % ACN to a final composition of 60 % ACN in water (Fig. 3.5). An optimum gradient time of 12 min was chosen based on peak efficiency and resolution (Fig. 3.6). The influence of column temperature was examined within the range of 15-35 °C; optimum resolution and retention time were obtained at 20 °C for all analytical peaks (Fig. 3.7). Hence, this temperature was kept constant in consecutive experiments. The effect of flow rate was evaluated by plotting corrected peak area against flow rate. The effect was variable for the major peaks but there was a correlation between the analytical peaks having a maximum at 0.8 mL/min (Fig. 3.8). Optimum chromatographic conditions are summarized in Table 3.4.

Again, initial chromatograms showed elevated baselines due to matrix effect posing a risk for error in quantitation especially when the analytes were present at low concentrations near the limit of quantitation (LOQ). To avoid this error DLLME technique was proposed which is primarily used for this purpose by many researchers (Maryam et al., 2019; Lee et al., 2019b). However, due to the polar nature of the compounds under consideration IDLLME was developed for the first time to overcome this problem. Contrary, to the conventional DLLME, in IDLLME potential matrix constituents, which would interfere with the analytical peaks, were extracted into the organic extraction phase, i.e., chloroform, while the compounds remained in the aqueous phase. The baselines obtained in both cases were compared (Fig.3.9). It was found that IDLLME was an efficient sample clean-up method, which significantly reduced the matrix effect.

From the chromatograms four peaks were identified as vitexin 2"-*O*-rhamnoside, rutin, vitexin, and hyperoside by comparing their retention times and the UV spectra with those obtained with the standard compounds. The percentage concentration of each compound in the samples under investigation is illustrated in Table 3.6 and Figure 3.11. *C.pallasii* (CpE and CpT) showed a concentration of hyperoside (4.8 %, 8.2 %) in respect to *C.azarolus* (4.4 %). Whereas, *C.azarolus* (4.4 %) and *C.pallasii* (CpE) both had a percentage concentration of 4.4 % for vitexin 2"-*O*-rhamnoside compared to *C.pallasii* CpT (3.8 %). It was also clear that *C.azarolus* recorded the same concentration (4.4 %) for both; vitexin 2"-*O*-rhamnoside and hyperoside.

On the other hand, *C.azarolus* revealed a slightly higher concentration of rutin (2.9 %) and vitexin (1.7 %) as regards to *C.pallasii* (CpE and CpT) (2.6 % and 2.4 %) and (1.4 % and 1.4 %) respectively. These results reveal evidently that there is a slight variation in the percentage concentration of flavonoids, vitexin 2"-*O*-rhamnoside, rutin, and vitexin between the *Crataegus* samples except for hyperoside in *C.pallasii* (CpT) (8.2 %) which showed a high concentration as compared to *C.azarlous* and *C.pallasii* (CpE) from El-merj city, which is related to environmental factors.

On the other hand, Crataegutt® Trophen showed a lower concentration towards all compounds as compared to *Crataegus* samples. According to Crataegutt®Tropfen the percentage concentration of vitexin 2"-*O*-rhamnoside (1.6 %) which is regarded as one of the most important constituents in *Crataegus* herbal preparations (Xixiang et al., 2007) is four times that of hyperoside (0.4 %). More information on Crataegutt®Tropfen and *Crataegus* related products is presented in Section 1.1.6.

Since flavonoids are active metabolites in *Crataegus* species, they are used for standardisation in many international Pharmacopoeias. According to the USP Monographs 2009, the standardised extract of *Crataegus* leaf and flowers is required to contain not less than 0.6% of C-glycosylated flavones, expressed as vitexin and not less than 0.45% of *O*-glycosylated flavones expressed as hyperoside. On the other hand, the European Pharmacopoeia 2008 states that the standardised hawthorn leaf and flower extract is required to contain at least 0.8% - 3% of total flavonoids based on hyperoside. Following these requirements and comparison with the quantitative results obtained in this study related to the herbal-derived product Crataegutt® Tropfen, the ethyl acetate extract of *C. pallasii* and *C. azarolus* meet the criteria stated by the

pharmacopeias. Consequently, The Libyan *C.pallasii* and Cypriot *C.azarolus* are proposed as a novel plant crude natural material for the production of *Crataegus* phytopharmaceuticals.

Mostly, pharmacological studies that have been conducted to evaluate the vasorelaxant effect of plant extracts or pure compounds on isolated tissues *invitro*. As mentioned earlier in Section 1.1.5.4.1., several studies referred to a significant vasorelaxant effect of *Crataegus* species extracts on rat aortic rings or human coronary artery. In this research study, the total ethanolic extracts of *C. pallasii* and *C. azarolus* was tested for the first time on the human saphenous vein segments with intact endothelium obtained from patients (3 males, aged 53±3) who had undergone coronary artery bypass surgery. However, the results displayed in Table 3.7 and Figure 3.12 demonstrated that the ethanolic extract of both *C.pallasii* and *C.azarolus* showed no relaxant effect on the phenylephrine contracted human saphenous vein segments. A result that could be proposed to factors as environmental species variability.

It is obvious that the Cytotoxic activity of *Crataegus* species does exist which is not only evident from scientific evidence but also surveys on traditional medicine. The scientific information and the ethnobotanical knowledge on *Crataegus* species reporting its anticancer activity are discussed in some detail in Section 1.1.5.2 and particularly for *C.azarolus* in Section 1.2.2.4.2. The fact that this activity is related to certain cell lines and not available to others is conventional as the sensitivity of a test substance could differ towards different cell lines. *Crataegus* species showed anticancer activity particularly from fruit extracts (Mortazavi-Derazkola et al.,2020), fruit peel (Li et al., 2013), and flower bud extract (Rodrigues et al., 2012) on MCF-7 cells. While *C.azarolus* fruit extracts showed moderate activity towards MCF-7 cell lines (Mraihi et al.,2015). In our study, the cell viability and cytotoxicity tests were also carried out on MCF-7 cells and treated with different concentrations of the total ethanolic leaf and flower extracts of *C. pallasii*, and *C. azarolus*. *C.pallasii* (CpE) showed a slight reduction of cell viability at 100 µg/mL doses, incubated for 48 h. Whereas, CaC and CpT relayed ineffective (Fig.: 3.13, 3.14, 3.15) It must be taken into consideration that this is the first report about Libyan *C.pallasii* and Cypriot *C.azarolus* total extracts evaluation towards MCF-7 cells.

CHAPTER 4

Conclusion

Crataegus is regarded as an important medicinal plant for the treatment of several ailments especially those related to cardiac problems. Many researchers had proved the therapeutic effects of some of these species and evaluated its chemical metabolites. But although the chemistry of the genus *Crataegus* has been described intensively, yet the chemical composition of many *Crataegus* species is to be characterized. In this research two species of *Crataegus* have been under investigation, *Crataegus pallasii* Griseb. and *Crataegus azarolus* L. *C. pallasii* was collected from two regions in Libya; (CpE) collected from Bata region in El-merj city and (CpT) purchased from attar from the old city in Tripoli. In Libya, *C.pallasii* is used by the natives for the treatment of cardiac problems (Jafri and EL- Gadi, 1977). However, the results of our study reported a variation in the results of these two *C.palllasii* samples, this diversity between the same species is attributed to adaptation and environmental variation. *Crataegus azarolus* which is distributed in different regions in North Cyprus is mainly used by the natives as an edible fruit and in preparation of jam (Ciftcioglu, 2015). On the other hand, *C.azarolus* is one of the *Crataegus* species that has been inscribed in the pharmacopeias (European Pharmacopoeia 6.0, 2008) and researchers from different regions in the world have pointed out to its phytochemical and the biological activity (Sections: 1.2.2.3. and 1.2.2.4).

In this study, we characterised the phytochemical content and the biological activity of the Libyan *C.pallasii* and Cypriot *C.azarolus* species since no work has been directed towards them so far. In a step to present these two species for the production of herbal drugs derived *Crataegus* phytomedicines.

For this purpose, simple preliminary phytochemical tests was the first step to scan important groups of metabolites in *C.pallasii* and *C.azarolus*. In accordance with the reported literature, the results revealed a high concentration of flavonoids and a moderate concentration of saponins and tannins (Table 3.1) and the absence of alkaloids, cardiac glycosides, and sterols in both water and ethanolic extracts.

Besides the scan for the non-volatile constituents, the volatile oil of *C.pallasii* and *C.azarolus* was also investigated by the hydrodistillation method. Following the reported literature about the volatile constituents of other *Crataegus* species, the major compounds identified in our study were the alkane series namely; tricosane, pentacosane, heptacosane, tetracosane, eicosane, and docosane, respectively (Table 3.2).

To prepare total extracts of *C.pallasii* and *C.azarolus* for identification and quantification of bioactive flavonoids by HPLC analysis and to perform the biological studies, Soxhlet apparatus extraction was selected as it revealed a higher recovery yield of total extract then percolation extraction method using ethanol as the solvent of extraction (Table 3.3).

Crataegus phytopharmaceutical drug Crataegutt®Tropfen was evaluated with *C.pallasii* and *C.azarolus* using RP-HPLC-DAD to determine the availability of *C.azarolus* and *C.pallasii* for production of phytopharmaceutical *Crataegus* derived products. The chromatograms revealed the presence of four peaks identified as vitexin 2"-*O*-rhamnoside, rutin, vitexin, and hyperoside by comparing their retention times and the UV spectra with those obtained with the standard compounds. The results indicated that the percentage concentration of the flavonoids detected are in correspondence with the pharmacopeias and since the standardised preparations of medicinal plants are compelling for future therapies, this research provides scientific evidence on the fact that the Libyan *C.pallasii* and Cypriot *C.azarolus* are a good source for the production of *Crataegus* derived herbal pharmaceutical drugs.

The biological part of this thesis was carried out with the total ethanolic extract to determine the relaxant effect on the human saphenous vein segments with intact endothelium and the cytotoxic activity on MCF-cells. The results obtained were negative which could be considered to many factors; the genotype, locality, and /or to extraction protocol, variability (Sahloul et al., 2009; Jurikova et al., 2012).

Finally, this research study displays for the first time scientific information regarding two *Crataegus* species; namely, *C.pallasii* from Libya and *C.azarolus.* These plant species might offer a novel promising therapy that is beneficial for general health. However, more clinical studies are needed to provide scientific data and

establish their effectiveness, and toxicity especially in humans, and emphasise their economic importance as for the production of herbal drugs.

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Volatile Oil Constituents of *Crataegus azarolus* L. and *Crataegus pallasii* Grisb.

Najat Agiel[*1,2], Duygu Yiğit Hanoğlu[3], Azmi Hanoğlu[1], Kemal H. C. Başer[1] and Filiz Mericli[1,4]

[1] *Near East University, Faculty of Pharmacy, Department of Pharmacognosy, Nicosia – Turkish Republic of Northern Cyprus*

[2] *The University of Tripoli, Faculty of Education, Department of Biology, Libya*

[3] *Near East University, Faculty of Pharmacy, Department of Pharmaceutical Botany, Nicosia – Turkish Republic of Northern Cyprus*

[4] *Near East University, Faculty of Pharmacy, Department of Phytotherapy, Nicosia – Turkish Republic of Northern Cyprus*

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Abstract: In this study the volatile oils constituents of the inflorescence and unripe fruits of *Crataegus azarolus* L. and *Crataegus pallasii* Grisb. were investigated. Fifty-four compounds were identified by GC and GC-MS analysis. The major outstanding constituents of the essential oil in all samples (dried and fresh *C. azarolus* and dried *C. pallasii*) were; tricosane (33.8%, 29.3%, 34.0 %), pentacosane (24.6%, 21.1%, 30.8%), heptacosane (12.5%, 10.2%, 11.7%), and tetracosane (6.0%, 5.6%, 5.7%), respectively. Besides these alkanes, ten compounds (nonanal, β-elemene, undecanal, β-caryophyllene, (*E, E*)-α-farnesene, eicosane, hexyl benzoate, (*Z*)-3-hexenyl benzoate, (*E*)-2-hexenyl benzoate, docasane) were determined in all samples. Carvacrol, carvacryl acetate, carvone, and thymol were determined for the first time from *C. pallasii* essential oil. (*E*)-β-damascenone was determined only in dried *C. azarolus* oil; sesquiterpene compounds valencene, α-selinene and β-selinene, δ-cadinene, germacrene D, selina-3,7(11)-diene, spathulenol, and δ-cadinol were determined only in fresh *C. azarolus* sample. On the other hand (2*E*,6*E*)-farnesol was determined in dried *C. azarolus* and *C. pallasii* samples.

Keywords: *Crataegus azarolus*; *Crataegus pallasii*; volatile constituents; GC-MS analysis.

1. Introduction

Crataegus species (Rosaceae), more commonly known as "Alıç", "Yemişen" or "Mosphilla" in Cyprus and as "Zaarour" in the Middle Eastern Countries, is a diverse genus of flowering, fruit bearing shrubs or small trees that grow mostly in temperate zones, including countries of North Africa, Europe and Mediterranean basin. Western Asia. India. China and North America [1,2]. *Crataegus* species (Hawthorn) have been used traditionally since ancient times and the first report ofpatients treated with *C. oxyacantha* that were sufferinz from various heart illnesses was in 1896 [3].

Researches documented that bioflavonoid-like complexes appeared to be primarily responsiblefor the cardiac actions of the plant which included oligomeric procyanidins (OPC) and

*Corresponding author: E-Mail: najataghil@yahoo.com; Phone: 090-533-8562632

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flavonoids, hyperoside, quercetin, and vitexin. The action of these compounds on the cardiovascular system hasled to the development of drugs from leaf and flower extracts, which are widely used in Europe [1,4].

Recent reports showed that numerous diverse chemical constituents were in the leaves, fruits, and flowers of *Crataegus* which include sugars and sugar alcohols, organic and phenolic acids, terpenes, essential oils (including mixtures of terpenoids and phenylpropanoids) [5]. While intensive work has been done on the major bioactive compounds in the chemical profile of *Crataegus* species little attention has been given to volatile constituents [6]. This could be due to the low concentrations of these metabolites but they could contribute to the synergic effect of the plant and explain some therapeutic effects such as sedative effect [4]. Therefore, we aimed to study the volatile constituents of the flowers and fruits of two *Crataegus* species. *C. azarolus* growing in Cyprus and *C. pallasii* growing in Libya.

2. Materials and Methods

2.1. Plant Material

Crataegus azarolus samples were collected from two regions in Northern Cyprus; Cengizköy / Lefke on March 1, 2018, and from Near East University Campus in Nicosia on March 26, 2018. Samples were deposited at the Herbarium of the Near East University with voucher numbers NEUN 6899 and NEUN 6900, respectively. *Crataegus pallasii* samples were collected from El-merj in Libya from Bata Region on March 23, 2018. Samples were authenticated by Dr. Mohammed Nuri Abuhadra and deposited in the Herbarium of the Faculty of Science, Botany Department, the University of Tripoli in Libya with voucher number: D6831131.

2.2. Isolation of Essential Oil

For the isolation of the essential oils, fresh and dry inflorescences and unripe fruits were separated from branches of *C. azarolus* and *C. pallasii*. 150 g of each fresh and dry *C. azarolus* and 85 g of dry *C. pallasii* inflorescences and unripe fruits were hydrodistilled in a Clevenger–type apparatus for 3h. The resulting oils were trapped with hexane and stored at 4 ℃ until used.

2.3. Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

2.3.1. GC-MS Analysis

The GC-MS analysis was carried out with an Agilent 5977B GC-MSD system. Innowax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) was used with helium as carrier gas (0.8 mL/min). GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250°C. Mass spectra were recorded at 70 eV. Mass range was from *m/z* 35 to 450. The sample was dissolved in 10% n-hexane and 1µL was injected.

2.3.2. GC Analysis

The GC analysis was carried out using an Agilent 7890B GC system. FID detector temperature was 300°C. To obtain the same elution order with GC-MS, simultaneous auto-injection was done on a triplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

2.4. Identification of Compounds

Identification of the essential oil components was carried out by comparison of their relative retention times with those of authentic samples or by comparison of their linear retention index (LRI)

to series of n-alkanes. Computer matching against commercial (Wiley GC/MS Library, NIST Chemistry WebBook) [7,8] and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data was used for the identification [9,10].

3. Results and Discussion

The essential oils isolated by hydrodistillation from the inflorescences and unripe fruits of *C. azarolus* and *C. pallasii* were analyzed simultaneously by GC-FID and GC-MS. The chemical profiles of the essential oils, the percentage contents and linear retention indices of the components are shown in table 1. The components identified from the distilled oil of fresh *C. azarolus* were thirty-three forming 91.1% of the total oil composition and twenty-seven were identified from dried C. *azarolus* and thirty-one were determined from *C. pallasii* comprising 85.5% and 97.6% of the total oil composition, respectively. Oil yield of all samples was measured less than 0.01% on dry weight basis. The moisture content of *C. azarolus* was calculated as 65.6%.

The major outstanding constituents and common to all samples of the essential oils of *C. azarolus* (fresh and dried) and *C. pallasii* were the alkanes namely; tricosane (33.8%, 29.3% and 34.0%), pentacosane (24.6%, 21.1% and 30.8%), heptacosane (12.5%, 10.2% and 11.7%) and tetracosane (6.0%, 5.6%, and 5.7%), respectively. Docosane (1.7%, 2.0%, and 1.3%), was also present in all three samples. Carvacrol (6.8%), carvacryl acetate (0.3%), carvone (t) and thymol (0.1%), were determined for the first time from *C. pallasii* essential oil. (*E*)-β-damascenone was determined only in dried *C. azarolus*; sesquiterpene compounds valencene, α-selinene and β-selinene, δ-cadinene, germacrene D, selina-3,7(11)-diene, spathulenol, and δ-cadinol were determined only in fresh *C. azarolus* sample. On the other hand (2*E*,6*E*)-farnesol was characterized in both dried *C. azarolus* (6.3%) and *C. pallasii* (0.4%) samples.

Analysis of our readings documented an apparent difference between both samples of *C. azarolus*, most predominate the presence of hexacosane (1.3%) in fresh *C. azarolus* and (2*E*,6*E*)-farnesol (63%) in dry *C. azarolus* samples. Eighteen compounds were common to both, this variation in chemical composition is suspected to be due to, mainly two reasons. Firstly, samples have been collected from different localities in Cyprus and secondly, the difference in sample conditions (fresh and dried). Reports on the essential oils of *C. azarolus* in different regions of the world also showed considerable qualitative and quantitative variations. This is in accordance with the fact that essential oil formation in the plants is highly dependent on climatic conditions, genetic background, biotic, and abiotic environmental factors, part of the plant distilled, stage of plant development as well as the extraction methods [11].

Comparison of our investigations with the published data regarding the essential oil of *C. azarolus;* Lakache and his colleagues reported sixty-one compounds from the essential oil of leaves and flowers of *C. azarolus* growing in Algeria by two extraction methods; hydrodistillation assisted by microwave heating (HD-MW) and hydrodistillation (HD). The main groups of detected volatiles were acids and esters (12 compounds) 27.5% for HD-MW, 50.7% for HD; alkanes and alkenes (17 components) comprised 40.8 % for HD-MW, 29.5% for HD. It is obvious that the latter group of compounds obtained by using HD-MW method is quite similar to our results [12]. The Algerian *C. azarolus* aerial parts were also investigated by Boudjada *et al.,* five volatile compounds were identified; 2,4-bis (1,1-dimethyl ethyl)-phenol, tridecanoic acid 12-methyl-methyl ester, pentadecanoic acid 14-methyl-methyl ester, 8-octadecanoic acid methyl ester, and isobutyl nonyl phthalate [13]. The water, methanolic and ethanolic extract of the fresh leaves of the Lebanese C. *azarolus* were examined by gas chromatography coupled with mass spectrometry. In total 11 compounds were determined in the water extract with the major compound pluchidiol (33.6%). The methanolic extract revealed 8 compounds with the major compound α-tocopherol-beta-d-mannoside (21.9%) and in the ethanolic extract, 7 compounds were determined with the major compounds γ-tocopheryl methyl (43.7%) and phytol isomer (20.5%) [14].

Table 1. Volatile oil composition of inflorescences and immature fruits of *C. azarolus* and *C. pallasii*

LRI_{EXP}	LRI_{LIT}*	Compound Name	A%	B%	C%
1020	1025 [b]	α-pinene	0.9	0.3	-
1190	1177 [b]	α-terpinene	-	-	0.1
1213	1212 [c]	Limonene	1.9	0.5	-
1259	1245 [b]	γ-terpinene	-	-	0.7
1288	1282 [c]	*p*-cymene	0.4	-	0.3
1408	1391 [b]	Nonanal	0.2	0.2	0.1
1514	1491 [b]	α-copaene	0.2	-	-
1555	1543 [b]	Linalool	-	-	0.4
1613	1591 [b]	β-elemene	0.1	0.2	0.2
1622	1617 [a]	Undecanal	0.1	0.3	0.2
1620	1617 [a]	Terpinen-4-ol	-	-	0.3
1624	1623 [a]	β-caryophyllene	0.3	0.2	0.2
1728	1719 [a]	Borneol	-	-	0.2
1744	1726 [a]	Germacrene D	0.2	-	-
1748	1741 [a]	β-bisabolene	-	-	0.3
1758	1742 [d]	β-selinene	0.2	-	-
1761	1744 [a]	α-selinene	0.1	-	-
1763	1760 [a]	(*E*, *E*)-α-farnesene	0.3	0.4	0.4
1770	1740 [a]	Valencene	0.5	-	-
1770	1751 [a]	Carvone	-	-	t
1786	1755 [b]	δ-cadinene	0.2	-	-
1817	1797 [a]	Selina-3,7(11)-diene	0.2	-	-
1835	1830 [a]	Tridecanal	-	0.2	0.1
1854	1838 [a]	(*E*)-β-damascenone	-	0.1	-
1875	1854 [b]	(*Z*)-geranyl acetone	-	0.1	-
1893	1880 [e]	2,2,4-trimethyl-3-carboxyisopropyl pentanoic acid isobutyl ester	-	0.1	-
1901	1890 [a]	Carvacryl acetate	-	-	0.3
1941	1931 [a]	Phenylethyl alcohol	-	-	0.1
1973	1954 [a]	(*E*)-β-Ionone	-	-	0.1
2033	2008 [a]	Caryophyllene oxide	0.5	-	-
2052	2053 [a]	(*E*)-nerolidol	0.8	1.0	-
2075	2055 [a]	Anisaldehyde	-	-	0.1
2100	2100 [a]	Eicosane	1.9	3.8	1.6
2110	2095 [a]	Hexyl benzoate	0.1	0.3	0.1
2138	2118 [a]	Hexahydrofarnesyl acetone	0.4	0.2	-
2159	2144 [a]	Spathulenol	0.2	-	-
2163	2148 [a]	(*Z*)-3-hexenyl benzoate	0.5	0.6	0.2
2182	2170 [f]	(*E*)-2-hexenyl benzoate	0.1	0.2	0.1
2197	2191 [a]	3,4-dimethyl-5-pentylidine-2(5H)-furanone	-	t	-
2200	2200 [g]	Docosane	1.7	2.0	1.3
2210	2198 [a]	Thymol	-	-	0.1
2228	2223 [a]	Methyl hexadecanoate	-	0.3	-
2230	2233 [a]	δ-cadinol	0.2	-	-
2242	2239 [a]	Carvacrol	-	-	6.8
2265	2278 [d]	Torilenol	0.3	-	-
2300	2300 [g]	Tricosane	33.8	29.3	34.0
2332	2351 [a]	Eudesma-4(15),7-dien-1-β-ol	0.2	-	-
2370	2369 [a]	(2*E*,6*E*)-farnesol	-	6.3	0.4
2402	2400 [g]	Tetracosane	6.0	5.6	5.7
2416	2376 [a]	Manoyl oxide	0.2	0.7	-
2500	2500 [g]	Pentacosane	24.6	21.1	30.8
2602	2600 [g]	Hexacosane	1.3	-	0.7
2623	2613 [a]	Phytol	-	1.6	-
2700	2700 [g]	Heptacosane	12.5	10.2	11.7
		Total	**91.1**	**85.8**	**97.6**

LRI*EXP*: Linear retention indices calculated against n-alkanes by using FID data. LRI *LIT**: LRI from literatures: [3]*, [19]*, [20]*, [21]*, [22]*. t: trace (<0.1 percent).

A: *Crataegus azarolus* fresh inflorescences and immature fruits, NEU campus,TRNC; B: *Crataegus azarolus* dried inflorescences and immature fruits, Çengiköy / Lefke, TRNC; C: *Crataegus pallasii* dried inflorescences and immature fruits, Bata region, Libya.

Using solid phase microextraction (SPME) method, analysis of the volatile components of the leaf and flower of 7 *Crataegus* taxa collected from the Western Anatolia part of Turkey was conducted by Özderin *et al*. Forty volatile components from two samples of *C. azarolus* var. *aronia* essential oil were reported with the major components; benzaldehyde (82.5%, 23.9%), 2-hexenal (21.7%, 2.5%), butyraldehyde (15.2%, 4.4%) [15]. Due to the high popularity of the fruit jam of *C. azarolus* in Cyprus, Hadjimitsi and Zabetakis performed a study that involved the identification and quantification of volatile compounds from the commercial jam of the species purchased from a local producer in Cyprus. In that study, simultaneous distillation–extraction method was used and 44 components were identified by GC-MS. The major constituent identified was 2-furaldehyde (21.4%) [16]. A study to evaluate the chemical profile of *C. azarolus* fruits in Northern Italy attempted an innovative approach, namely, a specific fingerprint, coupled to the multivariate data analysis (PCA), which was used to show the single bioactive class contribution to the total fruit phytocomplex. Five monoterpenes; phellandrene, sabinene, γ-terpinene, terpinolene and limonene constituting a total of (15.4%) were characterized [17]. Hydrosol beverage of *C. azarolus* leaf and fruits that are used for the treatment of cardiovascular diseases in Persian nutrition culture and folk medicine was investigated by Azadeh *et al.,* and analyzed by GC–MS, to reveal the presence of hexadecanoic acid (7.7%), *p*-xylene (20.1%), thymol (28.7%) and thymolethanoate (2.3%) [18].

Other *Crataegus* species have also been investigated for their essential oil constituents. *C. monogyna* inflorescence essential oil was evaluated by Kowalski *et al.,* and 65 compounds were identified, the major compounds were; tricosane (12% - 17%), heneicosane (11% - 16%), linalool (6% -11%), n-hexadecanoic acid (1%-11%), nonadecane (3%-7%), (*E*,*E*)-α-farnesene (1%-5%), caryophyllene oxide (1%-4%) and methyl eugenol (6%) [23]. Whereas in early 1993, Robertson *et al*., also identified the major volatile compounds of *C.monogyna* flowers as alcohols, ketones and aldehydes; 3-methyl-1-butanol (23.2%), benzaldehyde (16.1%), 2-butanone (10.9%), 3-methyl butanal (9.6%), 4-methoxybenzaldehyde (9.2%) 4-methoxy benzoic acid (9.6%) and 3-pyridinecarboxaldehyde (8.3%) [24]. The essential oil of the flowers of *C. jackii*, *C. robesoniana*, and *C. flabellata* was reported by Kovaleva *et al*., 46 compounds were identified. The major compounds were alkanes, mainly; tricosane (11.1%, 19.2%, 17.9%) which is in agreement with our results [25]. Özderin *et al*., identified fifty-three volatile components, from *C. orientalis* subsp. *orientalis*, collected from Muğla-Fethiye in Turkey. Major components were aldehydes; 2-hexenal (38.6%), capronaldehyde (6.8%) and from *C. orientalis* subsp. s*zovitzii* major components were propyl methyl ketone (26.6%), butyraldehyde (9.4%) and 2-hexenal (6.6%) [26]. Horvat and Chapman also investigated volatile oils from fruits of *C. opaca*, *C. aestivalis* and *C. rufula* from South Georgia. Twenty-four compounds were identified comprising mainly esters and aldehydes, constituting 70.4% of the volatiles [27]. The chemical characterization of *C. oxyacantha* essential oil from Algeria was determined by Chouitah and Meddah. Twenty-five compounds were detected, representing 97% of the total essential oil, eugenol (24.3%), longifolenaldehyde (17.5%), β-selinene (15.6 %) were the main components [28]. In a study by Nojima *et al.,* to identify volatile compounds from hawthorn fruit (*Crataegus* spp.) that act as behavioral attractants for hawthorn-infesting *Rhagoletis pomonella* flies. Six volatiles were mentioned: ethyl acetate (94.3%), 3-methylbutan1-ol (4%), isoamyl acetate (1.5%), 4,8-dimethyl-1,3(*E*),7-nonatriene (0.1%), butyl hexanoate (0.01%), and dihydro-β-ionone (0.1%) [29].

4. Conclusion

To the best of our knowledge, this is the first report regarding the volatile oil analysis of inflorescences and unripe fruits of two *Crataegus* species, *Crataegus azarolus* growing in Cyprus and *Crataegus pallasii* growing in Libya. Fifty-four compounds were identified by GC and GC-MS analysis. Scientific studies document that alkanes are considered important substances in practical and clinical uses with huge potential in the nutraceutical and pharmaceutical industries [30] and in pest management programs [31]. Therefore, the alkanes concentration of *C. azarolus* and *C. pallasii* can be evaluated for these purposes.

Moreover, the presence of monoterpenes, sesquiterpenes, aldehydes, esters, ketones and the important constituent (*E*)-β-damascenone which is regarded as a useful marker for characterisation of

the quality of rose oil and wine [32,33] that was isolated for the first time in minute amounts from Bulgarian rose oil [34] also suggests the use of *C. azarolus* and *C. pallasii* in food, cosmetics, and pharmaceutical industries to improve flavor and taste [35,36,37]. On the other hand, current literature documents the importance of flavonoids and oligomeric-proanthocyanidins in the treatment of cardiovascular disease by *Crataegus* species, it is noteworthy to investigate whether the volatile oil of *Crataegus* sp. would be responsible for other pharmacological properties of this genus *e.g.* anxiolytic, antiviral, antimicrobial, antioxidant, etc. [1,38]. Thus, further research is recommended to investigate the biological and economic importance of the essential oil of *C. azarolus* and *C. pallasii*.

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Conflict of interest statement

The authors have no conflict of interest to declare.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

ORCID
Najat Agiel: 0000-0003-4620-0019
Duygu Y. Hanoglu: 0000-0003-1345-4768
Azmi Hanoglu: 0000-000207586-9080
Kemal H. C. Baser : 0000-0003-2710-0231
Filiz Mericli : 0000-0002-4172-5417

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2. ENC: CURRICULUM VITAE

Name	Najat	**Surname**	Agiel
Place of Birth	Tripoli-Libya	**Date of Birth**	12.6.1965
Nationality	Libyan	**Tel**	05338562632
E-mail	najataghil@yahoo.com		

Educational Level

	Name of the Institution where he/she was graduated	**Graduation year**
Postgraduate/Specialization	Ph.D. in Pharmacognosy - Near East University / TRNC	2020
Masters	MSc in Pharmacognosy - University of Tripoli / Libya	2009
Undergraduate	Bachelor degree in Pharmaceutical Sciences - University of Tripoli / Libya	1989
High school	Pakistan Community School in Tripoli / Libya	1984

Job Experience

Duty	**Institution**	**Duration (Year - Year)**
Assistant Lecture	Faculty of Education -Biology Department - University of Tripoli	2010 - 2015
pharmacist	University of Tripoli Poly Clinic - Libya	1997 - 2010
pharmacist	The General Drug and Equipment Medical Company (DEMCO) in Drug Information Department, Tripoli -Libya	1990 - 1996

Foreign Languages	**Reading comprehension**	**Speaking***	**Writing***
Arabic	yes	Very good	Very good
English	yes	Very good	Very good

Foreign Language Examination Grade								
YDS	ÜDS	IELTS	TOEFL IBT	TOEFL PBT	TOEFL CBT	FCE	CAE	CPE
			77					

	Math	**Equally weighted**	**Non-math**
ALES Grade			
(Other) Grade			

Computer Knowledge

Program	**Use proficiency**
Word – excel-power point	Good