REPUBLIC OF TURKEY
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PHARMACOGNOSTICAL INVESTIGATIONS ON *PRUNUS MAHALEB* OIL AND ITS KERNELS

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Pharmacognosy Programme
THESIS OF MASTER OF SCIENCES

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Department : **Pharmacognosy**

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Thesis Title : **Pharmacognostical Investigation on *Prunus mahaleb* Oil and its Kernels**

Student Name : **Osama ALSHEHRI**

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This is to certify that this study is fully adequate in scope and quality, as a thesis for the degree of Master of Sciences in the Programme of Pharmacognosy.

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APPROVAL:

In the opinion of the above Examination Committee Members appointed by the Executive Council of the Institute of Health Sciences, this thesis was found to satisfy all the requirements as a thesis for the degree of Master of Sciences and therefore is accepted at the meeting of the Institute Executive Council of Health Sciences.

Prof. Dr. Ersin FADILLIOĞLU

Director of the Institute

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ABSTRACT

Alshehri, O. Pharmacognostical investigations on *Prunus mahaleb* L. oil and its kernels, Hacettepe University Institute of Health Sciences, M.Sc.Thesis in pharmacognosy, Ankara, 2014. Medicinal plants are getting worthy all over the world due to their therapeutic usage. Among medicinal plants in Turkey, *Prunus mahaleb* L. (syn. *Cerasus mahaleb* L. Mill.) is commonly known as mahlep, mehlep, idris agac, pis agac, in Turkey and its kernels are used for medicinal and nutritional purposes. Its oil also has a valuable important in medicinal and industrial purposes. Each step in collection and obtaining the kernels from the field are investigated and reported in detail. The results of the nutrition values of the kernels were given as carbohydrate (32.36%), fat (31.80%), protein (27.70%), ash (4.50%) and humidity (3.64%). Antimicrobial activities were determined using the micro dilution method against the bacteria (*E.coli* ATCC 25922, *E. faecalis* ATCC 29212, *S. aureus* ATCC 29213) and the fungi (*C. albicans* ATCC 90028, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 90018) and found no statistically significant results. The kernels are found safe enough and toxicity can be seen as dose dependant in the *in vitro* cytotoxicity assay. The fatty acid compositions of the oil were determined by GC-MS and GC. The main fatty acids of *Prunus mahaleb* kernels are found as oleic (34.5%), linoleic (31.0%) and α-eleostearic (24.0%) which shows that ratio of saturated and unsaturated fatty acid are good for medicinal and nutritional aspect.

Key words: *Prunus mahaleb*, kernel, oil, nutrition value, fatty acids, cytotoxicity, antimicrobial activity

ÖZET

Alshehri, O. *Prunus mahaleb* L. tohumları ve yağı üzerinde farmakognozi araştırmalar, Hacettepe Üniversity Sağlık Bilimleri Enistitusu, Farmakognozi Yüksek lisans Tezi, Ankara, 2014. Tıbbi bitkiler tüm dünyada tedavi edici özelliklerine bağlı olarak değer kazanmaktadır. Bu bitkiler arasında bulunan *Prunus mahaleb* L. (syn. *Cerasus mahaleb* L. Mill.) mahlep, mehlep, idris agac, pis agac olarak bilinmekte olup yağının tıbbi ve endüstriyel olarak önemli bir yeri vardır. Bu çalışmada meyvaları toplama ve tohumları elde etmek için kullanılan basamaklar yerinde incelenerek kayıt altına alınmıştır. *Prunus mahaleb* L. tohumları ile yapılan beslenme değerleri deneysel sonuçlarına göre karbohidrat (% 32.36), yağ (% 31.80), protein (% 27.70), kül (% 4.50) ve nem (% 3.64) olarak bulunmuştur. Antimikrobiyal aktivite deneyi (*E.coli* ATCC 25922, *E. faecalis* ATCC 29212, *S. aureus* ATCC 29213) bakterileri ve (*C. albicans* ATCC 90028, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 90018) mantar suşları denenerek mikro dilusyon yöntemiyle yapılmış ancak istatistiksel belirgin sonuçlar elde edilmemiştir. *In vitro* sitotoksisite deneyi tohumların güvenilir ve toksik etkinin doza bağlı olduğu tespit edilmiştir. Yağ asitleri aşağıdaki gibi bulunmuştur. Yapısı tayin edilen major yağ asitleri ve miktarları GC ve GC-MS yöntemiyle bulunmuştur. Buna göre *Prunus mahaleb* tohumları ana yağ asitleri olarak oleik (%34.5), linoleik (%31.0) ve α-eleostearik (%24.0) taşımaktadır ve doymuş doymamış yağ asit miktarları arasındaki oran tedavi ve besleyici yönden değerlidir.

Anahtar kelimleri: *Prunus mahaleb*, tohum, yağ, beslenme değeri, yağ asitler, sitotoksisite, antimikrobiyel aktivite

INDEX OF CONTENTS

ABBREVIATIONS

B. subtilis	*Bacillus subtilis*
BF3	Boron trifluoride
^{13}C-NMR	Carbon- nuclear magnetic resonance
℃	Celsius degree
C.albicans	*Candida albicans*
C. kruseri	*Candida kruseri*
C. parapsilosis	*Candida parapsilosis*
CLNA	Conjugated linolenic acid
cm	Centimeters
DAD	Diode-array detection
diam	Diameter
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
FID	The flame ionization detector
f	Fraction
FTIR	Fourier transform infrared
GC	Gas chromatography
GC-MS	Gas chromatography- mass spectrometry
GLC	Gas liquid chromatography
H_3BO_3	Boric acid
HCl	Hydrochloric acid
H_2SO_4	Sulfuric acid
hrs	Hours
^{1}H-NMR	Proton- nuclear magnetic resonance
HPLC	High-performance liquid chromatography

IC_{50}	The half maximum inhibitory concentration
i.p	Intraperitoneal injection
ICP-MS	Inductively coupled plasma mass spectrometry
K_2SO_4	Potassium sulphate
KOH	Potassium hydroxide
Kg	Kilograms
K. pneumonia	*Klebsiella pneumonia*
L	Liter
L.	Carl Linnaeus
LPS/ IFN-γ	Lipopolysaccharide induced interferon gamma
LDL	Low-density lipoprotein
MOPS	3-(N-morpholino)propanesulfonic acid
MIC	Minimal inhibition concentration
MEM	Complete modified Eagles medium
mg	Milligrams
m	Meters
mm	Millimeters
Mill.	Philip Miller
NaCl	Sodium chloride
N	Normality
PBS	Pre-warmed phosphate buffered saline tablets
ppm	Parts per million
P. aeruginosa	*Pseudomonas aeruginosa*
rpm	Rotary per minutes
RRI	Relative retention index
S. aureus	*Staphylococcus aureus*
syn.	Synonym
TLC	Thin layer chromatography
UV	Ultraviolet
V79	Chinese hamster lung fibroblast cell line

FIGURES

TABLES

INTRODUCTION

Medicinal plants are getting worthy all over the world due to their therapeutic usage. Although there is a great interest for their products, scientific studies about their chemical composition and their safety issues are still under investigation. Among medicinal plants in Turkey, *Prunus mahaleb* L. (syn. *Cerasus mahaleb* L. Mill.) is commonly known as mahlep, mehlep, idris agac, pis agac, in Turkey and its kernels are used for medicinal and nutritional purposes.

Prunus mahaleb L. (syn. *Cerasus mahaleb* L. Mill.) is a deciduous small tree or large perennial shrub and found in Mediterranean region, central and eastern of Europe, northern Africa, western and central of Asia (1). It is wild in nature and also cultivated for ornamental.

The kernels of Mahaleb are mainly produced in Central and Southeast of Anatolia, and Tokat and Amasya are main market centers in production and exportation. It is known that the fruits start with green colour at first then turn to red and then from dark purple to black colour in mid to late summer (1). Although there are different types of mahaleb in the region, the kernels of black mahaleb fruits are usually preferred and exported in the market (2,3). Kernels are mostly common in Arabic World and Turkey and used for different purposes (4-10). Native people use kernels for different purposes, as a folk medicine, in bakery as food, in liqueurs and special wines and in industry as varnishes and lacquers (11).

According to the statistical data of the Aegean Exporters Union of Turkey, the exported mahaleb products and the total income from this exportation increases year after year gradually. The main countries that import mahaleb products from Turkey are Germany, USA, Australia, Greece, Egypt and Thailand (12). These countries commonly use the kernels in many things as nutritional value as well as rootstock in grafting cherries (13).

There is no scientific approach or intention so far in standardization to isolate the kernels and evaluate the quality of the product. Moreover the isolation procedure is still not well established. Unfortunately the number of scientific studies done in identifying the chemical constituents and their biological activity of the kernels are still insufficient.

The purpose of this study is to establish the collection and production method of the material by going to the local region, to evaluate the nutrition value of the product, to establish safety of the kernels and the composition of the oil which is the main chemical group of the kernels.

1. THEORETICAL PRINCIPLES

1.1. Botany

1.1.1. Rosaceae

Rosaceae is about 2830 species in 95 genera. The name is derived from the type genus *Rosa*. Among the most species-rich genera are *Alchemilla* (270), *Sorbus* (260), *Crataegus* (260), *Cotoneaster* (260), and *Rubus* (250) but the largest genus by far is *Prunus* (plums, cherries, peaches, apricots, and almonds) with about 430 species. The Rosaceae can be herbs, shrubs, or trees. Most species are deciduous, but some are evergreen. The Rosaceae have a cosmopolitan distribution (found nearly everywhere except for Antarctica), but are primarily concentrated in the Northern Hemisphere in regions that are not desert or tropical rainforest (14).

The family was traditionally divided into 6 subfamilies: *Rosoideae, Spiraeoideae, Maloideae (Pomoideae), Amygdaloideae (Prunoideae), Neuradoideae,* and *Chrysobalanoideae*, and most of these were treated as families by various authors (15). Schulze-Menz, in Engler's Syllabus recognized *Rosoideae, Dryadoideae, Lyonothamnoideae, Spireoideae, Amygdaloideae,* and *Maloideae,* and were primarily diagnosed by the structure of the fruits (16). Takhtajan (1997) delimited 10 subfamilies: *Filipenduloideae, Rosoideae, Ruboideae, Potentilloideae, Coleogynoideae, Kerroideae, Amygdaloideae (Prunoideae), Spireoideae, Maloideae (Pyroideae), Dichotomanthoideae,* and 21 tribes. A more modern model comprises three subfamilies, one of which *(Rosoideae)* has largely remained the same while the boundaries of *Rosaceae* are not disputed, there is not general agreement as to how many genera into which it should be divided (17).

1.1.2. *Prunus* Species

Linnean classification:

In 1737, Linnaeus used four genera to include the species of modern *Prunus—Amygdalus, Cerasus, Prunus and Padus*—but simplified it to *Amygdalus*

and *Prunus* in 1758. Since then, the various genera of Linnaeus and others have become subgenera and sections, as it is clearer that all the species are more closely related (18).

Modern classification

A recent DNA study of 48 species concluded that *Prunus* is monophyletic and is descended from some Eurasian ancestor (19). Historical treatments break the genus into several different genera, but this segregation is not currently widely recognized other than at the subgeneric rank. Integrated Taxonomic Information System (ITIS) recognizes just the single genus *Prunus*, with a list of species (20). A modern treatment of the subgenera derives from the work in 1940 and six subgenera are described (21) as follows:

1. Subgenus *Amygdalus*, almonds and peaches axillary buds in threes (vegetative bud central, two flower buds to sides); flowers in early spring, sessile or nearly so, not on leafed shoots; fruit with a groove along one side; stone deeply grooved; type species: *Prunus dulcis* (almond).
2. Subgenus *Prunus*, plums and apricots: axillary buds solitary; flowers in early spring stalked, not on leafed shoots; fruit with a groove along one side, stone rough; type species: *Prunus domestica* (plum).
3. Subgenus *Cerasus*, cherries: axillary buds single; flowers in early spring in corymbs, long-stalked, not on leafed shoots; fruit not grooved, stone smooth; type species: *Prunus cerasus* (sour cherry).
4. Subgenus *Lithocerasus*: axillary buds in threes; flowers in early spring in corymbs, long-stalked, not on leafed shoots; fruit not grooved, stone smooth; type species: *Prunus pumila* (sand cherry).
5. Subgenus *Padus*, bird cherries: axillary buds single; flowers in late spring in racemes on leafy shoots, short-stalked; fruit not grooved, stone smooth; type species: *Prunus padus* (European bird cherry).
6. Subgenus *Laurocerasus*, cherry-laurels: mostly evergreen (all the other subgenera are deciduous); axillary buds single; flowers in early spring in racemes, not on leafed shoots, short-stalked; fruit not grooved, stone smooth; type species: *Prunus laurocerasus* (European cherry-laurel).

1.1.3. *Prunus mahaleb* L.

Figure 1.1. Flowering of mahaleb

Prunus mahaleb L. (syn. *Cerasus mahaleb* L. Mill.) is a member of the famous rose family (Rosaceae) subfamily Prunoidae . The tree is a deciduous small tree or large perennial shrub growing to 2-10 meters (m) (rarely up to 12 m) tall while the trunk up to 40 centimeters (cm) diameters (diam). The bark is grey-brown, notable lenticels on young stems and superficial fissured on old trunks. The leaves are 1.5-5 cm long, 1-4 cm wide, rotate, glabrous, green, pointed. It is clustered at the end of alternately arranged young twigs, looked ovate to cordate, serrate edges and longitudinal venation. The petiole is 5-20 millimeters (mm), may be or not have two glands. The flowers are sweet pure white, fragrant, 8-20 mm diam., with an 8-15 mm pedicel, and they are arranged 3-10 together on a 3-4 cm long raceme (22). Stones globular or ovoid, and its apex acuminate, 5.5-6.5 x 4.4-5 mm (23). Flowering time: April–May, Fruiting time: July (24).

A

B

C

Figure 1.2. The fruits of mahaleb; (A) During ripening (B) When matured (C) The kernels

2.2. Chemical Compositions

2.2.1. Chemical Constituents of The Oil

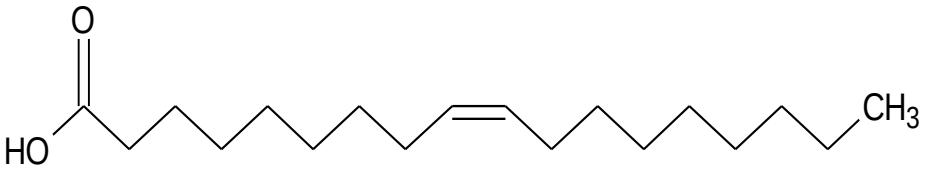
Özgül- Yücel et al.(25) reported that one of the selected oil seeds that grown in Turkey and rich in conjugated linolenic acid is mahaleb. They obtained the oil from the seeds by 4 hours extraction process with petroleum ether at room temperature. The solvent was then evaporated at 40^0C. During extraction and evaporation steps the seeds and the oil were not exposed to the light. It is basically oleic and linoleic acid-rich oil and therefore have little drying ability (semi dried oil). The fatty acid composition of the seeds was determined by gas liquid

chromatography (GLC). Fatty acid composition of mahaleb seed oil was given in (Table 2.1.).

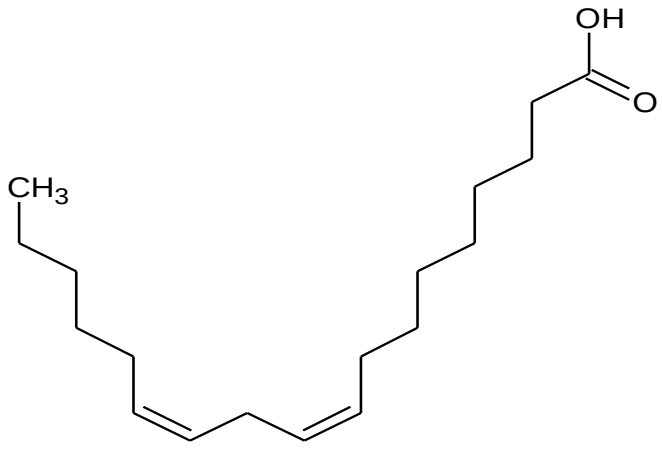
Table 2.1. Fatty acid composition of mahaleb seed oil

Fatty acid	**%**	**Fatty acid**	**%**
oleic acid	35.4	arachidic acid	0.7
linoleic acid	28.5	palmitoleic acid	0.3
α-eleostearic acid	21.0	gondoic acid	0.3
β-eleostearic acid	5.2	behenic acid	0.1
palmitic acid	4.6	lignoceric acid	0.1
stearic acid	1.8	linolenic acid	0.1
catalpic acid	1.3	pentadecanoic acid	0.1

Mahaleb seed oil contains a considerable amount of conjugated linolenic acid (27.6%) of the total fatty acids; it is rich in α-eleostearic acid which accounted for 76.1% of the total fatty acids. Others are β-eleostearic acid and catalpic acid were 19.0% and 4.9% respectively.



Oleic acid



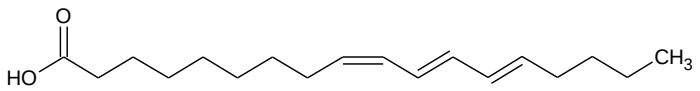
Linoleic acid

Shams, K. et al.(26) reported that GLC analysis was carried out to investigate the structure of the oil. The oil was obtained from 1 kilogram (kg) of kernels using percolation method with petroleum ether. The oil was then saponified by refluxing with alcoholic potassium hydroxide. The unsaponifiable part was extracted with ether which was washed and dehydrated with anhydrous sodium sulphate. The Hydrocarbon fraction applied to GLC and 12 hydrocarbons were identified (Table 2.2.).

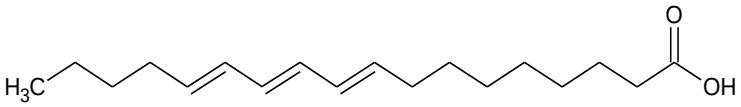
Table 2.2. Hydrocarbon fraction of the oil

(n-C_{10} - n-$C_{32\ unsat.}$)	**%**	**(n-C_{10} - n-$C_{32\ unsat.}$)**	**%**
C_{25}	25.93	C_{18}	4.86
C_{23}	25.00	C_{27}	4.77
C_{24}	9.97	C_{19}	4.56
C_{20}	9.10	$C_{10\text{-unsat}}$	1.75
C_{28}	7.15	$C_{18\text{-unsat}}$	0.47
$C_{32\text{-unsat}}$	6.27	C_{10}	0.17

The hydro-alcoholic soap solution was saponified and after addition of sulphuric acid extracted with ether. This fraction was then studied as fatty acid fraction. Major fatty acids composition of the fatty acid fraction were oleic acid (53.12%) and linoleic acid (35.04%). Also palmitic acid (8.20%) and traces for others like: pentadecanoic acid (0.99%), arachidic acid (0.60%), stearic acid (0.46%), heptadecanoic acid (0.41%), octanoic acid (0.27%), dodecanoic, tridecanoic and myristoleic acid (0.18%), nonanoic and decanoic acid (0.08%), hexanoic acid (0.06%), undecanoic acid (0.04%) and heptanoic acid (0.03%) were observed by gas chromatography-mass spectrometry (GC-MS). No evidence confirmed the presence of eleostearic acid.



∝- Eleostearic acid



β- Eleostearic acid

The major sterols from the sterol fraction of the seeds were identified by GC-MS and given in (Table 2.3.).

Table 2.3. Sterol content of the seeds

Sterol Content	**%**	**Sterol Content**	**%**
B-sitosterol	48.94	stigmasterol	17.47
campesterol	18.76	cholesterol	14.83

Different chemical composition of the seed oils from different colored mahaleb were given in a study done in 2008 (3). This may be due to their different genetic background. The fatty acid composition of oils was analyzed by GC with a capillary column (Table 2.4.).

Table 2.4. Fatty acid compositions of the seed oils from different colored *P. mahaleb* kernels

Fatty acid	**yellow**	**red**	**black**	**Fatty acid**	**yellow**	**red**	**Black**
Palmitic	7.38	7.91	8.66	Catalpic	0.70	0.55	0.59
Palmitoleic	0.51	0.45	0.50	Arachidic	-	0.31	0.25
Stearic	1.34	2.03	3.17	Eicosadieno-ic	1.80	-	-
Oleic	29.38	30.15	33.67	Behenic	Tr.	Tr.	Tr.
Linoleic	34.13	33.33	29.11	Lignoceric	Tr.	Tr.	Tr.
α-eleostearic	18.60	18.98	17.56	$\sum$	98.55	98.74	98.43
β-eleostearic	4.71	5.03	4.92	$\sum_{unsat.}/\sum_{sat.}$	88.83/ 8.72	88.49/ 10.25	86.33/ 12.10

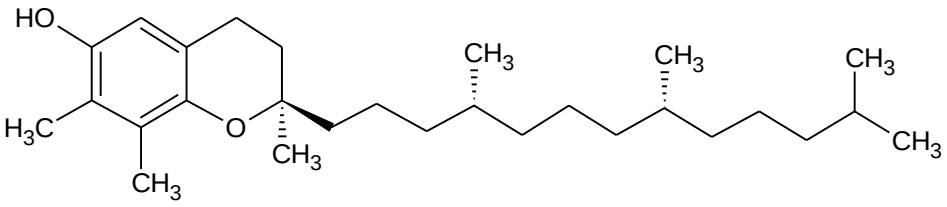
-: not determined

Tr.: trace

Fatty acid components of red and black colored types were characterized as 98.74% and 98.43% respectively. Fatty acid analysis has shown that the seeds from yellow, red and black colored fruits of mahaleb contained major compounds with

different percentage. Unsaturated fatty acids are the major constituents (average about 88%) and all samples were rich in conjugated linolenic acid (CLNA). α-eleostearic acid was the dominant conjugated linolenic acid among mahaleb types, accounted about 77% of the total CLNA. α-Eleostearic acid was found between (17.56-18.98%), followed by β-eleostearic acid as (4.71-5.03%) and catalpic acid (0.55-0.70%). Linoleic acid was the major fatty acid in yellow and red colored mahaleb seeds (33.13- 34.33% respectively), while oleic acid was the major fatty acid for black mahaleb seed oil (33.67%). Arachidic acid was found only in red and black types between (0.25-0.31 percent). While eicosadienoic acid was found only in the yellow types (1.8%).

Characterization of the seed oil and meal from mahaleb was reported in an another study (27). This study investigated the compositional properties including fatty acids of seeds, amino acids and trace elements. The seed oil was obtained by using soxhelet apparatus with petroleum ether. The fatty acid compositions were determined by GLC. The major fatty acids in the mahaleb oil were linoleic acid (47%), oleic acid (45%), palmitic acid (5.7%), linolenic acid (0.1%) and trace amounts less than 0.1% with myristic and lauric acid. Tocopherol composition of mahaleb oil by high-performance liquid chromatography HPLC was 28.5 mg / 100 g oil. The main isomer was γ-tocopherol. The seed oil of mahaleb was isolated by using soxhelet apparatus with n-hexane for 8 hours.



γ-tocopherol

Table 2.5. The compounds of mahaleb oil determined by ^{1}H-NMR, UV and FTIR

Fatty acid	**%**	**Fatty acid**	**%**
α- eleostearic	38.32	arachidic	0.31
Oleic	31.29	eicosadienoic	0.29
linoleic	22.96	palmitoleic	0.24
palmitic	3.87	gadoleic	0.12
stearic	1.86	myristic	0.04
Cis-vaccenic	0.69	$\sum_{unsat.}/\sum_{sat.}$	93.91/6.08

The fatty acid composition of seed oil was identified using proton nuclear magnetic resonance (^{1}H-NMR), ultraviolet (UV) and Fourier transform infrared(FTIR) (28). The oil contained polyunsaturated fatty acids in high amounts (Table-2.5). Also the oil contained high quantity of tocopherols (γ- tocopherol (main isomer), α- tocopherol, and δ- tocopherol) when applied to HPLC. The tocopherols in seed oil are extremely important because of their role in protecting against the oxidative deterioration of polyunsaturated fatty acids in plant materials. α- tocopherol is recommended because it has a higher biological activity than other tocopherols but γ- tocopherol exhibits a higher antioxidants than α- tocopherol.

Öztürk, I. et al. (29) reported that the fatty acid composition of mahaleb seed oil after methylation process and the results obtained by GC-MS were given in (Table 2.6.).

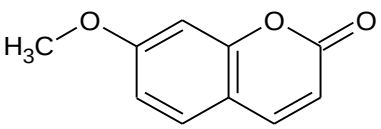
Table 2.6. The Methylated fatty acid composition

Fatty acids	**%**
conjugated linoleic	39.76
oleic	31.33
linoleic	23.01
palmitic	3.45
stearic	2.0
arachidic	0.29
palmitoleic	0.16

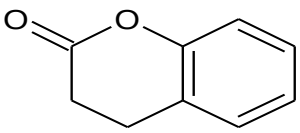
1.2.2. Phenolic compounds

Coumarins and coumarinic acid:

Mahaleb kernels were obtained from local market and defatted with petroleum ether and then extracted with ethanol in a continuous extractor (soxhlet). The ethanolic extract was concentrated and excess diethylether was added. The ether insoluble portion was fractionated on a silica gel column chromatography using chloroform: ethyl acetate: water ($CHCl_3$- EtOAc-H_2O 50:45:5), the extracts showed herniarin glycoside and free sugars as sucrose, fructose, glucose and mannose. Identification of the sugars was based on thin layer chromatography (TLC) analysis and GLC of its trimethylsilyl derivatives against authentic samples. The authors studied the mixture of n- hexane/ ether extracts of kernels by using same system in column chromatography on silica gel and isolated coumarin, dihydrocoumarin and herniarin (30).



Herniarin



Dihydrocoumarine

Shams,K.A. reported that (31) kernels were obtained from local market in Cairo and extracted and fractionated by using the standard method. The fraction rich in coumarins were purified by different chromatographic techniques and were identified by UV, MS, ^{1}H, ^{13}C-NMR as well as acid hydrolysis. Three coumarin compounds were identified, one of them totally novel. The new compound were 2-glucosyloxy-4-methoxy methyl trans cinnamate. The two known compounds from the fraction were coumarin and herniarin. From the mucilage fraction only arabinose was identified.

Ieri, F. et al. (32) studied fruits, kernels and local liquor of white mahaleb. The aim of the study is to identify and quantify of the secondary metabolites of the samples by using a single extraction and identification method. In the frsh tissues of mahaleb, 1-phenolic acid derivatives (main compound is *o*-coumaric acid glucoside) three hydroxycinnamic acid derivatives were tentatively identified: *o*-coumaric acid

diglucoside, dihydro-*o*-coumaric acid 2-O-glucoside and *o*-coumaric acid 2-O-glucoside were obtained. The data was obtained by using HPLC/(DAD)/MS and HPLC/MS/MS analysis. The kernels showed high content of coumarin which is the main class in the samples (0.87 mg g^{-1}). The other compounds were flavonols (main compounds are quercetin glycosides): quercetin derivative and quercetin-3-glucoside. From the liqueur sample mainly anthocyanins were isolated (main compounds are cyanidin glycosides): cyanidin 3,5 -diglucoside, cyanidin 3-sambubioside, cyanidin 3-xylosyl-rutinoside and cyanidin 3-rutinoside.

Öztürk, I. et al. (29) reported that a suitable solvent mixture for the efficient extraction of phenolics from the fruits and seeds of black mahaleb using simplex lattice mixture design was 64% water and 36% acetone. By using GC-MS the main compound in the seed of mahaleb is coumarin (49.08 %). The major sugars were glucose, fructose and sucrose. Total phenolics and flavonoids of the fruit were 2266 mg gallic acid equivalent/100 g of dry sample and 946.57 mg catechin equivalent/ 100 g of dry sample, respectively. Total anthocyanin content of the fruit was 505.7 mg/100g of dry sample.

1.2.3. Other Compounds

The amino acid profile was established by using amino acid analyser on the defatted seeds and the total amount of amino acids was found as 1,223 mg/g nitrogen (N). The percentages of essential amino acids were 50.9% in the mahlab. The total amount of the essential amino acids found in mahlab seed was 623.8 mg/g N. The total amount of non- essential amino acids was 599.4 mg/g N. The total aromatic amino acid levels (phenylalanine and tyrosine) found in mahalab was 117.0 mg/g N. All the essential amino acids except tryptophan were found very low comparing with other food additive. Mineral values of the seeds were analysed by inductively coupled plasma (ICP)-MS and major elements were found as calcium (Ca), potassium (K) and magnesium (Mg) as 133.7; 204.2 and 102.2 parts per million (ppm) respectively (27).

1.3. Activity Studies

1.3.1. Antimicrobial Activity

The n-hexane or methanolic extracts of different parts of mahaleb had been examined *in vitro* (flowers, leaves, seed kernels, fleshy seed coats, fruit stalks, branches and resins) for antibacterial and antifungal activity by micro dilution method (33). Standard strains of *Acinetobacter baumannii, Proteus mirabilis, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumoniae* and *Bacillus subtilis* with their pathogens as well as fungi; *Candida albicans, C. parapsilosis, C. tropicalis and C. kruseri*) were used for this purpose. All extracts showed antibacterial activity against gram-positive standard bacteria with concentration (16-64 μg ml^{-1}) and against gram-negative bacteria (8-64 μg ml^{-1}). The minimal inhibition concentration (MIC) value of the extracts against all gram-positive and gram-negative isolated strains was (250 μg ml^{-1}) excluding *B. subtilis* (64 μg ml^{-1}). The extracts demonstrated antifungal activity at concentration (16-64 μg ml^{-1}) and all extracts were found to inhibit *C. krusei* with MIC value (64 μg ml^{-1}). Preliminary phyochemical screening showed that phenolic and terpenic compounds from the plant parts were the best predictors of high antibacterial and microbial activity.

Bayramcı, N. et al.(34) checked the antibacterial activity of ethanolic extracts of mahaleb fruits and seeds by using disc diffusion and agar diffusion method against pathogens and non-pathogens strains of bacteria (*E. coli, S. aureus, P. aeruginosa, Agrobacterium tumefaciens, Chromobacterium violaceum, K. pneumoniae, Vibrio harveyi, B. subtilis, B. thuringiensis, B. megatorium*). The antibacterial activity of mahaleb fruits was not observed, but the seeds have shown antibacterial activity against all bacteria.

1.3.2. Antioxidant Activity

The studies showing the antioxidative activity of the seeds were done by several authors. The material used for this purpose was mainly methanolic extract of the seeds and the method used was 1, 1-diphenyl-2-picrylhydrazyl (DPPH)

scavenging method. The fractions obtained from the extract by using n-hexane, ethyl acetate and water were also used in a study (35). The extracts and the fractions were also applied to "β-carotene-linoleic acid assay", "ferric reducing antioxidant power activity" and "nitric oxide scavenging activity" and according to the results obtained the seed extract has a moderate antioxidant activity and the methanolic extract has better activity than others (33,35-37).

1.3.3. Anti-inflammatory Activity

The mahaleb kernels were extracted with ethanol and ethanolic fraction was applied to sensitized adult male guinea pigs to see the prophylactic efficacy. The 7 day long *in vivo* studies showed that total ethanolic extract was efficient in evoked complete anti-inflammatory activity among the survival animals receiving low and medium doses. The prophylactic anti-inflammatory activity of the total ethanolic extract was higher than that of the defatted ethanolic extract (26).

Oskoueian, A. et al. (37) showed that methanolic extract was a potent source of anti-inflammatory activity by an anti-inflammatory assay using lipopolysaccharide/induced interferon gamma (LPS /IFN-γ) stimulated murine monocyctic macrophage RAW 264.7 cells.

1.3.4. Others

In vivo pharmacotoxicity study was carried out on total and defatted ethanolic extracts of *P.mahaleb* seeds as well as its oil fraction. The oil fraction were safe and free from any acute lethal toxicity in intraperitoneal (*i.p.*) and oral doses up to 100 ml/ kg applied to sensitized adult male guinea pigs (26).

During their study on the antioxidant activity of the seed extracts, Oskoueian, A. et al.(37) showed strong tyrosinase inhibitory activity, xanthine oxidase inhibitory and anticholinesterase activity of the methanolic extract of mahaleb seed by *in vitro* methods.

2. EXPERIMENTAL SECTION

2.1. Materials

2.1.1. Plant Material

Kernels of *Prunus mahaleb* L. were bought from the ateliers working in the province of Tokat city in June 2012, after checking each production procedure starting from collection of the plant material from the field to prepare it for markets.

2.1.2. Chemical Compounds and Solvents

Boron tri fluoride (BF3) in Methanol:	Sigma-Aldrich
3-(*N*-morpholino)propane sulphonic acid (MOPS):	Sigma-Aldrich
Sulfuric acid (H_2SO_4):	Merck
4% boric acid (H_3BO_3):	Sigma-Aldrich
petroleum ether:	Riedel-de Haen
L-glutamine:	Biological industries
n-hexane:	Sigma-Aldrich
Trypsin EDTA:	Biological industries
Complete Modified Eagles Medium (MEM):	Sigma-Aldrich
Trypan blue:	Sigma-Aldrich
Pre-warmed Phosphate Buffered Saline (PBS) tablets:	Sigma-Aldrich
Neutral Red Dye:	Sigma-Aldrich
Ethanol:	Sigma-Aldrich
Methanol:	Sigma-Aldrich
Acetic Acid:	Sigma-Aldrich
Dimethylsulfoxide (DMSO):	Merck
10% fetal bovine serum:	Biological industries

1% penicillin's streptomycin solution: PAA The Cell Culture

0.1 N Potassium hydroxide (KOH): 5.6 g of KOH dissolved in 1000 ml water.

0.5 N Potassium hydroxide (KOH): 28 g of KOH dissolved in 1000 ml water.

0.5 N Hydrochloric acid (HCl): 18.25 ml HCl dissolved in 1 L of water.

0.5 N Sodium chloride (NaCl) /Methanol: dissolve 1 g of NaCl in 50 ml methanol.

0.5 N Sodium hydroxide (NaOH): 20 g of NaOH dissolve in 1000 ml water.

Saturated Sodium chloride (NaCl): Dissolve roughly 36 g of NaCl in 100 ml of warm water and leave to cool.

40 % sodium hydroxide (NaOH): dissolve 400 g of NaOH in 1 L of water.

0.2 N hydrochloric acid (HCl): dissolve 7.3 g of HCl in 1 L of water.

Ethanol: Ether (v: v, 1:1): mix 25 ml of ethanol and 25 ml of ether.

Kjedahl catalyst: Mix 9 parts of potassium sulphate (K_2SO_4) with 1 part of copper sulphate (CuSO4).

Indicator solution: Mix 100 ml of 0.1 % methyl red (in 95 % ethanol) with 200 ml of 0.2 % bromocresol green (in 95 % ethanol).

Phenolphthalein indicator: dissolve 0.05 g phenolphthalein in 50 ml of 95% ethanol, and then dilute the solution to 100 ml with distilled water.

2.1.3. Apparatus

Distilled H_2O device:	GFL
Rotary vapor:	Heidolph
Soxhelet	Heidolph
Lyophilisation :	Christ
titration apparatus:	Burette
Heater 100^0 C :	Heraeus
Water bath :	Nuve
Microscope:	Leica
Oven:	Shellab

2.2. Methods

2.2.1. Extraction of Kernels

200g of powdered kernels were added to 1000 ml of distilled water and extracted for 8 hours in an rotary evaporator at not above 40 ^{0}C. At the end of the extraction period the water part is filtered from a double filtrate paper and then centrifuged for 30 minutes at a speed of 5000 rotary per minutes (rpm). The supernatant part was used in the experiments.

2.2.2. Oil Yield

As it is shown in Figure 3, a continuous extraction method by using a soxhlet apparatus to extract oil from the kernels. The apparatus contains three basic parts; at the lower part, a round- bottom flask of capacity 10 L.; the medium part is a cylindrical extractor (capacity 1L) that is thimble holder with side tube and siphon device. At the top part, equipped with bulb condenser with two tubes for inlet and outlet water. As in (38,39) the sample (100 g) was loaded in the thick paper thimble and placed in the extractor. The solvent (n-hexane) was added to the flask, gently heating on heat path. The solvent heated to reflux and its vapor travels up through the side tube to the condenser. The vapor cools and drips back to the thimble holder. When the thimble holder is filled with the solvent, it is automatically emptied by siphon device to the bottom flask. The cycle was repeating for 12 hrs. The sample is always extracted with fresh solvents in each cycle.

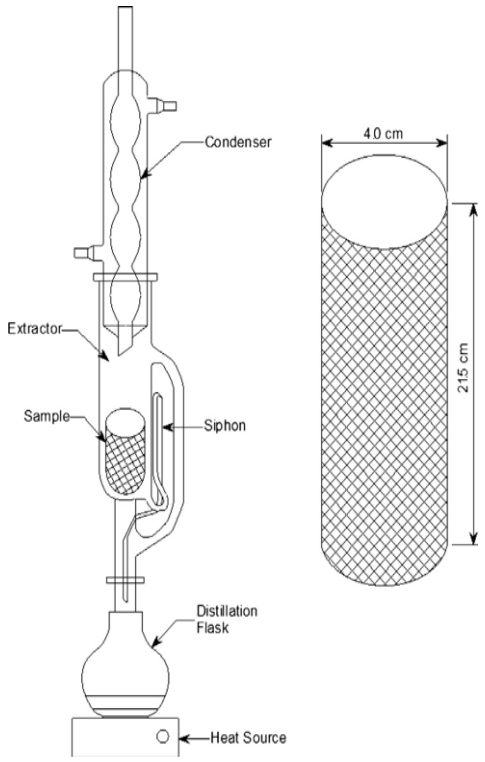


Figure 2.1. Simple draw of soxhlet apparatus combination.
From Dutta R, Sarkar U, Mukherjee (2014).

2.2.3. Pharmacopeia Analysis Of The Oil

Multiple quantitative tests are commonly used to evaluate the quality of fixed oils. Many validated methods are established in Pharmacopeias to standardize oils in pharmaceutical area. The index values are still important for evaluation of the oils and used in academic studies and industry. Gas liquid Chromatography is the most convenient method for qualitative and quantification of the oils and accepted method in pharmacopeias (40).

Acid index

Acidity is frequently expressed as the acid value or as free fatty acids, which is the number of mg of potassium hydroxide (KOH) required neutralizing the free acids in 1 g of the oil. It is determined to assess the rancidity of the oil. High acid values appear in rancidified oils.

Procedure: dissolve about 4 ml of the oil, accurately weighed, in 25 ml of a mixture

of equal volumes of ethanol and ether (1:1, v: v) contained in a flask. Shake it gently. Then neutralize with 0.1 N potassium hydroxide titrant and 1 ml of phenolphthalein indicator until pink coloration is observed. Calculated the acid value by the formula :

0.1 N 1ml KOH ⟶ 0.00561 g

0.1 N (a x f KOH) ⟶ X

a; ml of titrant (f) KOH; 0.9232

X; number of g of KOH to neutralize the amount of the oil used

then;

W ⟶ X

1 g of oil ⟶ Y

W; weight of the sample (oil) in g

Y; number of g of KOH require to neutralize the free acid in 1 g of the oil

Saponification index

The saponification index is the number of mg of potassium hydroxide (KOH) required neutralizing the free acids in, and hydrolyzing esters in, 1 g of the oil. It is a measure of the average molecular weight or chain length, the long chain fatty acids found have low saponification value because they have a few number of carboxylic groups per unit mass of fat and therefore high molecular weight.

Procedure: place 2.2 ml of the oil, weigh accurately, in a flask and add to it 25 ml of 0.5 N potassium hydroxide. Heat the flask on a steam bath, under suitable condenser to maintain reflux for 30 minutes. Then add 1 ml of phenolphthalein turns to pink , and titrate the excess potassium hydroxide with 0.5 N hydrochloric acid until become yellowish colour. Perform a blank determination under the same condition.

Calculation by the formula :

0.5 N 1ml KOH ⟶ 0.02805 g

0.5 N (b – a)ml HCl x f HCl KOH ⟶ X

b; volume consumed of HCl of the blank test in ml

a; volume consumed of HCl of the actual test in ml

f HCl: 1.0647 X; number of g of KOH to neutralize with HCl

then;

W ⟶ X

1 g of oil ⟶ Y

W; weight of the sample (oil) in g

Y; number of g of KOH required to neutralize the free acid and saponify the ester contained in 1 g of the oil

Ester index

The ester index is the number of mg potassium hydroxide (KOH) required saponifying the esters in 1 g of the oil. Also known it is the difference between the saponification and acid values. Ester value = Saponification value – acid value.

2.2.4. Preparation of fatty acid methyl ester

The residue (oil obtained by extraction using Soxhlet apparatus) was refluxed with 5 ml of 0.5 N sodium hydroxide in methanol for 10 min. Then, 5 ml of 14-20% BF3 in methanol solution was added through the condenser and boiled for a further 2 min. 5 ml n-hexane was added and boiled for a further 1 min. The solution was cooled and 5 ml of saturated NaCl solution was added and flask was rotated very gently several times. Additional saturated NaCl solution was added to float the hexane solution into the neck of a 1 ml flask and the upper hexane solution was transferred into a vial (41). Identification of the essential oil components were carried out by gas chromatography (GC) and gas chromatography-mass spectroscopy (GC-

MS) analysis. Each compound were identified by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to series of n-alkanes. Computer matching against commercial (Wiley and MassFinder 3), and in-house "Baser Library of Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data (42) was also used for the identification.
The GC-MS Chromatography: Agilent 5975 GC-MSD system.

Column : Innowax FSC

Carrier Gas : Helium (0.8 ml/min gas flow).

Oven temp. : initial temperature is 60°C for 10 min: to 220°C at a rate of 4°C/min; constant at 220°C for 10 min; to 240°C at a rate of 1°C/min and at 240°C for 20 min.

Split ratio : 40:1

The injection port : 250°C.

Mass spectra (MS) : 70 eV

selected mass range (*m/z*) : 35 - 450.

The GC Chromatograph: Agilent 6890N GC system.

Detector : The flame ionization (FID), 300°C.

Column : Innowax FSC, 60 m x 0.25 mm, 0.25 μm film thickness

2.2.5. Lyophilisation Method

The water extract of the *P.mahaleb* kernels were freezed in a fridge for a night and were lyophilisated by using special glass bottles for 24 hours. It has been used in nutrition values, antimicrobial and cytotoxicity methods.

2.2.6. Antimicrobial Activity Method

Antimicrobial activities were determined as minimum inhibitory concentration (MIC) values using the broth micro dilution method following the procedures reported by the Clinical and Laboratory Standards Institute (CLSI) against the bacteria (*E.coli* ATCC 25922, *E. faecalis* ATCC 29212, *S. aureus* ATCC 29213) and the fungi (*C. albicans* ATCC 90028, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 90018*).* The inoculums densities were approximately $5x10^5$ colony-forming units per milliliter (cfu/ml) and $0.5\text{-}2.5x10^3$ cfu/ml for bacteria and fungi, respectively. Ciprofloxacin and fluconazole were used as reference compounds for antibacterial and antifungal activities respectively. MICs were determined by broth micro dilution method reported by the CLSI. Antibacterial activity test was performed in Mueller-Hinton Broth (Difco, USA). RPMI-1640 medium with L-glutamine (ICN-Flow, USA) buffered with 3-(*N*-morpholino)propane sulphonic acid (MOPS) (ICN-Flow, USA) was used as the culture medium for antifungal activity test. The MIC values were recorded as the lowest concentrations of the substances that had no visible turbidity. All compounds were dissolved in dimethyl sulphoxide (43,44).

2.2.7. Nutrition Values of The Kernels

It is important to know the nutrition information of the foods in general, although it is important information for dietary specialists, but still it is important for relating with diseases.

Determination of Moisture Content (45)

Method:

a- Dry the empty dish and lid in the oven at 105°C for 3 hrs. Transfer it to desiccators to cool. Weigh the empty dish and lid.

b- Weigh about 3 g of sample to the dish and spread it to the uniformity.

c- Place the dish with sample in the oven and dry for 3 hrs at 105°C.

d- After drying, transfer the dish with partially covered lid to the desiccators to cool. Reweigh the dish and its dried sample.

Calculation: moisture (%) = $\frac{w1-w2}{w1}$ χ 100

where; w_1= weigh of sample before drying (g)

w_2= weigh of sample after drying (g)

Determination of Protein Content (45)

Method:

a- Place sample (0.5- 1g) in digestion flask.

b- Add 5 g of Kjedahl catalyst and 200 ml of conc. H_2SO_4.

c- Prepare a tube containing the above chemicals except the sample as blank. Place flasks in inclined position and heat gently unit frothing ceases. Boil briskly until solution clears.

d- Cool then add 60 ml of distilled water cautiously.

e- Immediately connect flask to digestion bulb on condenser and with tip of condenser immersed in standard acid (HCl) and 5-7 drops of mix indicator in receiver. Rotate flask to mix content thoroughly. After that heat until all NH_3 is distilled.

f- Take off receiver, wash tip of condenser and titrate excess standard acid distilled with standard NaOH solution.

Calculation: protein (%) = $\frac{(A-B)\ \chi\ N\ \chi\ 14.007\ \chi\ 6.25}{W}$

where; A= volume of 0.2 N HCl used sample titration (ml) .

B= volume of 0.2 N HCl used in blank titration (ml).

N= normality of HCl .

W= weigh of sample (g).

14.007= atomic weight of nitrogen.

6.25= the protein-nitrogen conversation factor for fish and its by-products

Determination of Ash Content (45)

Method:

a- Place the crucible and lid in the furnace at $550°C$ overnight to ensure that impurities on the surface of crucible are burned off.

b- Cool the crucible in the desiccators for 30 min .

c- Weigh the crucible and lid to 3 decimal places.

d- Weigh about 5 g sample into the crucible. Heat over low Bunsen flame with lid half covered. When fumes are not existed, place crucible and lid in furnace.

e- Heat at $550°C$ overnight. During heating, do not cover the lid. Place the lid after complete heating to prevent loss of fluffy ash. Cool down in the desiccator.

f- Weigh the ash with crucible and lid when the sample turns to gray. If not, return the crucible and lid to the furnace for the further ashing.

Calculation: $\text{ash}(\%) = \frac{weig\,ht\ of\ ash}{weig\,ht\ of\ sample}\ \chi\ 100$

Determination of Fat Content (45)

Method:

a- Place the bottle and lid in the incubator at $105°C$ overnight to ensure that weight of bottle is stable.

b- Weight about 3-5 g of sample to filter paper and wrap.

c- Take the sample into extraction thimble and transfer into soxhlet apparatus.

d- Fill petroleum ether about 250 ml into the bottle and take it on the heating mantle.

e- Connect the soxhlet apparatus and turn on the water to cool them and then switch on the heating mantle.

f- Heat the sample about 14 hrs (heat rate of 150 drop/ min).

g- Evaporate the solvent by using the vacuum condenser .

h- Incubate the bottle at 80-90°C until solvent is completely evaporate and bottle is completely dry.

i- After drying, transfer the bottle with partially covered lid to desiccator to cool. Reweight the bottle and its dried content.

Calculation: fat (%) = $\frac{weig\,ht\ of\ fat}{weig\,ht\ of\ sample}$ χ 100

Determination of Total Carbohydrates (46)

By difference and is calculated by this formula:

Total carbohydrates = 100- (moisture + protein + ash + fat) in g

2.2.8. Determination of *In vitro* cytotoxicity of The Kernels by Neutral Red Uptake Assay

The Neutral Red Uptake Assay is one of the most used cytotoxicity tests. The procedure is cheaper and more sensitive than other cytotoxicity tests. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. Most primary cells and cell lines from several origin may be successfully used (47) .

Procedure: Cells (V79 Chinese Hamster Fibroblast cell lines) are grown in MEM (modified Eagle's medium) supplemented with 10% fetal bovine serum and 1% penicillin's streptomycin solution. 1 ml of cells suspension and 9 ml of medium are centrifuge for 5 minutes at 1200 rpm. Then remove the supernatant and resuspend the pellet approximately 2 ml of medium (it is depend on the amount of cells). Cultured in 25 ml cell culture flasks .Incubate the cultures at 37ºC ± 1ºC, 90 % ± 5 % humidity, 5.0 % ± 1 % CO_2/air until the cells attach to the flask (within 4 to 24 h) at which time the Routine Culture Medium should be removed and replaced with fresh

Routine Culture Medium.

Day 1- setting up the flasks:

-Aspirate media from cells with the aspirator pump or disposable pipette. Wash cells by adding 5ml of warm Trypsin EDTA to each flask with a sterile 5 ml dispenser pump or disposable pipette then aspirate off.

-Trypsinise cells by adding 5 ml of Trypsin EDTA to each flask then incubate at 37(±1)°C for about 3 minutes. After incubation, the flasks should be removed from the incubator and tapped to detach cells.

-Check the flasks under the microscope to ensure cells have detached. If not, the flasks should be returned to the incubator for another minute.

-Add 10 ml of pre-warmed Medium with PBS to flasks to stop the reaction of Trypsin EDTA.

-Pellet the cells by centrifugation for 5 min at 1200 rpm. Remove the supernatant by aspiration .Resuspend the pellet in 2 ml of Routine Culture Medium.

-Mix 90 microliter (µl) of cells and 10 µl of Trypan Blue, then perform a cell count using Neubauer lam.

-Prepare a cell suspension: 96 wells-plates, 10.000 cells/well in 200 µl medium.

-Examine each plate under a phase contrast microscope.

-Incubate plates at 37(±1)°C for approx 24(±2) hours in a humidified atmosphere of 5% CO_2 in air.

Day 2- dosing plates:

- Examine plates microscopically for growth and absence of contamination. Typical signs of contamination are changes in colour or clouding of cell medium and changes in cell shape.

-Chemicals should be dissolved in 1 % DMSO and 99 % distilled water. Prepare appropriate dilutions of the test item in labeled plastic universals. Before use the

chemicals solution, filter the chemical solutions with mille pore filter. The last concentrations of chemicals / incubation time.

-Remove media from plates.

-Transfer each test item into the plate.

-Incubate plates at 37(±1)°C for approx 18 hrs in a humidified atmosphere of 5% CO_2 in air.

-Preparing Neutral Red Dye and Fixative Solution;

Neutral Red Stock solution: 20 mg Neutral red dye powder in 5 ml distilled water. (The NR Stock Solution (powder in water) should be stored in the dark at 4 °C temperature for up to one month).

Neutral Red Standard solution: 625 µl of Neutral Red Stock Solution in 50 ml of Routine Culture medium (pre-warmed to 37° C.). Then incubated in 37(±1)°C for approx 18 hrs in a humidified atmosphere of 5% CO_2 in air.

Fixative Solution (NR Desorb): 100 ml of ethanol, 2 ml of acetic acid, 98 ml of distilled water.

Day 3- applying Neutral Red Dye:

-Examine plates microscopically for growth and absence of contamination.

-In the safety cabinet, filter the Neutral Red dye into a sterile container with a 25ml plastic syringe and a disposable 0.2µm mille pore filter to remove any crystals. Change the filter on the syringe every time the syringe is refilled to prevent it becoming clogged.

-Add 200 µl Neutral Red dye to all wells with a multi-channel pipette.

-Incubate plates at 37(±1)°C for 3(±10mins) hours in a humidified atmosphere of 5% CO_2 in air.

-At the end of the incubation period, the medium is carefully removed and wash plates five times with pre-warmed PBS.

Washing plates: In the safety cabinet remove plate lid. Using a multi channel pipette add 200 µl of the PBS all plates wells and throw it. Repeat it five times. After that firmly tap each plate on absorbent paper cloth to remove any remaining liquid from the wells.

-Add 200 µl Fixative solution to all plate wells with a multichannel pipette.

-Shake plates on plate shaker for approx 10 minutes at 600 rpm until all Neutral Red dye has been extracted by the neutral red de-stain solution.

-Plates are to be kept in the dark between de-staining and reading, including whilst being read.

-Remove plate lids just before placing each plate on the plate reader and record the optical density of the Neutral Red solution at 540 nm wavelength.

3. RESULTS

3.1. Obtaining Mahaleb Kernels

Collection of the fruits: The Tokat Province is at the middle of Anatolia close to Black Sea Region and has an area of 10,470 km^2. The city is surrounded by the provinces of Amasya to the west, Sivas and Yozgat to the east, Ordu and Sivas to the south and Samsun and Ordu to the north. The climate is semi-arid and the annual average temperature is 11-12 ^{0}C (48). The countryside of Tokat has a lot of *Prunus mahaleb* trees which grow wild in nature. The plant is well known by local people and they are expertised in mahaleb collection. After time of ripening, simply they collect the fruits from the trees by hand and dried under the sun for a day before sending the fruits to Ateliers (Figure 3.1.).

Figure 3.1. Drying of the fruits at local areas

The half dried fruits are then transferred to ateliers or locally called “mahlep fabrikası”. The totally drying of the fruits is done in the shadow on hard floor, distributing the fruit on big areas for couple of days until it will be easy for handling (Figure 3.2.).

A

B

C

Figure 3.2. Drying of the fruits in Ateliers

To obtain kernels, the dried fruits are placed to a simple instrument which contains three basic steps, each one has a function to continue the operation of the separation.

1. Cracking step: This process (Figure 3.3.) is the beginning of separation and makes it easy for the other two steps. This process is used to crack the outer layer of the fruit.

A

B

Figure 3.3. The cracking process

2. Air Pumping Step: The pressure of the air confirms the splitting between the fruit and its seed (Figure .3.4).

A

B

C

Figure 3.4. The separation of kernels from cracked dried fruits by air pumping

3. Sieving Step: This procedure is to separate the seed from peels. The peels are used for agricultural purposes (Figure 3.5.). The sieved seeds are then stored for grinding.

A

B

Figure 3.5. The sieving procedure

After sieving, pure kernels are obtained by simple sackcloth (Figure 3.6.) and prepare for powdering .The kernels are pointed and egg-shaped. By chewing the kernels a special odor and taste develops.

A

B

Figure 3.6. The Kernels of *Prunus Mahaleb* L.

3.2. Chemical Properties of Mahaleb Oil

3.2.1. Results of Pharmacopeia Studies

The oil yield of the kernels and the results of chemical properties of the oil are given in Table 3.1.

Table 3.1. Results of pharmacopeia studies

Parameters	Values
Oil Yield	16.47
noitacificeps liO	Bright yellow, clear, fluid but viscose when waited
Density	1.83±0.1
Acid value	1.7*
Saponification value	216.5*
Ester value	214.8*

*All determination carried out in triplicate then average values were taken

3.2.2. GC-MS Results of Mahaleb Oil

Table 3.2. Fatty acid composition of mahaleb kernel oil

Compound	(%)
Palmitic (16: 0)	3.7
Stearic (18: 0)	1.4
Arachidic (20: 0)	0.4
∑ Saturated	5.5
Oleic (18: 1)	34.5
Linoleic (18:2)	31.0
Eleostearic acid**(18:3,9c,11t,13t)	24.0
Eleostearic acid**(18:3,9t,11t,13t)	3.6
Elaidic (18:1)	0.8
Palmitoleic (16:1)	0.2
∑ Unsaturated	94.1
Total	99.6
Unsaturated / saturated	17.1

%: calculated from FID data tr Trace (< 0.1 %).

**: tentative identification.

3.2.3. Nutrition Value of Mahaleb Kernels

The results were given in as g dry weighted of calculated sample. According to the Table 3.3. the results showed that kernels are rich in carbohydrates, fat and protein. And it is a good source of nutrition.

Table 3.3. Nutrition values of the kernels

Parameters	Average (%)
Moisture	3.64
Protein	27.70
Ash	4.50
Fat	31.80
Total carbohydrates	32.36

3.2.4. Antimicrobial Activity Results of Mahaleb Kernels and Its Oil

The Antibacterial and antifungal activities were investigated as explained in Section Material and Methods and the results were given in Table 3.4. and Table 3.5. as shown below. According to the microorganisms and the concentration of the kernels and oil studied, there is no statistically significant data observed in these concentrations.

Table 3.4. Antibacterial activity (MIC in µg/ ml).

	S. aureus	*E. coli*	*E. faecalis*
Kernel extract	> 1024	> 1024	> 1024
oil	1	1	1
control (ciprofloxacin)	0.5	0.004	1

Table 3.5. Antifungal activity (MIC in µg/ ml).

	C. albicans	*C. parapsilosis*	*C. krusei*
Kernel extract	>1024	> 1024	> 1024
oil	1	1	1
control (fluconazole)	1	1	32

3.2.5. *In vitro* Cytotoxicity Results of The Kernel

The data for cytotoxicity and viability in vitro tests was shown in Table 3.6.

Table 3.6. *In vitro* cytotoxicity results of the kernel

Concentration mg/ml	**Viability(PBS)**	**Viability (medium)**
1	100	100
5	61.75024369	57.93222578
10	43.30842269	40.63066267
25	31.97768873	30.00050805
50	31.39283007	29.45181121
100	30.75056861	28.84926078
250	26.77352973	25.11812224
500	25.95472761	24.34994665

IC_{50} (PBS control) = 8.18 mg/ml IC_{50} (medium control) = 7.29 mg/ml

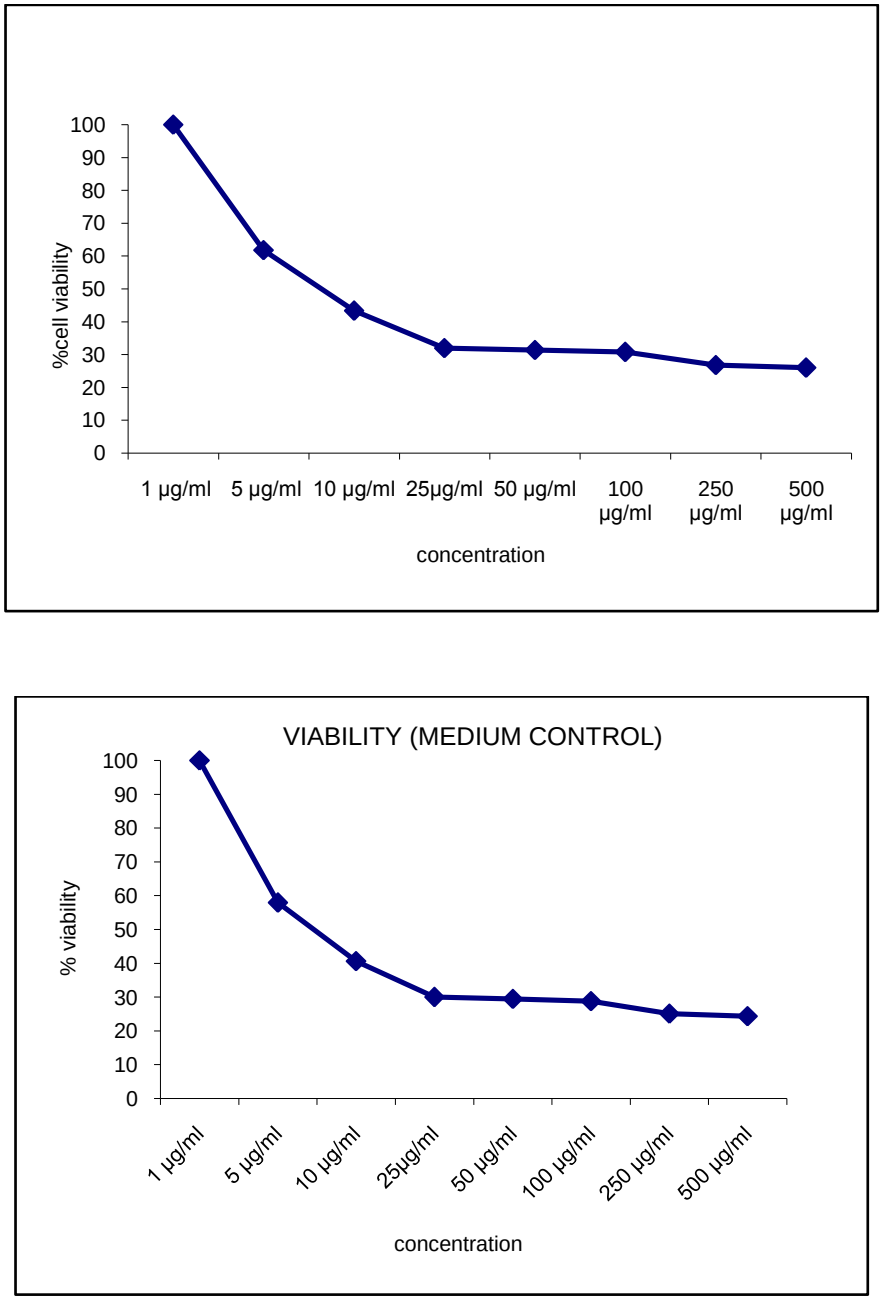


Figure 3.7. Cytotoxicity of *P. mahaleb* kernels

The data showed that there was no parameters found for cytotoxicity of *P. mahaleb* kernels.

4. DISCUSSION

Prunus mahaleb is a well known product grown wild in Turkey and the seeds are traditionally used for medicinal purposes and food. Its name is known as mahaleb, mehleb or mahlab in Turkey and is widely used either in Turkey or in foreign countries especially Arabic countries.

Prunus mahaleb kernels have economic value as they are exported to some American, Arabic and European countries. But there is very little information on obtaining process of kernels from the fruits. In this study, as a first step the collection and separation methods of kernels from the dried fruits were estimated. For this purpose being the major center in producing kernels, Tokat is selected and the process is recorded.

Table 4.1. Comparison of the results for kernel oils

lairetaM	**ssecorP noitalosI**	**Identification Method**	**sdnuopmoC niaM**	**tiL**
Our study	Soxhlet	GC GC-MS	Oleic, linoleic α -eleostearic acids	
Seed	Extraction	GLC	Oleic, linoleic	**(25)**
Seed	Percolation	GLC GC-MS	Oleic linoleic acids	**(26)**
Seed	Extraction	GC	Oleic, Linoleic acids	**(3)**
Seed	Soxhlet	GLC	Linoleic oleic acids	**(27)**
Seed	Soxhlet	^{1}H-NMR UV FTIR	α -eleostearic acids, oleic linoleic acids	**(28)**
Seed	Methylation	GC-MS	Conjugated linoleic, oleic linoleic acids	**(29)**

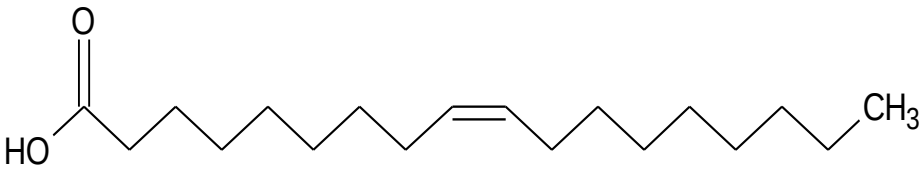
The references and our study showed that the oil of the kernels is the main compound of the kernels. The chemical structure of the oil has importance of containing both saturated and unsaturated fatty acids in large amounts. The studies

are given in Table 4.1. which shows the main structures as well as their isolation and identification methods.

The structure and the medicinal importance of the compounds of the oil:

1- Oleic acid (34.5%)

Definition: oleic acid is a monounsaturated (omega-9) fatty acid found in animal and plants. Also it may found in micro-organism. It is a 18-carbon chain compound with formula $C_{18}H_{34}O_2$ and has one double bond at position 9 from methyl end (18:1 Ω 9).



Occurrence in plants:

It has well known that oleic acid is directly related to olive oil. The main fatty acid in olive oil is oleic acid (55-83%) (49). Also it has been found in safflower oil (about 72%) (50), in soybean (85%) (51), in canola oil (about 55%) (52), in peanut oil (about 80%) (53) and many others.

Uses in health:

It is used in common diet especially Mediterranean area. It has been found that oleic acid is the major component to be used against breast cancer rather than the oil itself (54). Also it could be as an anti-inflammatory fatty acid which has a function of different pathways of immune component cells (55). Also it can have a beneficial effect against type II diabetes and reverse the negative effects of inflammatory cytokinase observed in obesity and non insulin dependent diabetes mellitus (56). Also it may be useful to treat adrenoleukodystrophy, deadly disease that affects the brain and adrenal glands (57). Also oleic acid have been found to could contribute the reduced of lipogenesis and cholesterologenesis (58). Also the oleic acid content in olive oil has been found to be the main reason to reduce the

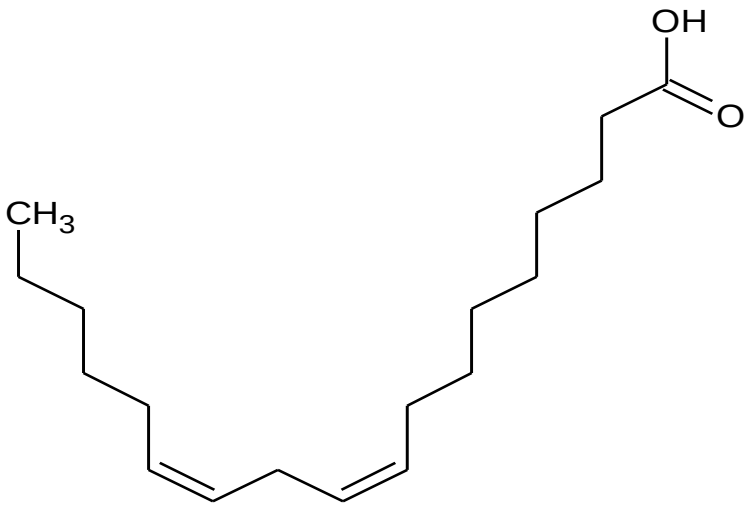
blood pressure (59). Also it has been found that oleic acid may contribute to the prevention of atherosclerosis (60).

Uses in industry:

It is well known that oleic acid has been used in many fields in industry. It is using as emulsifying agent in soap making (61), also it is a solubilizing agent in aerosol products (62), It is a coating material used in glass making (63). The esters of oleic acid may can be used as a lubricating oil (64). Also it has been used as excipient in pharmaceutical formulation (65). Also it is used in cosmetic products.

2- Linoleic acid (31%)

Definition: Linoleic acid is a polyunsaturated (omega-6) essential fatty acid. It is 18-carbon chain compound with formula $C_{18}H_{32}O_2$ and has two cis double bonds. The first double bond at position 6 from methyl end (18:2 Ω 6).



Occurrence in plants:

It is considering that linoleic acid is an essential fatty acid that human body cannot synthesized it and must be taken in normal diet from plants and deficiency of it cause health problems (66). It is mainly found in safflower oil (about 76%) (50),

in salicornia oil (74-79%) (67). Also in evening primrose oil (65-80%) (68), in poppy seed oil (about 74%) (69) and many others.

Uses in health:

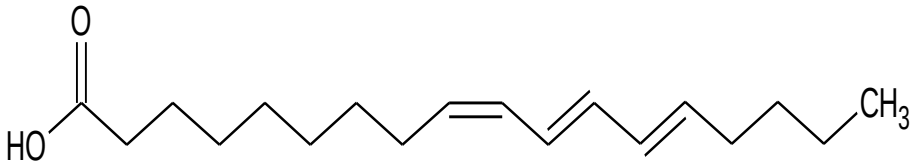
Linoleic acid showed a potential antioxidant activity (70,71). Also it is found that higher intake of linoleic acid may protect against ischemic stroke (72), also prevent from coronary artery disease (73), in diet is important for the safety of the skin, the immune system, cell membranes and synthesis for eicosanoids (74).

Uses in industry:

In cosmetics: acne reductive (75), anti-inflammatory (76), topical therapy to enhance skin barrier (77). In pharmaceutical: linoleic acid is a surfactant (78).

3- ∝- Eleostearic acid (24%)

Definition: ∝- eleostearic acid is a conjugated fatty acid (polyunsaturated fatty acids which at least one pair of double bonds are separated by only one single bond). It is an 18-carbon chain compound with formula $C_{18}H_{30}O_2$ and has three double bonds (18:3, 9c, 11t, 13t).



Occurrence in plants:

It is found in tung oil (67%), in bitter gourd oil (56%) (79). Also in cherry seed oil like *Prunus serotina* (27%) (80) and many others.

Uses in health:

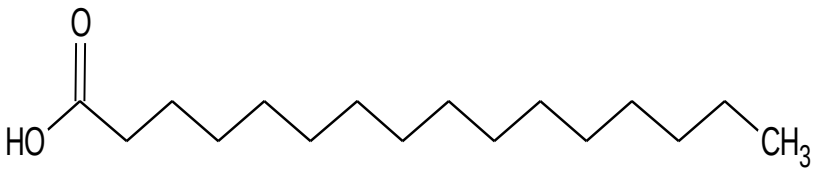
It has a potential antitumor effect (81,82). Also it has an antioxidant activity (83).

Uses in industry:

Drying agents in paints, varnishes and ink (84) and known as plasticizer.

4- Palmitic acid (3.7%)

Definition: palmitic acid is a long-chain saturated fatty acid. It is a 16-carbon chain with molecular formula $CH_3 (CH_2)_{14}COOH$ (16:0). It is the most common saturated fatty acid in animals, plants and micro-organism.



Occurrence in plants:

It is well known that palmitic acid is related to palm oil and animal fat. The main fatty acid in palm oil is palmitic acid (46%) (85). Also it is found in olive oil (12%) (49), in soybean oil (5%) (51) and many others.

Uses in health:

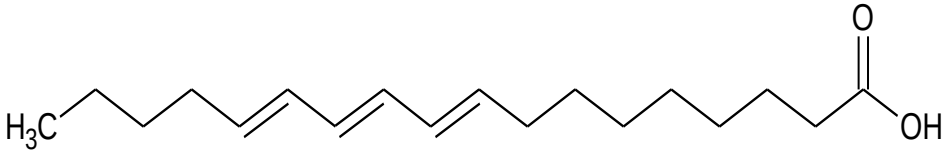
It has a beneficial effect in healthy infant's diet (86). Also in diet, it has a potential effect of lowering insulin activity (87). Also it has a potential antibacterial activity (88).

Uses in industry:

In cosmetics: soap , in pharmaceutical : it is a surfactant (89). In food manufactures (90).

5- β- Eleostearic acid (3.6%)

Definition: β- eleostearic acid is a conjugated fatty acid (polyunsaturated fatty acids which at least one pair of double bonds are separated by only one single bond). It is a 18-carbon chain compound with formula $C_{18}H_{30}O_2$ and has three trans double bonds (18:3, 9t, 11t, 13t).



Occurrence in plants:

It is found in very relative amount in tung oil, bitter gourd (79) and in cherry seed oil like *Prunus serotina* (80).

Uses in health:

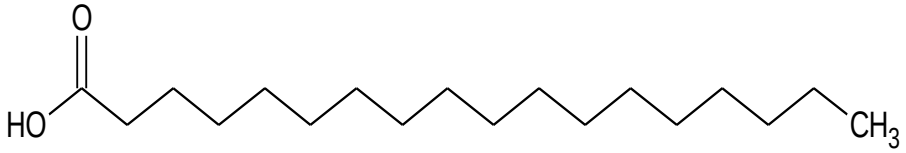
It has a potential effect in cancer disease (91).

Use in industry:

It is well known to use in gel formation and as plasticizer. β- eleostearic acid now is important manufacturing project in china (92).

6- Stearic acid (1.4%)

Definition: stearic acid is a long-chain saturated fatty acid. It is a 18-carbon chain with a molecular formula $CH_3\ (CH_2)_{16}COOH$ (18:0). Stearic acid is a major fatty acid in animals and some fungi, and a minor component in most plants.



Occurrence in plants:

Stearic acid has been found in many plants but in very relative amount equal or less than (5%) like palm oil (85), sunflower oil (93), soybean oil (94) and many others.

Uses in health :

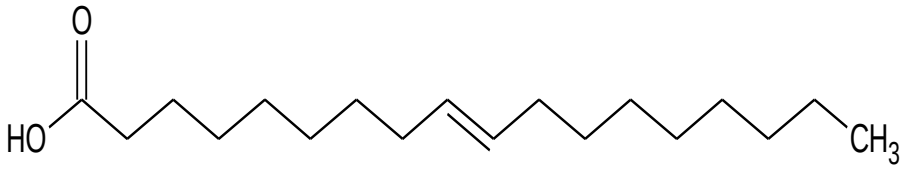
It has a potential effect to lower low-density-lipoprotein (LDL) that helps to treat cholesterol in normal diet (95). Also in diet has a beneficial effect on thrombogenic and atherogenic risk factors in males (96). In diet has a potential effect to reduce inflammation (97). In diet has a potential benefits on cardiovascular health (98).

Uses in industry:

It is well known to be used in cosmetics, detergent, soaps, candle making, lubricant and food ingredients. Also it is used in fireworks to coat metal powders (99).

7- Elaidic acid (0.8%)

Definition: elaidic acid is a monounsaturated (omega-9) fatty acid. It is the trans isomer of oleic acid. It is a trans fatty acid (a fatty acid that has been produced by hydrogenating an unsaturated fatty acid so changing its shape). It is found mainly in animal fat. It is a 18-carbon chain with molecular formula $C_{18}H_{34}O_2$ and has one trans double bond (18:1 Ω 9).



Occurrence in plants:

It is mainly found in partially hydrogenated vegetable oils like cottonseed, soybean, palm and peanut oils.

Uses in health:

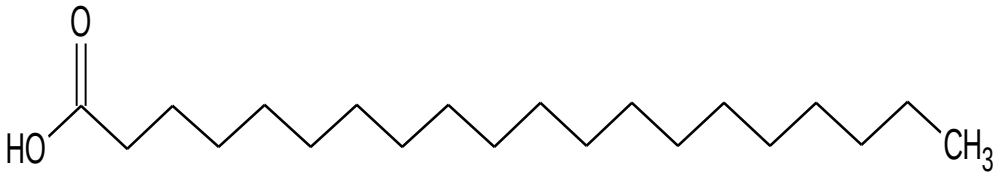
Diet with elaidic acid may have a serious of health problems and must be taken in specified amount (100).

Uses in industry:

In food manufacture to prevent rancidity. It gives a longer shelf life (101).

8- Arachidic acid (0.4%)

Definition: arachidic acid is a long-chain saturated fatty acid. It is a 20- carbon chain with molecular formula CH_3 $(CH2)_{18}COOH$ (20:0).



Occurrence in plants:

It is found in a relatively small amount in vegetable oils like peanut oil (102), jatropha curcas (Barbados nut) oil (103), mustard oil (104) and many others.

Uses in health:

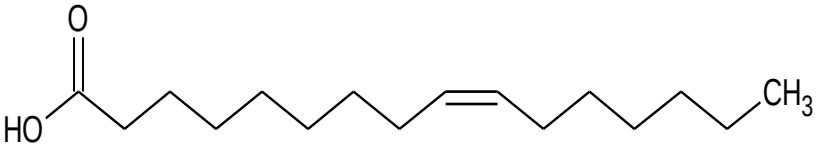
In diet, it may have a risk factor on health (105)

Uses in industry:

It is used in detergent, lubricant, food packaging, photographic material, cosmetics. In pharmaceutical used as nutritional supplements (106).

9- Palmitoleic acid (0.2%)

Definition: palmitoleic acid is a monounsaturated (omega-7) fatty acid. It is found in animal and plants. It is a 16-carbon chain with molecular formula $C_{16}H_{30}O_2$ and has one double bond at position 7 from methyl end (16:1 Ω 7).



Occurrence in plants:

It is found mainly in macadamia nut oil (16-24%) (107) and sea buckthorn berry oil (11-27%) (108). Also it is found in relative amount in olive oil (3%) (109) and many others.

Uses in health :

It is found that palmitoleic acid has a significant relationship in diet with obesity (110). Also it has a potential effect on body weight and anti diabetic (111). Also it has a potential beneficial effect on cholesterol (112).

Uses in industry :

It is used in beauty products such as skin, hair, nail and acne (113). In US army, palmitoleic acid has been used as a beneficial component in ready to eat meals (MRE), which is used in the field by troops for short-term benefits and also long term benefits (114). Troops with higher level of palmitoleic acid were found to have had lower risk of suicide (115).

Apart from the fatty acids the oil contains good amount of tocopherol and some sterols which is important in antioxidant activity. The tocopherols in seed oil are extremely important because of their role in protecting against the oxidative deterioration of polyunsaturated fatty acids in plant materials. The phenolic compounds of the kernels are also another important group in the usage. The studies showed that these compounds possess important biological and pharmacological properties and may have benefits for human health. Due to the usage of *Prunus mahaleb* kernels as food additives in Turkey and in other countries, the nutrition value and cytotoxicity become important to be discussed.

In the references there is no studies done for identifying the nutrition values of the kernels. In our study the nutritional value of the kernels were evaluated in various parameters such as moisture content, carbohydrate, protein, fat and ash contents by standard methods. According determination the nutritional values was found that *Prunus mahaleb* kernels were containing the contents of moisture was 3.6 %, ash was 4.5%, total carbohydrates was 32.36%, protein was 27.7% and fat was

31.8 % over dry weight. These results suggest that *Prunus mahaleb* kernels are good sources for carbohydrates, fats and proteins and that they may have positive effects on free radical scavenging and iron chelating which may use as preliminary information and develop further to be commercially useful in food industry or health products as medicinal food.

The important factors which must be taken into consideration in evaluating the safety of any herbal drug are the nature and significance of the adverse effect and in addition, the exposure level where the effect is observed. Cytotoxicity assays are performed to predict potential toxicity, using cultured cells which may be normal or transformed cells. These tests normally involve short term exposure of cultured cells to test substances, to detect how basal or specialized cell functions may be affected by the substance, prior to performing safety studies in whole organisms. It can also provide insight towards the carcinogenic and genotoxic dispositions of herb-derived compounds and extracts. The ability of a plant extract to inhibit cellular growth and viability can also be ascertained as an indication of its toxicity. Assessment parameters for cytotoxic effects include inhibition of cell proliferation, cell viability markers (metabolic and membrane), morphologic and intracellular differentiation markers. In our experiments the results showed a dose dependent viability of cells and least cytotoxicity due to the parameters of the assay.

In conclusion with this study the collection, isolation and marketing of *Prunus mahaleb* kernels were established and photographed from the main market place of Turkey. Due to the usage of the kernels in Turkey and in exported countries, the safety and the nutritional values are important. By using standard and validated methods, the kernels are found safe and valuable as food. The oil of the kernels has also importance in medicine by containing high amounts of total saturated and unsaturated fatty acids. The GC and GC-MS results of our study show that the kernel oil of Prunus *mahaleb* is rich in oleic, linoleic and ∝- eleostearic acid. These results are well matched with the references. The published studies show that the seeds of *Prunus mahaleb* also contain sterols, coumarins, flavonoids and other phenolics as well as nitrogen containing compounds. With this study we try to focus on the chemical compounds of the oil and to see any risk or benefit using as a health product. *Prunus mahaleb* seeds are found valuable as food additives and as medicinal

product. The scientific studies show that the kernels contain compounds having medicinal value in non oily fractions and need to be investigated by further studies.

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