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

EVALUATION OF ANTIOXIDANT POTENTIALS OF SOME FRUITS AND OTHER PLANT ORGANS WIDELY CONSUMED IN TURKEY

**IN THE PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**

Mehmet Engin Celep, Pharm.

Advisors
Prof. Dr. Erdem Yeşilada
Prof. Dr. Ahmet Aydın

ISTANBUL

FEBRUARY 2013

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

EVALUATION OF ANTIOXIDANT POTENTIALS OF SOME FRUITS AND OTHER PLANT ORGANS WIDELY CONSUMED IN TURKEY

**IN THE PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**

Mehmet Engin Celep, Pharm.

Advisors
Prof. Dr. Erdem Yeşilada
Prof. Dr. Ahmet Aydın

ISTANBUL

FEBRUARY 2013

Doktora öğrencisi Mehmet Engin Celep'in çalışması jürimiz tarafından Farmakognozi Anabilim Dalı doktora tezi olarak uygun görülmüştür.

İMZA

Başkan : Prof. Dr. Erdem YEŞİLADA
Üniversite : Yeditepe Üniversitesi .....

Üye :Prof. Dr. Ahmet AYDIN
Üniversite : Yeditepe Üniversitesi .....

Üye : Prof. Dr. Ekrem SEZİK
Üniversite : Yeditepe Üniversitesi .....

Üye : Prof. Dr. Hasan KIRMIZIBEKMEZ
Üniversite : Yeditepe Üniversitesi .....

Üye : Prof. Dr.İlhan GÜRBÜZ
Üniversite :Gazi Üniversitesi .....

ONAY

Yukarıdaki jüri kararı Enstitü Yönetim Kurulu'nun 21/02/2013 tarih ve ....3-1..... sayılı kararı ile onaylanmıştır.

Prof. Dr. Selçuk YILMAZ
Sağlık Bilimleri Enstitü Müdürü

Anne ve babama...

Sizin için...

Sizin sayenizde...

ACKNOWLEDGEMENTS

I am deeply grateful to the following individuals for their invaluable help and support. This work would be impossible without their generosity of knowledge and time.

First, I would like to express my sincerest gratitude to Prof. Dr. Erdem Yeşilada not only for his unlimited guidance and support, but also for his patience and understanding throughout my studies. I am also grateful for his continued interest and his precious suggestions. He has expanded my horizon and inspired me both as a researcher and an individual.

I also would like to convey my deepest gratitude to Prof. Dr. Ahmet Aydın for his invaluable advice, technical help and positive encouragement. He never hesitated to share his knowledge with me, and always motivated me during my studies. I will always be greatly indebted to him.

I would like to present my sincerest thanks to Prof. Dr. Hasan Kırmızıbekmez for his invaluable suggestions and ideas for the improvement of this study. He always showed me patience and was always generous to me since I started to work as a research associate.

I also wish to thank my colleagues in our faculty for their close friendship and kindness.

Lastly, I thank my mother and father, who made everything I dreamt of possible.

ABSTRACT

Oxidative stress is an inevitable consequence for aerobic organisms. It generates the production of reactive oxygen species which may cause detrimental effects to biological systems. It is known for playing a crucial role in the etiology of several diseases including diabetes, cancer and cardiovascular disorders. To increase the body's antioxidant potential for fighting against oxidative stress, it has been recommended that individuals increase their intake of dietary antioxidants. Regular consumption of fruits and vegetables containing natural antioxidants is associated with reducing the risk of various diseases. Hence, we chose three edible fruits consumed widely in Turkey: *Cornus mas* L., *Diospyros kaki* L. and *Laurocerasus officinalis* Roem. to evaluate their antioxidant capacity both *in vitro* and *in vivo*. We also studied the antioxidant effects of the leaves of *C. mas* under increased oxidative stress conditions, since they are traditionally used against diabetes, and oxidative stress is clearly attributed to diabetes. *In vitro* screening tests indicated that 80% methanolic extracts of all studied plants have high antioxidant activity in terms of free radical scavenging and metal reducing activity. *In vivo* antioxidant studies on healthy rats demonstrated that the antioxidant capacity of liver homogenates were increased, although no changes were observed in the activities of antioxidant enzymes SOD, CAT and GSH-Px or in the level of lipid peroxidation. Studies on CCl_4–induced oxidative damage also showed that 80% methanolic extract of *C. mas* leaves restored the activities of antioxidant enzymes, lowered the level of lipid peroxidation and elevated the total antioxidant capacities of both total blood and liver homogenates of the animals. Further activity guided fractionation studies led to the isolation of gallic acid, a well known antioxidant.

Key words: *Cornus mas* L., *Diospyros kaki* L., *Laurocerasus officinalis* Roem., antioxidant activity, gallic acid

ÖZET

TÜRKİYE'DE SIKÇA TÜKETİLEN BAZI MEVYE VE BAŞKA BİTKİ KISIMLARININ ANTİOKSİDAN POTANSİYELLERİNİN İNCELENMESİ

Oksidatif stres aerobik canlılar için kaçınılmaz bir sonuçtur. Biyolojik sistemler üzerinde hasara yol açması muhtemel reaktif oksijen türlerinin oluşmasına sebep olur. Bu durumun aralarında diyabet, kanser ve kalp–damar rahatsızlıklarının da aralarında bulunduğu bir çok hastalığın kökeninde bulunduğu bilinmektedir. Vücudun oksidatif strese karşı antioksidan savunma kapasitesini artırmak için antioksidan bakımından zengin gıdaların tüketilmesi tavsiye edilmektedir. Bu sebeple ülkemizde meyveleri sıkça tüketilen üç bitkinin, *Cornus mas* L., *Diospyros kaki* L. ve *Laurocerasus officinalis* Roem., *in vitro* ve *in vivo* antioksidan kapasitelerini araştırıldı. Bunun yanında diyabet ile oksidatif stres arasındaki ilişki bilindiğinden, ülkemizde halk arasında şeker hastalığına karşı kullanılan *C. mas* yapraklarının artan oksidatif strese karşı etkisi incelenmiştir. *In vitro* tarama testleri tüm bitki materyallerinden hazırlanan %80 metanol ekstrelerinin yüksek serbest radikal süpürücü etki ve metal bağlayıcı etkiye sahip olduğunu ortaya koymuştur. Sağlıklı hayvanlar üzerinde yapılan *in vivo* çalışmalar ise hayvanlardan alınan karaciğer örneklerinde antioksidan kapasitenin arttığını ancak antioksidan enzim aktivitesinde ya da lipit peroksidasyonunda herhangi bir değişikliğe yol açmadığı gözlenmiştir. CCl_4 ile indüklenmiş oksidatif hasar üzerinde yapılmış çalışmalarda *C. mas* yapraklarının azalmış antioksidan enzim aktivitesini düzelttiği, lipit peroksidasyon seviyesini azalttığı, aynı zamanda toplam kan ve karaciğer örneklerinde antioksidan kapasiteyi arttırdığı gözlenmiştir. Aktivite ile yönlendirilmiş fraksiyonlama çalışmaları sonucunda antioksidan etkisi iyi bilinen bir bileşik olan gallik asit elde edilmiştir.

Anahtar kelimeler: *Cornus mas* L., *Diospyros kaki* L., *Laurocerasus officinalis* Roem., antioksidan aktivite, gallik asit

CONTENTS

***Cornus mas* L.**

***Diospyros kaki* L.**

Laurocerasus officinalis

Abbreviations

AAPH	:2,2'–azobis(2–amidino–propane) dihydrochloride
ABTS	:2.2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid)
AGE	:Advanced Glycation End–Product
ArOH	:Phenolic compound
ATP	:Adenosine Triphosphate
BDE	:Bond Dissociation Enthalpy
CAPS	:3-(Cyclohexylamino)-1-propanesulfonic acid
CAT	:Catalase
CL	:Chemiluminescence
CM	:*Cornus mas*
CoQ	:Coenzyme Q
CUPRAC	:Cupric Reducing Antioxidant Capacity
CYP	:Cytochromes
DHLA	:Dihydrolipoic acid
DNA	:Deoxyribonucleic acid
DPPH	:2,2-Diphenyl-1-picrylhydrazyl
EDTA	:Ethylenediaminetetraacetic acid
ER	:Endoplasmic Reticulum
EtOAc	:Ethyl acetate
FAD	:Flavin Adenine Dinucleotide
FCR	:Folin Ciocalteu Reagent
FMNH	:Flavin Mononucleotide
Fr.	:Fraction
FRAP	:Ferric Reducing Antioxidant Power
GLUT 4	:Glucose Transporter 4
GR	:Glutathione reductase
GSH	:Reduced Glutathione
GSH-Px	:Glutathione Peroxidase
GSSG	:Oxidized Glutathione
HAT	:Hydrogen Atom Transfer
HNE	:4-Hydroxy-2-*trans*-nonenal

$L^{\cdot}$	:Lipid Radical
LDL	:Low Density Lipoprotein
L-DOPA	:Dihydroxyphenylalanine
MDA	:Malondialdehyde
MeOH	:Methanol
NADP	:Nicotinamide Adenine Dinucleotide Phosphate
NADPH	:Reduced Nicotinamide Adenine Dinucleotide Phosphate
n-BuOH	:*n*-Butanol
ORAC	:Oxygen Radical Absorbance Capacity
PPAR-γ	:Peroxisome Proliferator–Activated Receptor–γ
PUFA	:Polyunsaturated Fatty Acid
$R^{\cdot}$	:Free Radical
$R–H_2O$	:Remaining Aqueous Phase
RNS	:Reactive Nitrogen Species
ROS	:Reactive Oxygen Species
RS	:Reactive Species
SET	:Single Electron Transfer
SOD	:Superoxide Dismutase
STZ	:Streptozotocin
TBARS	:Thiobarbituric acid Reactive Substances
TEAC	:Trolox Equivalent Antioxidant Capacity
TOAC	:Total Antioxidant Capacity
TOSC	:Total Antioxidant Scavenging Capacity
TPTZ	:2,4,6-tripyridyl-s-triazine
YEF	:Yeditepe University Faculty of Pharmacy Herbarium

Figures

Tables

Spectra

1. INTRODUCTION & AIM

There has been a growing interest on natural products not only for the maintenance of health but also for the prevention of diseases and assisting the treatment. The possible risks and side effects of synthetic drugs play a major role in this phenomenon. Hence, it has become more and more important to investigate new natural remedies and/or leading natural drug molecules with fewer side effects.

Although oxygen is indispensible for all of the aerobic organisms on earth, it also may be toxic and mutagenic for these organisms. The consumption of oxygen by cells generates the formation of a series of reactive oxygen species (ROS). The deleterious effect of these ROS resulting in potential damage in biological systems is termed as oxidative stress, which may lead to damage in DNA, lipids or proteins. Oxidative stress is an inevitable consequence of aerobic life and it is clearly related to the etiology of a wide range of chronic and acute diseases such as cancer, diabetes, cardiovascular disorders and the aging process, etc. Living organisms generate both enzymatic (superoxide dismutase, catalase, etc.) and non – enzymatic (glutathione, uric acid, ceruloplasmin, etc.) antioxidant defense systems for providing protection against oxidative stress. However, their amount may be inadequate in the cases of excess production of ROS. Therefore, it is important to protect the balance between oxidative stress and antioxidant mechanisms in order to prevent the harmful effects caused by oxidative stress (1,2).

Epidemiological surveys have demonstrated the benefits of consuming antioxidant–rich diets, in the prevention of such diseases mentioned above (3). Numerous studies indicate that plants are a great source for natural antioxidants such as phenolic compounds, vitamins E and C, etc., which may increase the antioxidant potential of the body to protect from the damaging effects of free oxygen radicals. However, most of these researches were carried out on *in vitro* antioxidant screening assays. Although these tests may also provide valuable mechanistic data about the activity, they may conflict with *in*

vivo studies due to the possible problems in the bioavailability of bioactive compounds.

In the light of this information, we have planned to investigate the antioxidant potentials of widely consumed three edible fruits, *i.e.*, *Cornus mas* L., *Diospyros kaki* L. and *Laurocerasus officinalis* Roem. by both *in vitro* and *in vivo* tests in detail.

Cornus mas L. is one of the two species of genus *Cornus* growing in Turkey (4). The ethnobotanical data report that the leaves of this plant have been used against diabetes in Turkey (5,6). Due to the direct relationship between diabetes and oxidative stress, we first aimed to evaluate the antioxidant potential of *C. mas* leaves in detail and to isolate antioxidant ingredient(s) by using the bioactivity guided fractionation procedures and to elucidate their chemical structures through spectroscopic techniques.

2. GENERAL DESCRIPTION

2.1. *Cornus* L.

2.1.1. Botanical Information

2.1.1.1. Cornaceae

Trees or shrubs. Leaves simple, entire, usually opposite, exstipulate. Flowers in corymbose cymes or umbels, hermaphrodite, actinomorphic, epigynous. Sepals small or absent. Petals usually 4. Stamens usually 4, alternating with petals. Ovary inferior, 2-locular; ovules 1 per loculus, pendulous from apex, anatropous; style 1. Fruit a drupe with a single 2-locular pyrene; seeds with copious endosperm (4).

2.1.1.2. *Cornus* species

Deciduous shrubs or trees. Leaves opposite, entire. Inflorescence a terminal cymose corymb or an axillary umbel. Flowers 4-merous, usually hermaphrodite; ovary bilocular. Fruit drupaceous with a bony stone.

The genus *Cornus* is represented by 2 species in “Flora of Turkey and The East Aegean Islands”. Dichotomous key for the determination of these species is as follows (4):

1. Inflorescence a terminal cymose corymb; drupe spherical **1. *sanguinea***

1. Inflorescence an axillary umbel; drupe elongate **2. *mas***

2.1.1.3. *Cornus mas* L.

Shrub or small tree, 3-5 m. Leaves lanceolate to broadly elliptic, 2.5 – 8.5 cm, with 3 – 5 pairs of veins. Inflorescence 1.5 – 2.5 cm diameter, 15 – 20-

flowered, subsessile; bracts 6 – 10 mm, ovate-lanceolate. Flowers greenish to pale yellow; petals 2 – 3 mm; sepals c. 0.5 mm. Drupe 12 – 15 x c. 7 mm, ellipsoid to cylindrical, yellow at first, becoming red (4).

Flowering season: 3rd – 5th months
Habitat: Broad – leaved forrest, scrub
Altitude: 20 – 1500 m.
Distribution in Turkey: Regions of Marmara, Black Sea, Mediterranean, Eastern Anatolia and Anatolia Interior.
Worldwide distribution: Central and Southeastern Europe, Crimea, Southern Russia, Caucasia, Northern and Northwestern Iran.

2.1.1.4. Local names of *Cornus* species

In Turkish: Kızılcık, beyaz kızılcık, çalı kızılcığı, çum, eğren, ergen, eyir, güren, kevren, kıran, kıren, şefit, zağal, zangal, zaval, zavrak, zoğal, zoval, zuğal, zuhal, zuval (7), kiren (5,7).

In Other Languages: Cornelian cherry (8), dogwood (9) (English), corn (10) (Istro – Romania), drijen (11) (Serbian), thanak (12) (Albanian).

2.1.1.5. Traditional Usage of *Cornus* Species

C. mas and *C. sanguinea* are the two species naturally growing in Turkey. The fresh or dried fruits of *C. mas* are frequently reported to be used against diarrhea (13). Stewed fruits of *C. mas* are also eaten to treat diarrhea as well as to treat sunstroke. Boiled fruits are homogenized after removing seeds and sieved; later a thick soup is prepared by the addition of flour, and administered orally to treat common cold and bronchitis. In addition, the decoction of fruits is drunk as hypoglycemic and the fruit juice obtained by pressing is used against diarrhea in the same region (5). The tonic prepared from the fruits of *C. mas* is also reported to be used against mild diarrhea in Montenegro (11). The fruits are prepared as syrup or macerated in raki and used as nutraceutical in Albania (12). The fruits of the same species are

fermented to produce vinegar and used for losing weight in Croatia (10). *C. sanguinea* fruits are used as dye and the fixed oil obtained from its seeds in soap making (13). The fruits of *C. officinalis*, a Fareastern species, are traditionally used as to treat tuberculosis, allergy, hepatitis, lumbago and chronic nephritis in China, Korea and Japan (14). It is also widely used against diabetes in those countries (15). *C.* kousa, a deciduous tree, is known for its colorful and attractive flowers and fruits. Therefore, it is widely grown for ornamental purposes (9).

The infusion prepared from *C. mas* leaves are used against diabetic complaints in Northwestern Anatolia (5). The dried powder of *C. mas* leaves are known to be used for wound healing (13). Its decoction was also reported to be used for the same purpose among Turkish population living in Germany (6). The decoction is also used against hypertension in the Marmara region (5).

The fixed oil from the seeds of *C. sanguine*a is used to treat toothache. The oil obtained by burning a piece of fresh branch is placed on a small cotton plug and inserted in the tooth cavity (5).

The barks of *C. flourida*, a native plant to eastern North America, were commonly used against malaria among the early American settlers. They claimed the activity to be equal or greater than *Cinchona* (16). The infusion prepared from the barks of *C. mas* is used for treating fever and against parasitic infections in Turkey (13).

2.1.2. Bioactivity Studies on *Cornus* Species

2.1.2.1. Studies on Antidiabetic Activity

Yamahara et al. found that the ether extracts of the seeds of *C. officinalis* exhibited good antidiabetic activity on streptozotocin–induced diabetic rats. They have also stated that bioactivity–guided fractionation of the ether extract yielded ursolic acid as the antidiabetic constituent, while oleanolic acid decreased the amount of water consumption and urine volume (15).

In a recent study conducted by Gao et al. (17), streptozotocin–induced diabetic rats were orally administered with 70% ethanolic extract of *C. officinalis* fruits at different doses for 40 days. At the end of the experimental period, blood glucose levels, urinary protein levels and water consumption were significantly decreased. The treatment also provided reduction in serum total cholesterol, low-density lipoprotein cholesterol and total triglyceride levels in diabetic rats. The levels of serum creatinine and albumin were also significantly decreased. *C. officinalis* also increased the activities of the antioxidant enzymes SOD, CAT and GSH-Px in the kidneys of diabetic rats. Besides, the treatment enhanced the expression of peroxisome proliferator–activated receptor–γ (PPAR- γ).

Yamabe et al. (18) demonstrated that 10 days of treatment with fruits of *C. officinalis* attenuated the levels hyperglycemia and proteinuria in STZ–induced diabetic rats. More importantly, the treatment inhibited the advanced glycation end–product (AGE) formation and also the expression of proteins related to this formation in a same way as with aminoguanidine, a known inhibitor of AGEs. In addition to these results, they indicated that the treatment with the fruit extract showed better improvement on serum creatinine and creatinine clearance levels than aminoguanidine. Yamabe et al. investigated the effects of loganin, a major iridoid glycoside found in fruits of *C.officinalis*, on type-2 diabetic db/db mice after 8 weeks of treatment. The results stated that hyperglycemia and dyslipidemia were ameliorated in both serum and hepatic tissue. Loganin alleviated the increased oxidative stress via a reduction in thiobarbituric acid reactive substances and serum reactive oxygen species. It

also showed lipid regulating effect in the livers of diabetic mice through suppressing mRNA expressions associated with lipid synthesis and adjusting the abnormal expression of peroxisome proliferator–activated receptor-α sterol binding protein in nucleus. Additionally, loganin inhibited AGE formation and the expression of its receptor. It also suppressed the hepatic tissue inflammation induced by nuclear factor- kappa B (19).

A similar study by Park et al. also investigated the *in vitro* inhibitory effects of the water extract of *C. officinalis* fruits on AGE products formation. Surprisingly, they found poor activity compared to that of aminoguanidine. However, they suggested that the polyphenolic fraction of the water extract showed much better activity than the extract itself (20).

Lin et al. (21) found that *C. officinalis* fruits significantly increased the glucose uptake from muscles and prevented cytokine–mediated β–cell death. Another study by Chen et al. (22) exhibited that methanolic extract of *C. officinalis* fruits suppressed the gene transcription related to hepatic gluconeogenesis and enhanced glucose responsiveness of pancreatic β–cells.

Jayaprakasam et al. studied the effects of anthocyanins and ursolic acid isolated from *C. mas* fruits on high fat fed mice. After 8 weeks of treatment, they found that these compounds prevented the high fat diet induced glucose intolerance. Also, the liver lipid accumulation and triacyl concentration were decreased (23).

Kim et al. (24) investigated the peroxisome proliferator–activated receptor-γ binding activity of *C. kousa* leaf extract in cell cultures. The results showed that there was a dose–dependent increase in the activity. Furthermore, the extract elevated adipogenesis and the expression of target proteins, including glucose transporter 4 (GLUT 4). It also enhanced the glucose uptake and stimulated insulin signaling.

2.1.2.2. Studies on Antioxidant Activity

Antioxidant activity of water extract of *C. mas* fruits was investigated by Gülçin et al. (25). They showed that the extract had significant DPPH radical, hydrogen peroxide and superoxide radical scavenging, metal reducing and chelating activities.

Another study on antioxidant properties of different *C. mas* fruit types was performed by Ersoy et al. (26). Their results suggest that the methanolic extracts of all types showed good DPPH radical and hydrogen peroxide scavenging activity and metal ion chelating potential.

Pantelidis et al. (8) studied the *C. mas* fruits grown in Greece. They measured the ferric reducing antioxidant power of the fruits and found a significant activity.

Hamid et al. assessed the antioxidant capacities of different *C. mas* genotypes with DPPH radical scavenging methods and reported high activity for all types (27).

Permanganate reducing activity of 80% ethanolic extract of *C. mas* fruits was determined by Popovic et al. They also measured ascorbic acid content of the fruits and found a good correlation between antioxidant activity and ascorbic acid content (28).

Tanaka et al. (29) evaluated the antioxidant activity of *C. capitata* roots by DPPH and superoxide radical tests. They also investigated the lipid peroxidation activity in linoleic acid system and found that ethyl acetate fraction demonstrated the highest activity. Several galloylglucoses were isolated from this fraction and among these compounds, tetra- and pentagalloylglucoses showed the highest activity.

Sultana and Lee studied DPPH radical scavenging activity of *C. kousa* stems and reported a significant activity (9).

In addition to these *in vitro* antioxidant activity experiments, Gao et al., as already mentioned, investigated the effect of *C. officinalis* fruit extract on antioxidant enzyme levels, SOD, CAT and GSHPx, in kidneys of streptozotocin–induced diabetic rats. After 40 days of treatment with the extract, the levels of all three enzymes were significantly increased (17).

Lee et al. studied the effects of *C. officinalis* fruit extract on SOD and CAT levels of acetaminophen–treated mice. After pretreatment for 7 days with the extract, the levels of these enzymes were restored (30).

2.1.2.3. Studies on Antimicrobial Activity

Graziose et al. (16) studied the antiparasitic activity of 95% ethanolic extract of *C. florida* barks. Through bioactivity-guided fractionation studies eight triterpenic compounds, betulinic acid and derivatives were isolated. These compounds were active on both *Plasmodium falciparum* and *Leishmania tarentolae*, pointing both antiplasmoidal and antileishmenial activity.

Türker et al. (31) investigated the antibacterial activity of different extracts of *C. mas* fruits. The ethanolic extract of fresh fruits had moderate activity against *Staphylococcus aureus, S. epidermidis, S. pyogenes* and *Enterobacter cloacae.*

Another study revealed that ethanolic extract of *C. mas* barks showed moderate inhibition against *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus vulgaris* and *Micrococcus luteus* (32).

2.1.2.4. Studies on Anticancer Activity

In an antitumor activity screening study, Türker et al. studied the effect of different extracts prepared from *C. mas* fruits. They reported that *in vitro* antitumor activity of the ethanol extract of *C. mas* was more prominent than that of aqueous extract (31).

The study performed by Savikin et al. was about the cytotoxic properties of the methanolic extracts of leaves and flowers of *C. mas*. The results indicated

that each of the extracts possessed significant cytotoxic activity against HeLa and LS174 human cancer cell lines *in vitro* (33).

Vareed et al. also demonstrated the inhibitory effect of *C. kousa* fruits against colon, breast, lung and central nervous system and stomach tumor cell lines (34).

Kwon et al. (14) performed a study revealing the apoptotic mechanism of ursolic acid isolated from the fruits of *C. officinalis.* They reported that ursolic acid significantly inhibited the growth of primary malignant tumor–derived prostate cells in dose– and time–dependent manners. Ursolic acid was also found to induce apoptosis as evidenced by an increased proportion of cells in sub–G1 phase, the formation of apoptotic cells, DNA fragmentation and nuclear condensation. Another evidence from the study showed that the treatment increased the activity of caspase-3, -8, -9 activities.

2.1.2.5. Studies on the Hepatoprotective Activity

Lee et al. (30) investigated the hepatoprotective activity of the ethanolic extract of the fruits of *C. officinalis* on acetaminophen–treated mice. They reported that pretreatment of mice with the extract for 7 days alleviated the hepatic damage caused by acetaminophen as evidenced by the serum marker enzymes. The treatment also inhibited lipid peroxidation and restored the levels of antioxidant enzymes.

Kwon et al. (14) demonstrated that the administration of loganin isolated from the fruits of *C. officinalis* had a protective effect against hepatic oxidative stress due to type-2 diabetes via regulations of protein expressions associated with oxidative stress, inflammation and apoptosis.

2.1.2.6. Studies on Other Activities

Gao et al. (17) studied the insecticidal effect of methanolic extract of *C. officinalis* fruits against larvae of *Drosophila melanogaster*. Fractionation studies

on the extract revealed that CH_2Cl_2 extract had the most potent activity against larvae of *D. melanogaster.*

Neuroprotective effects of the methanolic extract of the fruits of *C. officinalis* were investigated by Jeong et al. (35). The results demonstrated that the extract had significant neuroprotective activity against glutamate–induced toxicity in HT22 hippocampal cells.

2.1.3. Compounds Isolated from *Cornus* Species

2.1.3.1. Phenolic Compounds

a) Phenolic Acids and Derivatives

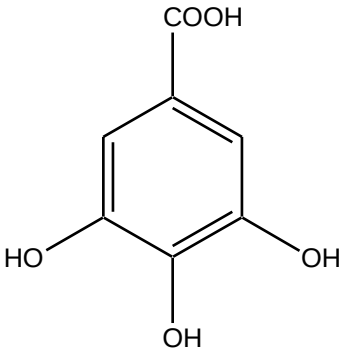


Table 1: Phenolic acids isolated from *Cornus* species

Compound	Species	Ref
Gallic acid	*officinalis*	(21)

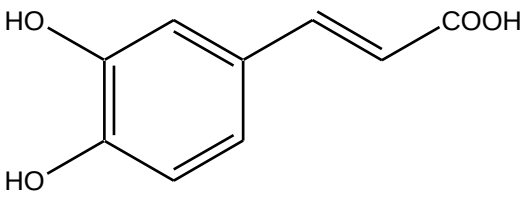


Table 2: Phenolic acids isolated from *Cornus* species

Compound	Species	Ref
Caffeic acid	*officinalis*	(21)

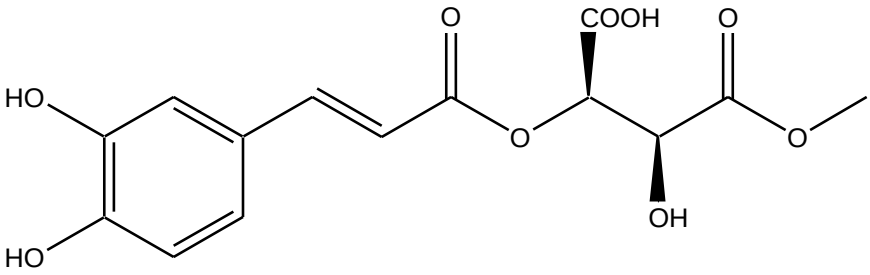


Table 3: Phenolic acid derivatives isolated from *Cornus* species

Compound	Species	Ref
Caftaric acid monomethyl ester	*officinalis*	(21)

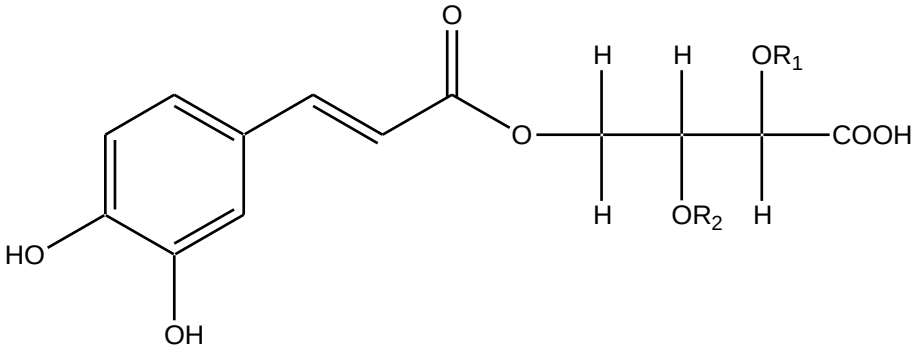


Table 4: Phenolic acid derivatives isolated from *Cornus* species

Compound	R_1	R_2	Species	Ref
4-caffeoyl-L-threonic acid	H	H	*controversa*	(36)
2-galloyl-4-caffeoyl-L-threonic acid	GA	H	*controversa*	(36)
2,3-digalloyl-4-caffeoyl-L-threonic acid	GA	GA	*controversa*	(36)

GA: Galloyl

b) Flavonoids

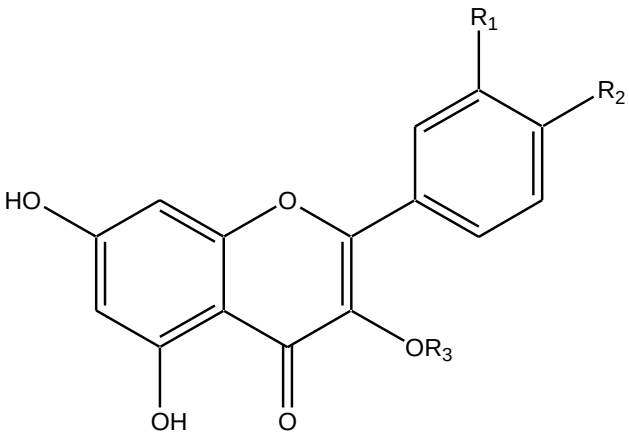


Table 5: Flavonoids isolated from *Cornus* species

Compound	R_1	R_2	R_3	Species	Ref
Kaempferol	H	OH	H	*officinalis*	(37)
4'-*O*-methyl-kaempferol	H	OMe	Glc	*officinalis*	(37)
Kaempferol-3-*O*-β-galactoside	H	OH	Gal	*mas*	(38)
Kaempferol-3-*O*-rhamnoside	H	OH	Rha	*kousa*	(34)
Kaempferol-3-*O*-glucoside	H	OH	Glc	*kousa*	(34)

Quercetin	OH	OH	H	*officinalis* *controversa* *momum*	(37) (39) (40)
Quercetin-3-*O*-β-glucoronide	OH	OH	GluA	*officinalis*	(21)
Isoquercitrin	OH	OH	Glc	*officinalis* *controversa* *mas*	(37) (39) (38)
Quercitrin	OH	OH	Rha	*controversa*	(38,39)
Hyperoside	OH	OH	Gal	*officinalis* *controversa* *mas*	(37) (39) (38)
Rutin	OH	OH	Rut	*controversa* *mas*	(39) (38)

GluA: Glucuronic Acid

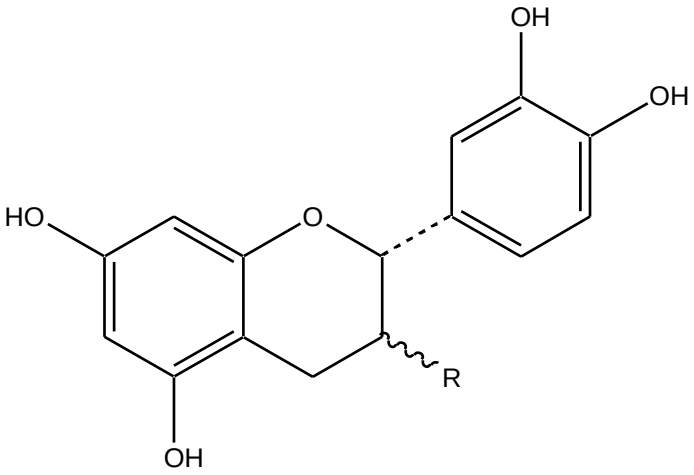


Table 6: Flavan-3-ols isolated from *Cornus* species

Compound	**R**	**Species**	**Ref**
Catechin	β-OH	*kousa*	(9)
Epicatechin	α-OH	*kousa*	(9)
Epicatechin-3-*O*-gallate	α-OGA	*officinalis*	(21)

GA: Galloyl

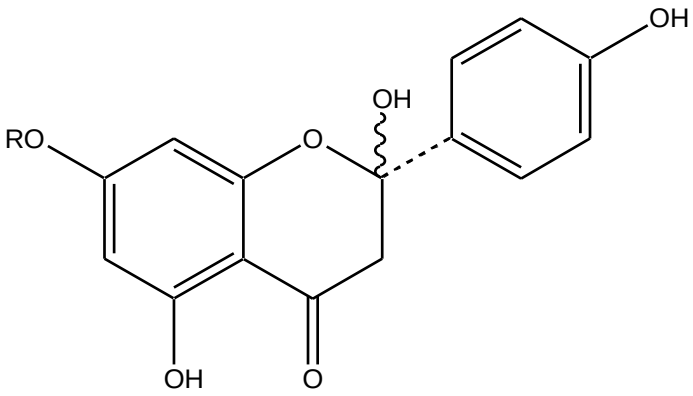


Table 7: Flavonoids isolated from *Cornus* species

Compound	R	Species	Ref
2-hydroxynaringenin	H	*kousa*	(9)
2-hydroxynaringenin-7-*O*-β-glucoside	Glc	*kousa*	(9)

c) Anthocyanins

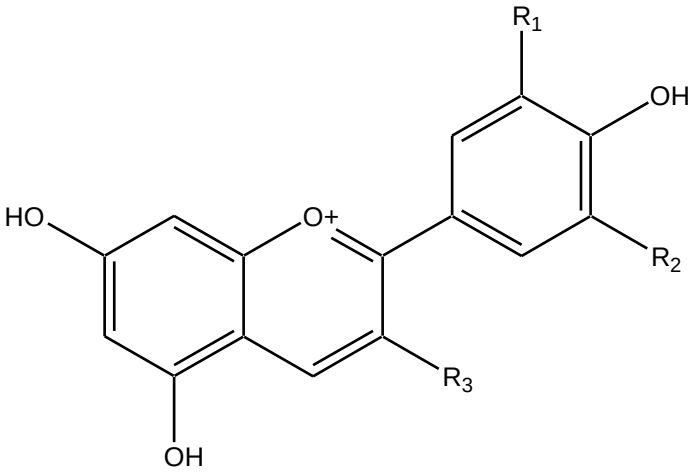


Table 8: Anthocyanins isolated from *Cornus* species

Compound	R_1	R_2	R_3	Species	Ref
Delphinidin-3-*O*-β-glucopyranoside	OH	OH	OGlc	*controversa*	(41)
Delphinidin-3-*O*-β-galactopyranoside	OH	OH	OGal	*mas* *officinalis* *alba* *controversa*	(23,41) (41,42) (43) (42)
Delphinidin-3-*O*-β-rutinoside	OH	OH	ORut	*alternifolia*	(41)
Delphinidin-3-*O*-β-galactopyranoside-3'-*O*-β-glucopyranoside	OGlc	OH	OGal	*alba*	(43)

Delphinidin-3-*O*-β-galactopyranoside-3', 5'-di-*O*-β-glucopyranoside	OGlu	OGlu	OGal	*alba*	(43)
Cyanidin-3-*O*-β-glucopyranoside	OH	H	OGlc	*florida* *suecica*	(41) (44)
Cyanidin-3-*O*-β-galactopyranoside	OH	H	OGal	*kousa* *mas* *officinalis* *controversa* *alba* *suecica*	(41) (23) (42) (42) (43) (44)
Cyanidin-3-*O*-β-galactopyranoside-3'-*O*-β-glucopyranoside	OGlc	H	OGal	*alba*	(43)
Cyanidin-3-(2-glucosylgalactopyranoside)	OH	H	OGlcGal	*suecica*	(44)
Pelargonidin-3-*O*-β-glucopyranoside	H	H	OGlc	*mas* *officinalis* *controversa*	(23,41) (41,42) (42)

d) Hydrolyzable Tannins

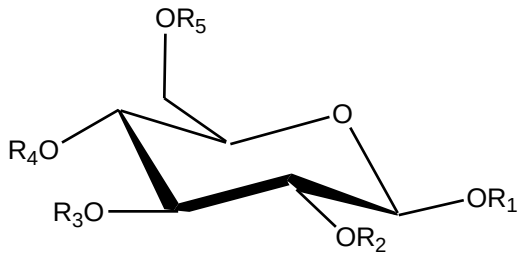


Table 9: Hydrolyzable tannins isolated from *Cornus* species

Compound	R_1	R_2	R_3	R_4	R_5	Species	Ref
1-*O*-galloyl-β-D-glucose	GA	H	H	H	H	*controversa*	(39)
1,6-di-*O*-galloyl-β-D-glucose	GA	H	H	H	GA	*controversa*	(39)
1,2,3-tri-*O*-galloyl-β-D-glucose	GA	GA	GA	H	H	*controversa*	(39)
1,2,6-tri-*O*-galloyl-β-D-glucose	GA	GA	H	H	GA	*controversa*	(39)
3,4,6-tri-*O*-galloyl-β-D-glucose	H	H	GA	GA	GA	*controversa*	(39)
1,2,3,6-tetra-*O*-galloyl-β-D-glucose	GA	GA	GA	H	GA	*capitata*	(29)
1,2,3,4,6-penta-*O*-galloyl-β-D-glucose	GA	GA	GA	GA	GA	*capitata*	(29)

GA: Galloyl

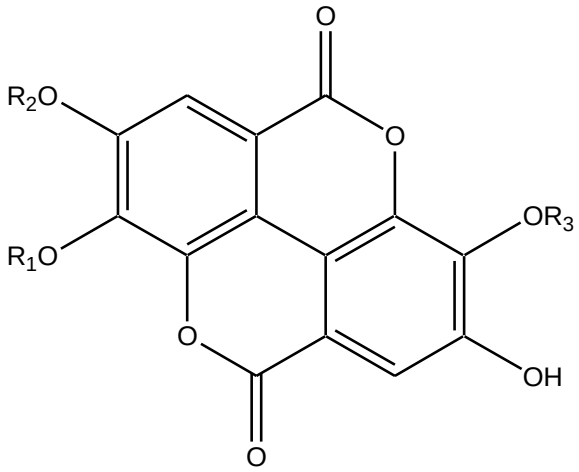


Table 10: Hydrolyzable tannins isolated from *Cornus* species

Compound	R_1	R_2	R_3	Species	Ref
3,4,3'-tri-*O*-methylellagic acid	Me	Me	Me	*kousa*	(9)
3,4-di-*O*-methylellagic acid	Me	Me	H	*kousa*	(9)

2.1.3.2. Terpenic Compounds

a) Iridoids

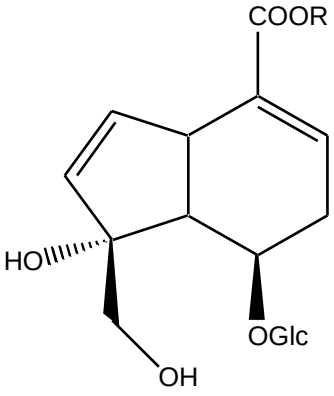


Table 11: Iridoids isolated from *Cornus* species

Compound	R	Species	Ref
Monotropein	H	*canadensis*	(45)
Galioside	Me	*canadensis*	(45)

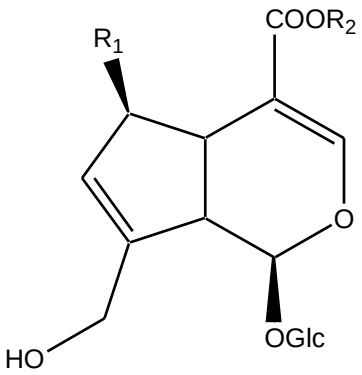


Table 12: Iridoids isolated from *Cornus* species

Compound	R_1	R_2	Species	Ref
Geniposide	H	Me	*suecica*	(45)
Scandoside	OH	H	*canadensis*	(45)
Scandoside methyl ester	OH	Me	*canadensis*	(45)

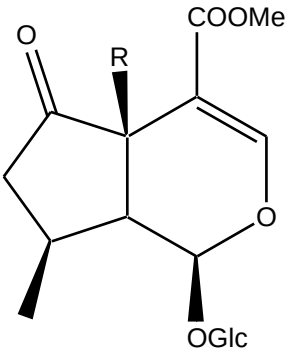


Table 13: Iridoids isolated from *Cornus* species

Compound	R	Species	Ref
Cornin	H	*nuttallii* *capitata*	(45) (29)
Hastatoside	OH	*nuttallii*	(45)

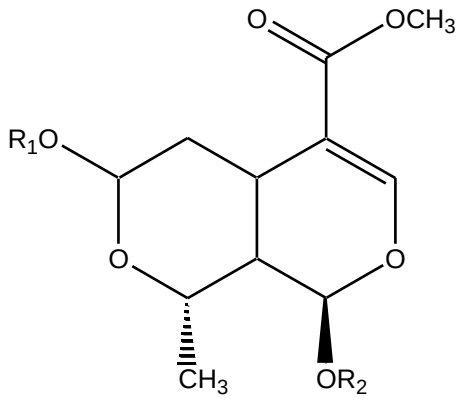


Table 14: Iridoids isolated from *Cornus* species

Compound	R_1	R_2	Species	Ref
Morroniside	H	Glc	*officinalis*	(35,37)
7-*O*-methyl-morroniside	Me	Glc	*officinalis*	(35,37)
7-*O*-butyl-morroniside	Me-C_3H_7	Glc	*officinalis*	(21,35)

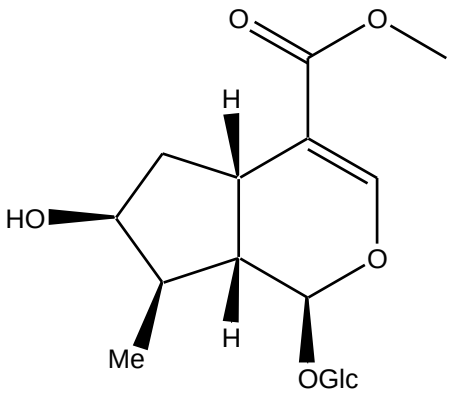


Table 15: Iridoids isolated from *Cornus* species

Compound	Species	Ref
Loganin	*officinalis*	(21)

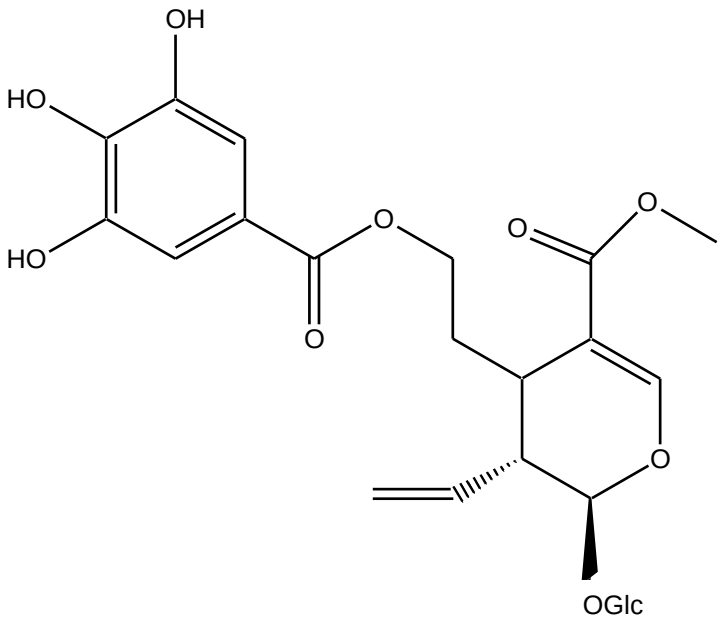


Table 16: Iridoids isolated from *Cornus* species

Compound	Species	Ref
Cornuside	*officinalis*	(21)

b) Triterpenes

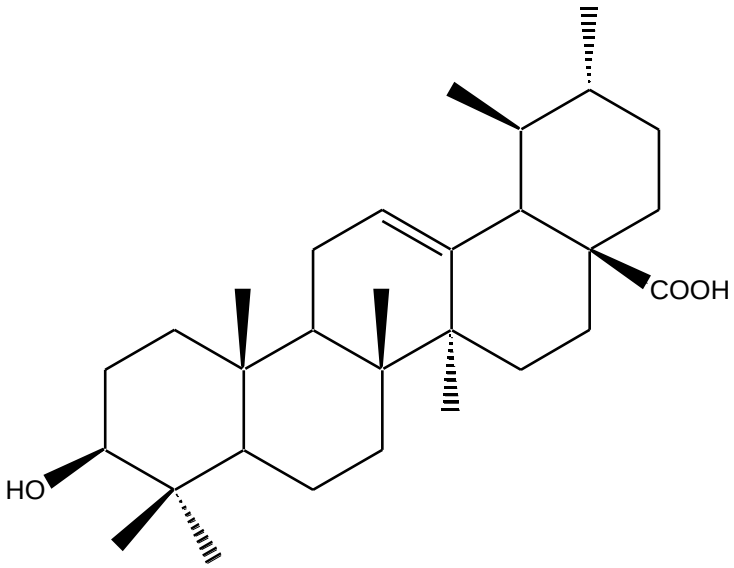


Table 17: Triterpenes isolated from *Cornus* species

Compound	Species	Ref
Ursolic acid	*kousa*	(34)
	mas	(23)
	florida	(16)
	officinalis	(14)

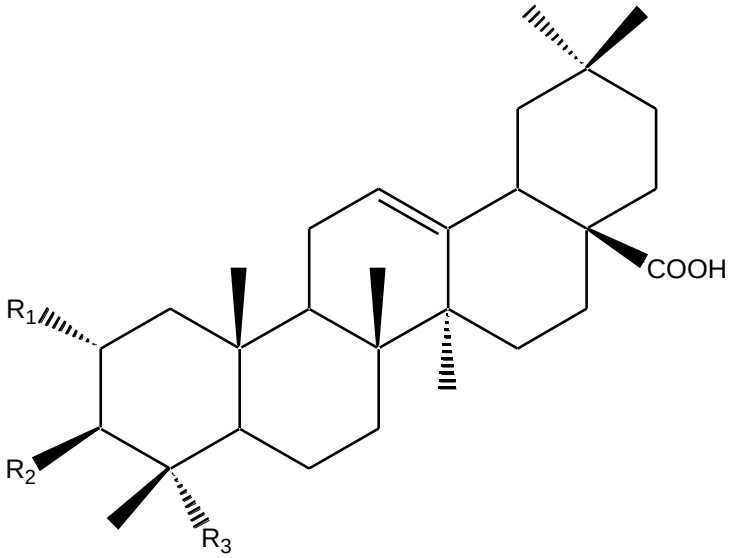


Table 18: Triterpenes isolated from *Cornus* species

Compound	R_1	R_2	R_3	Species	Ref
Oleanolic acid	H	OH	Me	*officinalis*	(21)
Maslinic acid	OH	OH	Me	*kousa*	(9)
Arjunolic acid	OH	OH	CH_2OH	*kousa*	(9)

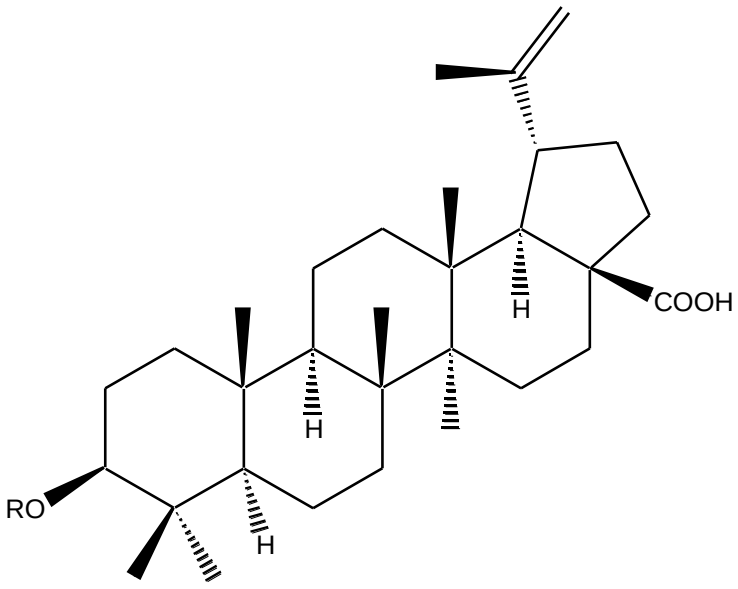


Table 19: Triterpenes isolated from *Cornus* species

Compound	R	Species	Ref
Betulinic acid	H	*kousa* *florida*	(9) (16)

3-β-*O*-acetyl betulinic acid	$COCH_3$	*florida*	(16)
3-β-*O*-*cis*-coumaroyl betulinic acid	*cis*-coumaroyl	*florida*	(16)
3-β-*O*-*trans*-coumaroyl betulinic acid	*trans*-coumaroyl	*florida*	(16)

2.1.3.3. Steroidal Compounds

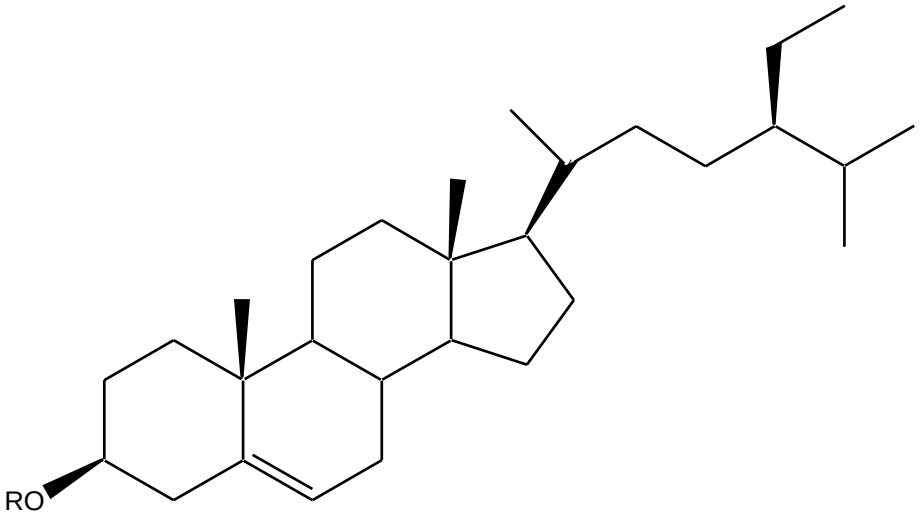


Table 20: Steroidal compounds isolated from *Cornus* species

Compound	R	Species	Ref
β-sitosterol	H	*officinalis* *florida*	(21) (16)
Daucosterol	Glc	*kousa*	(9)

2.1.3.4. Other Compounds

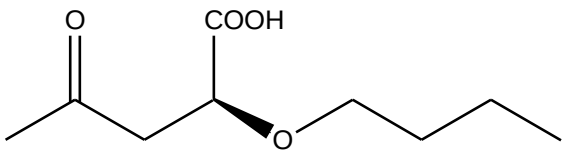


Table 21: Other compounds isolated from *Cornus* species

Compound	Species	Ref
Butoxysuccinic acid	*officinalis*	(21)

2.2. *Laurocerasus* Duhamel

2.2.1. Botanical Information

2.2.1.1. Rosaceae

Woody or herbaceous, sometimes armed. Leaves alternate, usually stipulate, simple or compound, often with a toothed margin. Infloresence diverse, in trees and shrubs often borne on spur shoots. Flowers hermaphrodite or unisexual, actinomorphic, perigynous or epigynous. Sepals 4–5, free; epicalyx present or absent. Petals 4–5, free or absent. Stamens 1–many. Ovary superior to inferior, of 1–many carpels, the carpels only united in genera ± inferior ovary. Fruit with a dry or fleshy receptacle or hypanthium of diverse form, bearing follicles, achene(s) or drupe or forming a pome. Seeds usually without endosperm (46).

2.2.1.2. *Laurocerasus* species

Trees or shrubs. Leaves evergreen. Flowers in narrow leafless, axillary racemes; sepals petals 5; ovary superior. Stamens 20, arranges in two whorls. Fruit a dark purple or blackish, juicy drupe (46).

2.2.1.3. *Laurocerasus officinalis* Roem.

Shrub or small tree up to 6 m; annual shoots glabrous. Leaves oblong-elliptic to oblong-obovate, up to 20 x 8 cm, remotely dentate or entire, dark green and lustrous above, paler beneath, glabrous; petioles up to 15 mm, channeled. Racemes compact, usually shorter than leaves, lacking leaves but with some small bracts. Flowers white, pedicels up to 8 mm; hypanthium campanulate with inflexed lobes; petals c. 4 mm. Drupes ovoid, c. 8 mm diam. dark purple or black; stones smooth (46).

Flowering season: 4^{th} – 6^{th} months
Habitat: Undergrowth in forests, mostly with *Fagus* and *Rhododendron*
Altitude: 20–2000 m.

Distribution in Turkey: Regions of Marmara, Black Sea and Southeastern Mediterranean

Worldwide distribution: Bulgaria, Central Europe, Western Caucasia, Northern Iran.

2.2.1.4. Local names of *Laurocerasus* species

In Turkish: Taflan, karayemiş, karamış, kattak, Laz kirazı, Laz üzümü (7).

In English: Cherry laurel

2.2.1.5. Traditional Usage of *Laurocerasus* Species

The fruits of *L. officinalis* are widely ingested as food in the Black Sea region of Turkey. They are consumed as fresh fruits in local markets, and also as dried, pickled, jam, marmalade or as fresh fruit juice (47). The fruits are also quick–dried in oven before eating, and named as “sira” by local people (7).

In addition to the consumption as food, the fruits are also utilized in Turkish folk medicine. They are employed against eczema, hemorhhoids and as diuretic (47). Fresh fruits are also used to pass kidney stone (13).

The seeds are known to be used for the treatment of stomach ulcer and against bronchitis (47). Besides, they are utilized against hyperglycemia. The oven–dried seeds are powdered and eaten in the morning (13).

Fresh leaves are externally used against pain and high fever. Fresh broad leaves are applied on the forehead, after being wilted over open fire (5). The infusion prepared from leaves is known to have sedative effects. It is also employed against cough and nausea (13).

2.2.2. Bioactivity Studies on *Laurocerasus* Species

2.2.2.1. Studies on Antiinflammatory Activity

Erdemoglu et al. investigated the anti-inflammatory and antinociceptive activity of both ethanolic and aqueous extracts of *L. officinalis* leaves in a preliminary screening study. They reported that the ethanolic extract exerted significant activity against carrageenan–induced paw edema in mice, whereas the aqueous extract was reported to be in effective. Moreover, *p*-benzoquinone–induced abdominal constriction test revealed that the ethanolic extract had significantly high antinociceptive activity (48).

In another study about the same activities, Akkol et al. stated that the ethanolic extract of *L. officinalis* leaves was shown to possess significant anti-inflammatory activity without causing any gastric damage. The bioassay–guided fractionation studies led to the isolation of three phenolic molecules as the active constituents such as 2-*O*-β-D-glucopyranosyl-2-hydroxyphenyl-acetic acid, kaempferol-3-*O*-β-D-xylopyranosyl-*O*-β-D-glucopyranoside and (+)-cathecin (49).

2.2.2.2. Studies on Neuroprotective Activity

Orhan and Akkol studied the neuroprotective effects of both the leaves and fruits of *L. officinalis* through cholinesterase inhibitory activity against acetylcholinesterase and butrylcholinesterase. Their results indicated that the methanolic extract of leaves had significant activity in terms of inhibition of both cholinesterase enzymes (50).

2.2.2.3. Studies on Antioxidant Activity

Kolaylı et al. studied the comparative antioxidant activity of *L. officinalis* fruits with different solvents i.e. water, 70% ethanol and acetone. The aqueous extract showed the best radical scavenging activity In addition to the antioxidant activity, they calculated the amount of some trace elements such as

manganese, iron, zinc and copper. The results postulated that the amounts of all these elements were below dietary recommended allowance (51).

In another study by Orhan et al., the polyphenolic extract prepared from the seeds exerted good superoxide radical scavenging activity (52).

2.2.3. Compounds Isolated from *Laurocerasus* Species

In a previously mentioned bioassay–guided fractionation study by Akkol et al., three phenolic compounds were isolated: 2-*O*-β-D-glucopyranosyl-2-hydroxyphenyl-acetic acid, kaempferol-3-*O*-β-D-xylopyranosyl-*O*-β-D-glucopyranoside and (+)-cathecin (49).

In another study two kaempferol glycosides from the chloroform fraction of the hydroalcoholic extract of *L. officinalis*; kaempferol-3-*O*-(6-*O*-α-D-xylofuranosyl- β-D-galactopyranoside and kaempferol-3-*O*-(6-*O*-β-D-glucofuranosyl- β-D-galactopyranoside (53).

Weinges et al. isolated benzyl-β-D-primveroside from the green fruits of *L. officinalis* (54).

Ayaz et al. postulated that the fruits contain vanillic acid, procatechuic acid, *p*-hydroxybenzoic acid, caffeic acid and *p*-coumaric acid as phenolic acid. The same study revealed that they contain palmitic acid, stearic acid, oleic acid and linoleic acid (55).

2.3. *Diospyros* L.

2.3.1. Botanical Information

2.3.1.1 Ebenaceae

Trees or shrubs. Leaves alternate, subentire, exstipulate. Flowers usually unisexual, dioecious. Calyx 3–6–lobed, hypogynous, persistent and often accrescent in fruit. Corolla gamopetalous, Calyx 3–6–lobed, imbricate in bud. Male flowers: stamens 4–many, hypogynous or attached to base of corolla. Female flowers with or without staminoides. Ovary superior, 3– or more locular; placentation axile, ovules 1–2 in each loculus. Fruit a berry (56).

2.3.1.2. *Diospyros* species

Trees or shrubs. Leaves deciduous (in Turkey), alternate, entire. Buds ovoid with 2–3 outer scales. Flowers dioecious, yellowish, reddish or whitish; female flowers usually solitary, male flowers in few to many–flowered axillary cymes. Calyx and corolla (in Turkey) usually 4–lobed; stamens (in male) 4–many; staminodes present in female flowers. Ovary 4–12–locular. Berry large and juicy with 1–10 large flattened seeds; calyx persistent and accrescent (56).

The genus *Diospyros* is represented by 2 species in “Flora of Turkey and The East Aegean Islands”. Dichotomous key for the determination of these species is as follows:

1. Fruit globose, c. 2 cm diam.; male flowers c. 5 mm; young shoots pubescent to glabrescent **1. *lotus***

1. Fruit ovoid to depressed–globose, 5–8 cm diam.; male flowers c. 10 mm; young shoots persistently brownish–pubescent **2. *mas***

2.3.1.3. *Diospyros kaki* L.

Decidous tree to 15 m, with rounded crown. Bark scaly on old trunk. Young shoots and buds conspicuously pubescent. Leaves entire, elliptic–ovate, oblong–ovate or obovate, to 18 x 9 cm, usually broadly cuneateat base, dark green, glabrous and shining above, lighter green and pubescent. Flowers yellowish–white; male flowers in threes, c. 10 mm, with 16–24 stamens; female flowers 12–20 mm. Fruit ovoid to deprresed–globose, 5–8 cm diam. with 4 or 8 furrows, orange–red to bright yellow (56).

Flowering season: 6th month.
Habitat: Cultivated in gardens and orchards (in Turkey)
Distribution in Turkey: Cultivated in throughout N. Turkey for its edible fruit and ornament; less commonly grown in S. Anatolia.
Worldwide distribution: Native in China and Japan.

2.3.1.4. Local names of *Diospyros* species

In Turkish: Trabzon hurması, kara hurma, hırnık, hırniyh, hurma eriği, kara yemiş, Yahudi hurması, amme, Japon hurması (7).

In English: Japanese persimmon, persimmon.

2.3.1.5. Traditional usage of *Diosypros kaki* L.

The fresh or dried leaves of *D. kaki* are used in the treatment of hypertension, angina and internal haemorrhage in China. They are also utilized in the treatment of resistant hiccups in traditional Chinese medicine, and have been used traditionally in Korea and Japan to promote maternal health (57,58).

Both fruits and leaves are used to treat coughs, hypertension and dyspnea in Japanese folk medicine. They are also used for the same purpose in Malaysia (57).

2.3.2. Bioactivity Studies on *Diospyros* species

A vast number of studies was performed in the literature about the biological activities of *Diospyros* species. A wide review about the pharmacology and chemotaxonomy of *Diospyros* genus by Mallavadhani et al. gives detailed information (59). Here, we concentrated on the recent studies about *D. kaki.*

Matsushita et al. studied the anticancer and cytotoxic effects of *D. kaki* heartwood and isolated active constituents from the methanolic extract (60). Chen et al. also isolated cytotoxic compounds from the leaf extract of *D. kaki* (58).

Kawase et al. investigated the cytotoxic activity, multidrug resistance reversal activity, anti-human immunodeficiency virus activity and anti-*Helicobacter pylori* activity. They reported that acetone fractions exerted potent cytotoxic activity, whereas none of the tested fractions were effective for anti-*H. pylori* and anti-human immunodeficiency virus activities. They also stated that both hexane and methanolic fractions showed a remarkable multidrug resistance reversal activity (57).

Another study on the anticancer activity by Jo et al. demonstrated that methanolic extracts of *D. kaki* calyx had cytotoxic effects against various human cancer cells (61).

Sanchetti et al. found that the methanolic extract of *D. kaki* leaves had potent α-glucosidase inhibitory effect besides its antioxidant effects (62).

2.3.3. Compounds isolated from *Diospyros* species

As the same review mentioned before gives detailed information about the compounds isolated from *Diospyros* species (59), recent compounds from *D. kaki* are given here.

Matsushita et al. isolated 4,8-dihydroxy-5-methoxy-2-naphthaldehyde from the blackened heartwood of *D. kaki* (60). Chen et al. reported the isolation of a new biphenyl derivative, 4,5-dimethoxy-3-β-D-glucopyranosyloxy-4-hydroxy-biphenyl, along with vitexin and isorhamnetin (58). In another study by Chen et al., 4,6-dihydroxy-2-glucopyranosylbenzophenone, 4,4-dihydroxy truxillic acid, myricetin, annulatin, hyperoside, isoquercetin, rutin, quercetin and kaempferol were isolated from the leaves of D. kaki (63). Ohguchi et al. also isolated isoquercitrin and hyperoside from the peel extract of *D. kaki* (64)

Thuong et al. reported the isolation of two new ursane type triterpenoids from the methanolic leaf extract: 3,19-dihydroxyurs-12,20(30)-dien-24,28-dioic acid and 3,19-dihydroxyurs-12-en-24,28-dioic acid (65).

2.4. OXIDATIVE STRESS

2.4.1. Oxygen and Oxidative Stress

Oxygen is the commonest element in the Earth's crust, and composes 21% of the atmosphere (66). It is also found in the seas, lakes, rivers and other bodies of water. Except for some anaerobic species, all organisms require molecular O_2 as an electron acceptor for efficient production of energy (66,67).

When living organisms first appeared on Earth, they were all anaerobes because there was a very little amount of oxygen. Over 2.2 billion years ago, cyanobacteria evolved to make photosynthesis, leading to the appearance of oxygen in significant amounts in the Earth's atmosphere. As the O_2 content rose, many primitive species became extinct. Some organisms followed the evolutionary path of adapting to rising atmospheric O_2 by restricting themselves to environments into which the O_2 did not penetrate. Other organisms followed a different path and began to evolve defense systems to be protected against O_2 toxicity (66). The toxicity of oxygen to the living organism was first described by Joseph Priestley in 1775. He compared its effect on the body as similar to that of burning a candle; as a candle burns much faster in oxygen than in air, the body becomes quickly exhausted in this pure kind of air (68).

Although the poisonous side effects of oxygen were known for a long time, the causes of them remained obscure until mid 19's. The earliest suggestion was that O_2 directly inhibits essential enzymes (66). However, it was later understood that most enzymes in aerobes are unaffected by O_2. In 1954, Daniel Gilbert and Rebecca Gersham showed the similarities between the effects of oxygen and those of ionizing radiation and proposed that the toxicity of oxygen is due to partially reduced forms of oxygen, in other words reactive oxygen species (ROS) leading to oxidative stress (66,68,69).

Reactive oxygen species (ROS), as well as reactive nitrogen species (RNS) are products of normal cellular metabolism. They are both well recognized for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems (66). The beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular responses to noxia, as for example in defense against infectious agents and in the function of a number of cellular signaling systems. One further beneficial example of ROS at low/moderate concentrations is the induction of a mitogenic response (69).

The detrimental effect of ROS causing potential biological damage is termed oxidative stress and nitrosative stress. This occurs in biological systems when there is an overproduction of ROS/RNS on one side and a deficiency of enzymatic and non-enzymatic defense systems on the other. Briefly, oxidative stress arises from the metabolic reactions that use oxygen and represents a disturbance in the balance of oxidant/antioxidant reactions in living organisms (69).

2.4.2. Free Radicals

Free radicals can be defined as molecules or molecular fragments, capable of independent existence (the reason for “free"), that contain one or more unpaired electrons in atomic or molecular orbitals (66,69). For this reason they promote electron transfers, such as oxidation and reduction (70). The presence of one or more unpaired electrons usually causes free radicals to be attracted to a magnetic field and makes them highly reactive (66,71). As Halliwell & Gutteridge states “All oxygen radicals are ROS, but not all ROS are oxygen radicals.” (66).

Free radicals can be formed by losing a single electron from a non–radical atom or molecule or by gaining one (66). Another pathway for radical formation is the breaking of a covalent bond. If one electron remains on one atom, and the other electron remains on the other atom, this process is known as **homolytic fission**. The cleavage of these covalent bonds is an endothermic

reaction, and requires heat or electromagnetic radiation (71). Homolytic fission can be described as below;

(Assuming that **A** and **B** atoms are covalently bonded)

$$A : B \longrightarrow A^{\cdot} + B^{\cdot}$$

In **heterolytic fission**, the opposite of homolytic fission, one atom receives both electrons when a covalent bond breaks. It can be described as below;

$$A : B \longrightarrow A^{-} + B^{+}$$

The atom “A” receives both electrons and therefore gains negative charge, whereas the atom “B” is left with a positive charge. But it should be expressed that neither of these species is a free radical, and heterolytic fission yields only charged ions (66).

2.4.2.1. Reaction mechanisms of free radicals (66)**:**

A) In the case of meeting of two radicals, they form a covalent bond and join their unpaired electrons. As a result, they lose their radical properties.

$$H^{\cdot} + H^{\cdot} \longrightarrow H_2$$

B) But, most biological molecules are non-radicals. When a radical meets a non-radical, a chain of reactions may occur.

1. A radical may add on to another molecule. The resulting product still has an unpaired electron, and behaves as a radical.

$$A^{\cdot} + B \longrightarrow [A - B]^{\cdot}$$

2. A radical may donate a single electron to a non-radical. The radical acts as a reducing agent, and the recipient has an unpaired electron.

$$A^{\cdot} + B \longrightarrow A^{+} + B^{\cdot -}$$

3. A radical may take a single electron from a non-radical. The radical acts as an oxidizing agent, and again the recipient has an unpaired electron.

$$A^{\cdot} + B \longrightarrow A^{-} + B^{\cdot +}$$

4. A radical may abstract a hydrogen atom from a C–H bond. As $H^{\cdot}$ has only one electron, an unpaired electron must be left on the carbon.

$$(C - H) + OH^{\cdot} \longrightarrow C^{\cdot} + H_2O$$

This reaction may lead to the propagation of the chain reaction of lipid peroxidation.

2.4.2.2. Sources of ROS

Oxidation and formation of reactive oxygen species (ROS) is an inevitable consequence in human aerobic mechanism. The cell is exposed to a large variety of endogenous and exogenous sources (68):

A) Endogenous sources:

1. Mitochondrial electron transport chain:

Endogenous formation of ROS is a continuous process during the life span of every cell in the organism (68). The mitochondrial electron transport chain has been long known to be capable of generating ROS (72). This chain is the main source of ATP in the mammalian cell and therefore essential for life (69). As a consequence of normal aerobic respiration (73), the reduction of oxygen to water in the mitochondria for ATP production occurs through the donation of 4 electrons to oxygen to produce water (68), such as:

$$O_2 + 4e^{-} + 4H^{+} \longrightarrow 2H_2O$$

During this process, several reduced oxygen derivatives are formed (73,74). In many cases, there is “leakage” of ROS from mitochondria into the cellular environment (74). Moreover, the production of ROS by mitochondria involves NADH-coenzyme Q (complex I), succinate-coenzyme Q (complex II),

QH_2 – cytochrome *c* reductases (complex III). Although, most of the electron transfer is tightly coupled, a small amount of leakage occurs, as mentioned above. This leakage primarily occurs from the NADH-coenzyme Q reductase complex, from the autooxidation of coenzyme Q, itself (72,75). About 1 to 3% of the oxygen reduced in the mitochondria may form superoxide radical (66).

2. Enzymes:

Enzymes comprise another important source of endogenous ROS production (68). There are 2 different mechanisms of ROS production by enzymes such as: designated production of ROS by certain enzymes, and production of ROS as a by-product of normal enzyme activity (68). Phagocytosis can be given as an example of the former mechanism. Phagocytic cells destroy bacteria or virus-infected cells with “oxidative burst” of ROS. This reaction is catalyzed by a plasma membrane-bound NADPH oxidase with the extracellular production of large amounts of ROS (68,72,73).

In addition, there is a large variety of enzymes that can cause oxidation, and formation of free radicals. The most well known of these enzymes is xanthine oxidase. This enzyme can directly cause the reduction of molecular oxygen to superoxide (72). In fact, xanthine oxidase catalyzes the oxidation of hypoxanthine and xanthine to uric acid, and the formation of ROS is a by-product (76).

Other oxidases such as diamine oxidase, dopamine-β-hydroxylase, D-amino acid oxidase, tryptophan dioxygenase, and fatty acyl-CoA oxidase also can generate ROS (66,67,72,77).

3. Auto-oxidation reactions:

Several numbers of important biological molecules are oxidized in the presence of oxygen (O_2) and yield superoxide radical ($O_2^{\cdot -}$). These molecules are adrenalin, noradrenalin, L-DOPA (dihydroxyphenylalanine), dopamine,

glyceraldehyde, reduced form of flavin mononucleotide ($FMNH_2$), reduced form of flavin adenine dinucleotide ($FADH_2$) and thiol compounds such as cysteine.

Since O_2 is poorly reactive, the rate of the auto-oxidation reactions is accelerated by the addition of transition metal ions. In other words, auto-oxidation reactions are catalyzed by metal ions (66).

4. Haem proteins:

Iron is in the Fe^{2+} state in the haem of haemoglobin and myglobin, and remains in this state until the binding of O_2. After that, some electron delocalization takes place.

$$\mathbf{HAEM - Fe^{2+} - O_2 \longleftrightarrow HAEM - Fe(III) - O_2^{\cdot -}}$$

There is an intermediate bonding between Fe^{2+} bonded to O_2, and Fe(III) bonded to $O_2^{\cdot -}$. This causes the release of $O_2^{\cdot -}$.

$$\mathbf{HAEM - Fe^{2+} - O_2 \longrightarrow O_2^{\cdot -} + HAEM - Fe(III)}$$

It has been estimated that about 3% of the haemoglobin present in human erythrocytes undergoes oxidation every day. Haemoglobin and myoglobin oxidation is accelerated by nitrite ions or by certain transition metal ions, especially copper (66,78).

5. Endoplasmic reticulum:

The endoplasmic reticulum (ER) of many animal and plant tissues contains cytochromes (CYPs), basically known as cytochromes P450. The name P450 was given because reduced cytochromes form a complex with carbon monoxide and this complex absorbs light strongly at 450 nm. The CYPs catalyse oxidation of the substrates by O_2. One atom of oxygen binds the substrate and the other atom forms water, such a reaction known as **mono-oxygenase** or **mixed function-oxidase**. CYPs require a reducing agent (RH_2) and they utilize NADPH for this purpose. The most important mechanism in the formation of ROS from chemical agents is the activation of xenobiotics by the

microsomal CYP450 system. This system produces ROS by adding (reduction) or removing (oxidation) one electron from target molecules (66,67,79).

B) Exogenous Sources:

a) Irradiation: Exposure of living organisms to either ionizing or nonionizing irradiation is a major exogenous source of ROS (48,60). Exposure of the cell to γ-radiation results in the production of a variety of ROS from ionization of the intracellular water. Even exposure to non–ionizing irradiation can indirectly produce a variety of ROS (68).

b) Air pollutants: Air pollutants such as car exhaust, cigarette smoke, and industrial wastes are a major class of exogenous sources of ROS which can be formed by skin interaction or by inhalation (68).

c) Drugs: Narcotic drugs and anesthetizing gases are considered major contributors of ROS formation. Also a large number of xenobiotics such as toxins, pesticides, and herbicides, chemicals such as mustard gas, carbon tetrachloride and alcohol, including alcoholic beverages also contribute to ROS production (68).

d) Food: A large portion of the daily consumed food is oxidized and contains different kinds of oxidants such as peroxides, aldehydes, oxidized fatty acids, and transition metals (68).

2.4.2.3. Superoxide radical ($O_2^{\cdot -}$):

Superoxide radical is formed by the addition of one atom to the ground state O_2 molecule (66,67). It is formed almost in all aerobic cells by “leakage” of electrons onto O_2 from various components of the cellular electron transport chains (81). The production of superoxide occurs mostly within the mitochondria of the cell. During energy transduction, a small number of electrons “leak” to oxygen prematurely, forming superoxide (69). Another pathway for superoxide formation is the respiratory burst of phagocytic cells such as neutrophils and

macrophages (81). The role of haem proteins in superoxide formation is mentioned above.

Superoxide radical has a very short half-life, only in millisecond range. It is a weak oxidant, but rather a potent reductant. Although it is well known that it plays a major role in the toxicity of oxygen molecule, superoxide radical, itself, does not induce large cell damage due to its weak oxidant effect (67,82). It may react with nucleophilic thiol (-SH) groups, resulting in the depletion of reduced glutathione (GSH). This causes further oxidative stress. If it reacts with –SH groups of enzymes, this may induce its inactivation (82). Much of the damage caused by superoxide radical is caused by commencing a chain of reactions which cause remarkable oxidative stress. In one of these reactions, known as **Haber–Weiss** reaction, superoxide radical reacts with hydrogen peroxide in the presence of iron. Despite the fact that iron regulation mechanism of the body ensures that there is no free intracellular iron, under stress conditions, an excess of superoxide radical releases “free iron” from iron containing compounds. This reaction yields an extremely reactive radical, hydroxyl radical ($OH^{\bullet}$) (66,67,69) such as:

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

As mentioned before, $O_2^{\bullet -}$ has a very limited half-life, and it is readily converted to O_2 and H_2O_2 in a process, called **dismutation reaction**.

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

Although this reaction can occur spontaneously in mild acidic conditions, it is also catalyzed by the enzyme, **superoxide dismutase** (66,67,81).

2.4.2.4. Hydrogen peroxide (H_2O_2):

H_2O_2 has no unpaired electrons, so it is not chemically a radical. But it is considered as ROS. It can be formed either directly with the reduction of O_2 or indirectly as a result of the dismutation reaction from $O_2^{\bullet -}$ (67,68,82–84).

$$O_2^{\cdot -} + 2\,H^+ + e \longrightarrow H_2O_2 \text{ (direct formation)}$$

$$2\,O_2^{\cdot -} + 2\,H^+ \longrightarrow O_2 + H_2O_2 \text{ (dismutation)}$$

Although H_2O_2 has limited reactivity (67,84), it can cause damage to the cell at a relatively low concentration (10 μM) (68). It is freely soluble in aqueous media, therefore it can travel through the cell compartments, and easily penetrate biological membranes (68,84). H_2O_2 exerts its deleterious effect directly with regard to its oxidizing property, or indirectly by serving as a source for more deleterious ROS (i.e. $OH^{\cdot}$, HOCl) (68). H_2O_2 can oxidize the thiol groups of proteins, and it can cause chain-breakage in DNA (67,68). High concentrations of H_2O_2 can inactivate one of the glycolytic pathway enzymes, glyceraldehyde-3-phosphate dehydrogenase in mammalian cells (85).

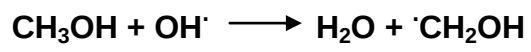
As stated earlier, H_2O_2 can cross biological membranes. It reacts with cellular Fe^{2+} and forms a very reactive and extremely oxidant radical, $OH^{\cdot}$ (66). This reaction is known as **Fenton reaction**, which is discussed later. Besides, it also yields $OH^{\bullet}$ by reacting with $O_2^{\cdot -}$ (Haber–Weiss Reaction) (81,86). Accordingly, the formation of $OH^{\bullet}$ lies in the origin of toxicity mechanism of H_2O_2. Simultaneous exposure to H_2O_2 and UV radiation increases the influence, because UV light induces the homolytic fission of H_2O_2 to $OH^{\cdot}$. Enzymes catalase and glutathione peroxidase avoids the damage caused by H_2O_2 (82).

2.4.2.5. Hydroxyl radical ($OH^{\cdot}$):

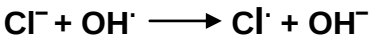
The hydroxyl radical is the neutral forms of the hydroxide ion (69). It is the most reactive radical and the most potent oxidant in biological systems (68,82), such that it can react with almost every type of biological molecules in living cell (86). It has a very short half-life of approximately 10^{-9} seconds (87). Thus, it reacts close to its site of formation (69). Therefore, the type of damage depends on the site of formation such as; production of $OH^{\cdot}$ close to DNA could lead to modification of purines or pyrimidines or to strand breakage (88), whereas production of $OH^{\bullet}$ close to an enzyme molecule present in excess in the cell, such as lactate dehydrogenase, might have no biological consequence

(81). There are three main types of chemical reactions that are induced by hydroxyl radicals, such as (86):

- Hydrogen atom abstraction, e.g. for methanol or lipid peroxidation:



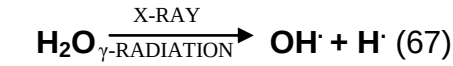
- Addition, e.g. it has the ability to add on to the aromatic purine and pyrimidine bases on the structure of DNA.
- Electron transfer, e.g. with Cl^- ion:



$OH^{\cdot}$ radical has such a massive reactivity that, if it is formed in living systems, it has the capability of reacting immediately with every biological molecule which is in its vicinity, and producing secondary radicals of variable reactivity. For example, it reacts with carbonate ion (CO_3^{2-}), and causes the formation of carbonate radical ($CO_3^{-\cdot}$) (66,67,86).

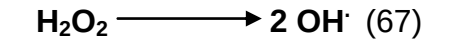
There are several reactions that $OH^{\cdot}$ can be generated, such as:

1. Since water is one of the major constituents of living cells, exposure to high energy radiation such as X–rays or γ–rays induces homolytic fission of water, and subsequent formation of $OH^{\cdot}$ (66).



It is also known that ultrasonication of aqueous solutions also produces $OH^{\cdot}$ (66,67).

2. $OH^{\cdot}$ can also be generated by heat or ionizing radiation-induced homolytic fission of O–O bond in H_2O_2 (66,86).



3. H_2O_2 reacts with Fe^{2+} and generates $OH^{\cdot}$. The same reaction is valid for copper (Cu^{+}). This interaction was first described by Fenton in 1894, and known as **Fenton reaction** (68,86).

$$\mathbf{Fe^{2+} + H_2O_2 \longrightarrow FeOH^{3+}\ (or\ FeO^{2+}) \longrightarrow\ Fe^{3+} + OH^{\cdot} + OH^{-}}\ (85)$$

$$\mathbf{Cu^{+} + H_2O_2 \longrightarrow Cu^{2+}\ OH^{\cdot} + OH^{-}}\ (66)$$

4. Another mechanism for $OH^{\cdot}$ production is the reaction of $O_2^{\cdot -}$ with H_2O_2. The reaction is known as **Haber–Weiss** reaction and named after Fritz Haber and Joseph Joshua Weiss, who described the reaction for the first time in 1932 (89). It is a two-step process and catalyzed in the presence of iron or copper ions.

$$\mathbf{1^{st}\ step:\ O_2^{\cdot -} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}}$$

$$\mathbf{2^{nd}\ step:\ Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}\ (Fenton\ reaction)}$$

$$\mathbf{Net\ reaction:\ O_2^{\cdot -} + H_2O_2 \longrightarrow OH^{\cdot} + {}^{-}OH + O_2}$$

5. Hypochlorous acid (HOCl) also generates $OH^{\cdot}$ by reacting with $O_2^{\cdot -}$ (66).

6. Hydroxyl radicals can also be generated from ozone and peroxynitrite radical (66).

2.4.2.6. Hypochlorous acid (HOCl):

Although HOCl is not a radical, it is considered as ROS, because it is a powerful two-electron oxidizing agent (66,90). It is generated by the reaction of chloride ion (Cl^{-}) with H_2O_2, which is produced by the dismutation of $O_2^{\cdot -}$ (90). This reaction generally occurs in phagocytic cells (67) and catalyzed by the enzyme **myeloperoxidase** (66), such as:

$$\mathbf{H_2O_2 + Cl^{-} \longrightarrow HOCl + OH^{-}}\ (67)$$

It is a very strong antibacterial agent such that it damages electron transport chain components and the ATP synthase, and subsequently inhibits ATP synthesis in bacteria (91). HOCl readily oxidizes thiols, ascorbates, NADPH and DNA. It also causes the chlorination of DNA bases, especially pyrimidines (92). Another target for HOCl damage is proteins. It directly causes side-chain damage, fragmentation and aggregation of proteins by multiple reactions. It also has the capability of crossing cellular membranes, and causes damage to membrane proteins and lipids on the passage (66).

As stated above, HOCl produces $OH^{\cdot}$ by reacting with $O_2^{\cdot -}$ (90). It also takes part in formation of singlet O_2 (66). It attacks lipids by adding across double bonds in unsaturated fatty acid residues in phospholipids (66). GSH and N-acetylcysteine protects the human body from the deleterious effects of HOCl (67).

2.4.2.7. Singlet oxygen ($^1O_2$):

Singlet O_2 is the more reactive form of molecular oxygen (67). It is generated by a mechanism such as: One of the electrons of the molecular oxygen gains energy and passes to an orbital which is in the opposite of its own spin direction (86). This situation removes the spin restriction that slows the reaction of O_2 with non–radicals. Singlet oxygens are much more oxidizing than ground state O_2 (66). They have two states: **delta** and **sigma**. Sigma state has energy of 37.5 kcal, while delta state has 22.4 kcal. Because the sigma state is more reactive, it rapidly decoys to the delta state. Therefore, only the delta state is considered in biological systems. It should be noted that delta state O_2 is not a free radical (66,67,79,86).

Singlet oxygen is often generated by photosensitization reactions. If several biological pigments such as **chlorophylls *a*** and ***b*, retinal, bilirubin, flavins (FAD, FMN) or porphyrins** are illuminated in the presence of O_2 with light of the correct wavelength, they absorb it and the energy raises the molecule into an **excited state.** This excitation energy can be transferred to an adjacent O_2, converting it to delta state.

By this way, flavins lose their orange colors, and chlorophylls lose their green colors. Singlet oxygen causes damage in the mitochondria, which is rich with haem proteins and other proteins containing flavins. This may cause the inhibition of catalase enzyme, which contains haem proteins. In case of continuous and consistent exposure of retina by light in certain wavelength, the retinal pigment found in retina may induce the generation of $^1O_2$, causing damage in the eye (66,67).

2.3.3. Lipid peroxidation:

Polyunsaturated fatty acids (PUFAs) are found in the entire supporting system of cells, including cell membranes, endoplasmic reticulum, and mitochondria (66,82,93). They are one of the major components of the phospholipids found in mammalian cell membrane. Some common, naturally occurring PUFAs (in the order of number of carbon atoms) are oleic acid, linoleic acid, α-linolenic acid, arachidonic acid, and eicosapentaneoic acid (66). The effects of $OH^{\cdot}$ on the fatty acid component of membrane phospholipids have been suggested to be the essential event in cellular oxidative damage (82).

Lipid peroxidation is the free radical oxidation of PUFAs in biological systems and it is composed of three stages: initiation, propagation, and termination (84). The process is initiated by the attack of $OH^{\cdot}$ on the fatty acyl side chain of the membrane phospholipids (LH). It abstracts an allylic hydrogen atom from a methylene carbon in the side chain and generates a lipid radical ($L^{\cdot}$). This step is called **initiation**, such as:

$$\mathbf{LH + OH^{\cdot} \longrightarrow L^{\cdot} + H_2O}$$ (66,67,82,84,85,93)

$OH^{\cdot}$ can also attack extrinsic carbohydrates and proteins like cell surface glycoproteins and phospholipids. Nitrogen dioxide can initiate similar reactions, however HOCl is more likely to chlorinate lipids by addition across double

bonds (66). $O_2^{\cdot -}$ is not reactive enough to abstract hydrogen atom from lipids, besides it cannot readily cross biological membranes (84,85).

As the number of the double bonds increase in a fatty acid side chain, the removal of a hydrogen atom becomes easier. This is the reason why PUFAs are more susceptible to lipid peroxidation than monounsaturated or saturated fatty acids (85).

The hydrogen atom possesses a single electron and its removal leaves behind an unpaired electron on the carbon atom to which it was originally attached. The resulting carbon–centered lipid radicals undergo **molecular rearrangement** to form a more stable **conjugated diene** configuration (82 84,85) such as:

$$\mathbf{L^{\cdot} + L^{\cdot} \longrightarrow L{-}L}$$

These conjugated dienes react with O_2 in aerobic medium to form peroxyl radicals ($LOO^{\cdot}$). Peroxyl radicals have the ability to combine with each other or they can attack membrane proteins, but they are also capable of abstracting hydrogen from adjacent fatty acid side chains and so propagating the chain reactions of lipid peroxidation. This step is called **propagation**. This single initiation event can cause the conversion of hundreds of fatty acid side chains into lipid peroxides (84,85,94). The length of the propagation chain depends on the lipid-protein ratio in a membrane, the fatty acid composition, the oxygen concentration and the presence of chain-breaking antioxidants within the membrane (85).

2.4.3.1. The role of transition metals and lipid peroxidation:

As mentioned before, Fe^{2+} takes part in the formation of $OH^{\cdot}$ in Fenton and Haber–Weiss reactions. Therefore, addition of Fe^{2+} to a peroxide–free unsaturated lipid in the presence of O_2 initiates lipid peroxidation via $OH^{\cdot}$ formation. This resulting lipid peroxidation can be inhibited by H_2O_2–removing enzymes, iron chelating agents, or hydroxyl radical scavengers (66,82,84).

Pure lipid radicals are usually stable at body temperature, but they are readily decomposed in the presence of transition metals (i.e. iron or copper). In this way, Fe^{2+} or its chelates react with lipid peroxides in a similar way to their reaction with H_2O_2, splitting O–O bond. This causes the generation of **alkoxyl radical** ($RO^{\cdot}$), such as:

$$\mathbf{ROOH + Fe^{2+} \longrightarrow Fe^{3+} + OH^{-} + RO^{\cdot}} \quad (66)$$

2.4.3.2. The Consequences of Lipid Peroxidation:

The occurrence of lipid peroxidation in biological membranes results in the impairment of normal membrane functioning, changes in fluidity, damage membrane proteins, inactivation of membrane-bound enzymes and ion-channels, and increased permeability to ions such as Ca^{2+} (66,85). Rises in Ca^{2+} induced by oxidative stress can activate phospholipase A_2, which causes the release of arachidonic acid from membrane phospholipids. The freed arachidonic acid can undergo lipid peroxidation and triggers the stimulation of eicosanoid system, which is also linked to lipid peroxidation.

Continued oxidation of fatty acid side chains and their further fragmentation eventually leads to the loss of membrane integrity. For instance, when lysosomal membranes are ruptured, hydrolytic enzymes are spilled into the rest of the cell. Peroxidation of erythrocyte membranes results in the losing of their ability to change shape and squeeze through the smallest capillaries. Loss of viability of spermatozoa on prolonged incubation at 37 ^{o}C can initiate lipid peroxidation, and the loss of germinating ability of seeds stored under damp, warm conditions. Besides, damage to endoplasmic reticulum or Golgi apparatus by peroxidation induces the decrease in the capability of cells to synthesize and export proteins, as happens in CCl_4 toxicity.

Peroxyl and alkoxyl radicals, aldehydes and singlet O_2 can damage receptors, enzymes such as glucose 6-phosphatase, the Ca^{2+}-ATPase of the endoplasmic reticulum, the Na^{+}, K^{+}-ATPase, and K^{+} channels. Since voltage-regulated K^{+} channels take part in generation of electrical activity in nerve

tissues and heart, damage to them can cause irregularities in heart beat, and death of neurons. Matrix enzymes and the constituents of electron-transport chain can be damaged within mitochondria. Lipid peroxidation in foods can change the taste, alter the texture of food proteins and destroy essential amino acids, which is a common problem in the storage of marine food (66,67,84,85,95).

2.4.3.3. By-products of Lipid Peroxidation

Isoprostanes: They are important products of lipid peroxidation with a prostaglandin-like structure. They are formed from PUFAs with at least three double bonds, including linolenic acid and arachidonic acid (66). They are produced in humans *in vivo* by a non–cyclooxygenase free–radical catalyzed mechanism involving peroxidation of PUFAs (95). Their formation involves the formation of positional peroxyl radical PUFA isomers by the endocyclization of the peroxyl radical followed by a further oxygen attack (95,96). Increased oxidative stress in the case of multiple human diseases or exposure to a wide range of toxins induces an increase in the formation of isoprostanes (66).

Decomposition products from lipid peroxides: Decomposition of lipid peroxides by metal ions or by heating results in the generation of a wide variety of products, including epoxides, **hexanal**, ***trans, trans*-2,4-decadienal,** ketones and hydrocarbons. Cleavage of aldehyde, hydrocarbon or other fragments from peroxidized lipids still leaves an oxidized fragment attached to the main lipid by an ester bond.

Ethane and pentane: Transition metal ions, especially iron and copper, and haem proteins can cause hydrocarbon formation from peroxides. In the case of reaction of Fe^{2+} with a hydroperoxide on the fifth carbon from the methyl end of a PUFA (linoleic or arachidonic acid), **pentane** can be generated (66).

Ethane and **ethylene** gases are produced by **β-cleavage** from linolenic acid. Formation of these gases is used in the estimation of lipid peroxidation rates *in vivo* (66,97).

Malondialdehyde (MDA): It arises from the peroxidation of PUFAs with more than two double bonds, such as linolenic, arachidonic or docosahexanoic acids. It exists in various forms, depending on the pH, i.e. at physiological pH, free MDA exists as an enolate anion which has low reactivity toward most amino groups. As pH declines, reactivity increases and proteins can be attacked by MDA, causing in the modification of several residues and formation of intra– and inter–molecular cross–links. MDA can also react with DNA bases (preferably guanine) and can induce mutagenic lesions. MDA is rapidly metabolized in mammalian tissues. Aldehyde dehydrogenases oxidize it to malonic semialdehyde, which decarboxylates to acetaldehyde, in turn oxidized by aldehyde dehydrogenases to acetate (66).

4-Hydroxy-2-*trans*-nonenal (HNE): It is generated during the peroxidation of linoleic and arachidonic acids. It is one of the several unsaturated aldehydes formed during lipid peroxidation. These aldehydes have the ability to react with –SH groups at physiological pH. Therefore, -SH groups on proteins as well as amino groups on DNA bases and phospholipids can react with HNE. When it is produced *in vivo*, it readily reacts with proteins and other biomolecules containing $–NH_2$ or –SH groups (66).

2.4.4. Protein Oxidation

Proteins are also major constituents of membranes and they are considered to be possible targets for ROS attack (68). Protein oxidation results in the malfunctioning of receptors, enzymes, antibodies, signal transduction and transport proteins. The protein damage caused by oxidation can lead to secondary damage to other biologically important molecules by raising the intracellular Ca^{2+} levels and activating nucleases. Increases in the amount of

oxidized proteins may be associated with age–related losses of some biochemical and physiological functions, and may also reflect unpaired damage to DNA. When DNA repair enzymes are damaged, oxidative DNA damage levels and mutation frequency are raised (66,68,72,98). Oxidized proteins are generally recognized as “antigens” by the immune system, triggering antibody formation (99), and they are more susceptible to proteolysis (72).

Protein damage can occur by direct attack of ROS/RNS, or by indirect (or secondary) damage involving the attack by by-products of lipid peroxidation such as MDA and HNE. Another mechanism of protein damage is glycation (66). These damages can cause changes in tertiary structure, degradation and fragmentation (68,98). The consequences of protein damage as a response mechanism are loss of enzyme activity, altered cellular functions such as energy production, interference with the creation of membrane potentials, and changes in the type and level of cellular proteins (68).

Among the several ROS/RNS, $OH^{\bullet}$, $RO^{\bullet}$, reactive nitrogen and halogen species predominantly cause protein damage. The attack of these species (e.g. peroxynitrite, HOCl, HOBr, *etc.*) leads to the production of 3-nitro-, bromo-, and chloro- tyrosines. 3-nitrotyrosine is the major marker for oxidative protein damage. Another result of the damage is the nitration of phenylalanine and tryptophan. Attack of $OH^{\bullet}$ or singlet O_2 upon proteins generates a wide range of end-products, such as *ortho*-tyrosine, *meta*-tyrosine, DOPA, hydroxyproline, glutamyl semialdehyde, *etc*. $OH^{\bullet}$ can also abstract $H^{\cdot}$ from peptide bonds. On the contrary, H_2O_2 or $O_2^{\cdot -}$ generally does not have direct effect on proteins at physiological levels. However, H_2O_2 is capable of attacking easily-accessible –SH groups (66,68,98).

First wave of $OH^{\bullet}$ attack generates free radicals that are in combination with O_2 to give peroxyl and alkoxyl radicals. Alkoxyl radicals can fragment by *β*-cleavage, releasing carbonyls from proteins. Since most of the proteins are capable of binding metal ions, especially iron and copper, subsequent exposure

to H_2O_2 generates $OH^{\cdot}$ which induces selective damage to the amino acid residues at the binding site (66).

2.4.5. DNA Damage

Reactive species are involved in the development of cancer, both by direct effects on DNA or by modulating signal transduction, cell proliferation and cell death. Direct damage to DNA by ROS can have negative effect on purine or pyrimidine bases, or the deoxysugar (66,72,100).

As stated before, DNA damage occurs in the form of strand breakage and chemical modifications to the DNA bases or to deoxyribose (66). Among the ROS, $OH^{\cdot}$ reacts with DNA by addition to double bonds of DNA bases and by abstraction of an H atom from the methyl group of thymine and each of C–H bonds of deoxyribose (100). It also can add to guanine at positions 4, 5 or 8 in the purine ring. Addition to C–8 produces an OH-adduct radical that is oxidized to **8-hydroxy guanine** (66)**.**

Deoxyribose and ribose are fragmented by $OH^{\cdot}$, yielding various products. All positions are prone to H abstraction by $OH^{\cdot}$ forming carbon-centered radicals. They are readily transformed to sugar peroxyl radicals in the presence of O_2. Afterwards, they experience a range of reactions, including disproportionation, rearrangement, elimination of water and C–C bond fragmentation, to yield a variety of carbonyl products (66,101). Another mechanism about DNA damage includes the enzyme **poly (ADP-ribose) polymerase,** which is activated following the peroxide-mediated DNA damage. Once activated, this enzyme uses vast amounts of NAD to repair DNA damage, caused by ROS (72).

2.4.6. Antioxidant Defense Systems Against Free Radicals

Ongoing exposure to numerous types of oxidative stress from various sources has lead both the cell and the whole organism to develop defense mechanisms for protection against reactive species (RS) (68). Halliwell defines the term antioxidant as “any substance that when present at low concentrations

compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate". The term "oxidizable substrate" includes almost everything within a living cell including proteins, lipids and DNA (102).

Antioxidant defense mechanisms are classified into 3 sub-groups according to a mechanistic approach (103):

1. **Preventing antioxidants:** As already mentioned, transition metals including iron and copper take part in the production of certain RS. Therefore, ferric or cupric ion reducers or metal chelators are supposed to prevent the production of RS (68,103).

2. **Scavenging antioxidants:** They discard RS swiftly before they attack biologically essential molecules (103). Primary/secondary antioxidant enzymes and non–enzymatic, low–molecular–weight antioxidants are included in this sub–group (68,103).

3. **Repairing antioxidants:** They scavenge the wastes of oxidant attack and reconstitute the lost cellular function.

2.4.6.1. Enzymatic Antioxidant Defense Mechanisms

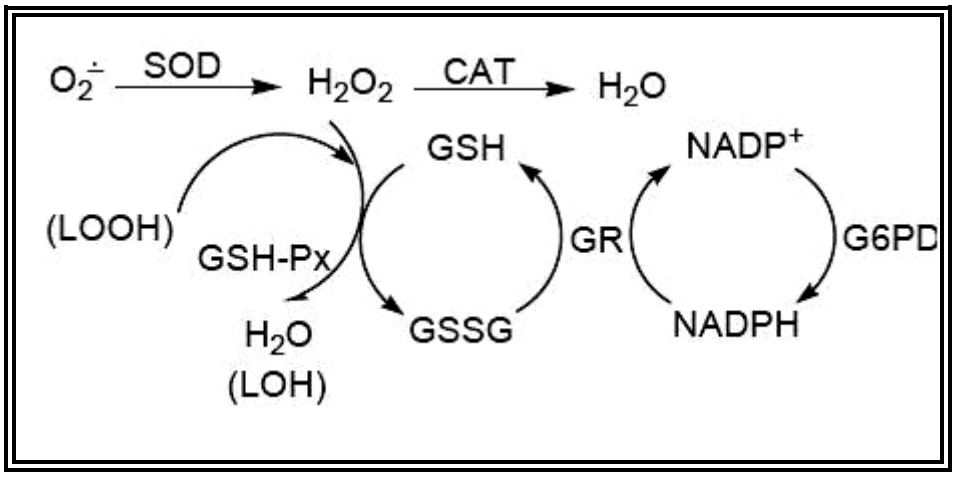


Figure 1: The mechanism of enzymatic antioxidant defences [Adapted from Aydin et al., 2001] (67)

2.4.6.1.1. Superoxide Dismutase (SOD)

a. Copper-Zinc SOD (CuZnSOD)

T. Mann and D. Keillin isolated a blue-green protein containing copper, and named it **haemocuprein** in 1938. Later, in 1953, a similar protein was isolated from horse liver and named **hepatocuprein**. This type of protein was again isolated from brain (**cerebrocuprein**). In 1970, it was discovered that the erythrocyte protein contains zinc in addition to copper. The enzymatic function of this protein was not realized at the beginning, and thought to serve as metal depots (66). It was in 1969 when Joe M. McCord and Irwin Fridovich first realized that the erythrocyte protein is able to remove $O_2^{\cdot -}$ catalytically. They reported that this protein acts as an enzyme, **superoxide dismutase** containing both copper and zinc (104).

CuZnSOD is an enzyme of molecular mass of approximately 32.000 (68). It contains two protein subunits each of which possesses an active site containing one copper and one zinc ion (105). CuZn-SOD is widely distributed in almost all eukaryotic cells and mostly localized in the cytosol of animal cells (66,68), but it can also be found in lysosomes, nucleus and the space between inner and outer mitochondrial membrane (105). The CuZn-SODs are exceptionally stable enzymes. They are fairly resistant to heat, denaturation by organic solvents and detergents, and attack by proteinases (66).

The primary function of CuZnSOD is to catalyze the dismutation reaction of $O_2^{\cdot -}$ as shown below:

$$\mathbf{O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow{CuZnSOD} H_2O_2 + O_2}\ (105)$$

This reaction states that the process results in the formation of H_2O_2, which is also a strong oxidant, as already mentioned. The produced H_2O_2 is detoxified in body by catalase enzyme, which is discussed later (67).

CuZnSOD inhibits the formation of $OH^{\cdot}$ by removing $O_2^{\cdot -}$ and preventing it to react with H_2O_2 in the presence of transition metals (77).

Cyanide is a powerful and non-specific inhibitor of CuZnSODs. They are also inactivated by incubating with **diethyldithiocarbamate**, a compound that binds to copper and removes it from the active sites (66).

b. Manganese SOD (MnSOD)

The SOD first isolated from *E.coli* was realized to be entirely different from CuZnSOD. It was understood that it had a molecular mass of 40.000 rather than 32.000, and its color was pink rather than blue-green. It was not inhibited by cyanide or diethyldithiocarbamate; nevertheless it was destroyed by treating with chloroform plus ethanol. It contained Mn^{3+} as its active site, thus it was named as MnSOD (or sometimes SOD2) (66,105,106).

Despite these differences, MnSODs catalyze essentially the same reaction as CuZnSODs. But unlike CuZnSODs, the reaction rate for MnSODs decreases at alkaline pH (66).

The MnSODs are widespread in bacteria, plants and animals. They are almost entirely located in the mitochondria in animal cells (105). Because the mammalian erythrocyte lacks mitochondria, it does not contain MnSOD; however it has been reported that the enzyme exists in mammalian liver. The relative amounts of MnSOD and CuZnSOD vary depending on the number of mitochondria present in the tissues they exist.

The MnSODs from higher organisms generally consist of four protein subunits, and have 0.5 or 1 ions of Mn per subunit. Removal of Mn from the active sites results in the loss of catalytic activity (66,90,106).

c. Iron SOD (FeSOD)

Four SODs have been purified from *E.coli* including periplasmic CuZnSOD and MnSOD. A third SOD was found to be an iron-containing

dimmer, and identical enzymes were later isolated from many bacteria, algae and higher plants. This fourth enzyme is a hybrid containing subunits of both manganese and iron enzymes in the same molecule. FeSOD is generally found in the cell cytoplasm (105).

FeSODs usually contain two protein subunits. There are generally one or two iron ions per molecule in dimeric enzymes. The iron is found as Fe^{3+} in FeSOD which is in “resting” state (107). Fe-SODs are also capable of catalyzing the dismutation reaction, but in a relatively slower manner than other subtypes (67). In addition, it is reported that fluctuations are possible between Fe^{3+} and Fe^{2+} states during catalyses. The catalytic activity of FeSODs tends to decrease at high pH and is not inhibited by cyanide, like MnSODs (107).

The amino acid sequences of FeSODs are similar to those of MnSODs, which explains the hybridization, mentioned before (66).

No animal tissues have yet been found to contain FeSOD, but some algae, yeasts (67) and higher plants like *Ginkgo biloba* L. (108).

d. Nickel SODs (NiSOD)

A new type of SOD with only nickel at the active site was recently purified from several aerobic bacteria of *Streptomyces* species and also in *Actinomycetes* and *Micromonospora* species. The amino acid sequence of Ni-SOD is also different from other SODs (109).

2.4.6.1.2. Catalase (CAT)

CAT was first purely isolated in the form of crystalline from beef liver by James B. Sumner and Alexander L. Dounce in 1937 (110). It consists of four protein subunits, and each of them contains Fe^{3+}-haem (Fe^{3+}-protoporphyrin) at its active site (111). Each subunit usually has one molecule of NADPH bound to it (66), which stabilizes the enzyme by protecting it from its own substrate H_2O_2 (67). CAT is likely to dissociate into its subunits during storage, lyophilization or exposure to acid or alkali, resulting in the loss of activity (66).

Dismutation of $O_2^{\cdot -}$ results in the formation of H_2O_2 . As already stated, H_2O_2, itself, causes oxidative damage to tissues, and also plays a major role in the generation of $OH^{\cdot}$. CAT (also known as “hydrogen peroxide oxido reductase”) catalyzes the direct disintegration of H_2O_2 to O_2, as shown:

$$\mathbf{2H_2O_2} \xrightarrow{\textbf{CATALASE}} \mathbf{2H_2O + O_2} \quad (66)$$

Most aerobes contain catalase with the exception of some bacteria and blue-green algae. Even though, catalase is located in all organs of animals; it is especially concentrated in erythrocytes and liver cells. Brain, heart and skeletal muscle have relatively lower levels of CAT. Besides, maize plant (*Zea mays* L.) is reported to contain multiple catalases (66).

CAT, like SOD, catalyzes a dismutation reaction; one H_2O_2 is reduced to H_2O and the other is oxidized toO_2. The reaction mechanism is shown below (66):

$$\mathbf{Catalase\text{-}Fe^{3+} + H_2O_2 \longrightarrow Compound\ I + H_2O}$$

$$\mathbf{Compound\ I + H_2O_2 \longrightarrow Catalase\text{-}Fe^{3+} + H_2O + O_2}$$

$$\mathbf{Net\ reaction:\ 2H_2O_2 \longrightarrow 2\ H_2O + O_2}$$

The structure of “compound I” has not yet been fully revealed. It is known for receiving two electrons from H_2O_2 to reform ferric catalase (66). Mammalian catalases can also catalyze peroxidase-type reactions, but limited to the accessibility of substrates (H_2O_2) (112). Compound I has the ability to oxidize methanol and ethanol to formaldehyde and acetaldehyde. It can also oxidize formic acid to carbon dioxide and nitrite to nitrate. It has been suggested that CAT can oxidize elemental mercury (Hg) absorbed into the human body to Hg^{2+} ions (66). CAT can be non-specifically inhibited by azide, cyanide, peroxynitrite, and HOCl. These can inhibit the functions of other enzymes. **Aminotriazole** inhibits CAT *in vivo*.

2.4.6.1.3. Selenium-dependent Glutathione Peroxidase (SeGPx)

SeGPx was first discovered in animal tissues in 1957 by Gordon C. Mills (113). Although it is found rarely in bacteria and higher plants, it also exists in some algae and fungi (114).

SeGPx removes H_2O_2 by coupling its reduction to H_2O with the oxidation of a thiol-containing tripeptide, **reduced glutathione** (GSH) (66). Free glutathione usually exists in the form of GSH, whereas a small portion exists in oxidized form (GSSG).The ratios of reduced to oxidized glutathione in normal cells are high.

In the presence of excessive H_2O_2, the oxidation of GSH to GSSG is catalyzed by SeGPx; and in this case, H_2O_2 is detoxified by being converted to water (67), as shown below:

$$\mathbf{H_2O_2\ (LOOH) + 2GSH \longrightarrow GSSG + 2H_2O}$$

SeGPx is a tetrameric enzyme and composed of four protein sub-units. They contain **selenium** at their active sites. Its molecular weight is approximately 85.000. It is mainly located in cytosol and mitochondria. Selenium is found as selenocystein in normal cystein instead of sulfur. GSH reduces the selenium portion of the enzyme, later this reduced enzyme reacts with H_2O_2. While, the selenolate form of SeGPx reduces the peroxide substrate (LOOH) to alcohol (LOH). GSH attends the reaction at this step and generates selenosulfide. A second GSH binds to selenosulfide and converts it to the active form of the enzyme, and by this way, GSH is oxidized to GSSG (66,67,114).

SeGPx can be inhibited by **mercaptosuccinate** (66).

2.4.6.1.4. Glutathione Reductase (GR)

As stated before, GSH is converted to GSSG by SeGPx during the reduction of H_2O_2 or other lipid peroxides. Since GSH amount of the organism is limited, GSSG is required to be re-converted to its reduced form, GSH. This conversion is catalyzed by GR in the presence of NADPH (67), such as:

GSSG + NADPH + H^+ ⟶ 2GSH + $NADP^+$

NADPH required is provided by the **pentose phosphate pathway**. The first enzyme in this pathway is **glucose-6-phosphate dehydrogenase.**

GR is composed of two protein subunits, each of which contains flavin adenine dinucleotide (FAD) at its active site. NADPH reduces FAD, which later donates its electrons onto a disulphide bridge in the active site. The two –SH groups, formed by this way, interact with GSSG and reduce it to 2 GSH, reforming the protein disulphide (66).

Both *in vitro* and *in vivo* inhibition of GR can be achieved by ***N,N*-bis(2-chloroethyl)-*N*-nitrosurea** (66).

2.4.6.2. Non–enzymatic Antioxidant Defense Mechanisms

2.4.6.2.1. Glutathione

Glutathione, γ-glutamylcysteinyl glycine, is a tripeptide found ubiquitously in microorganisms, most plants and all mammalian tissues. Glutathione exist in two different forms, “thiol–reduced” (GSH) and “disulfide-oxidized” (GSSG). GSH is the most abundant non-protein thiol, which is found in millimolar concentrations in most cells. Eukaryotic cells have three major depots of GSH. Nearly 90% of the cellular GSH is found in the cytosol, 10% in the mitochondria, and a small portion of it in the endoplasmic reticulum. The GSH/GSSG ratio may vary depending on the cellular location.

Glutathione has numerous vital functions, including detoxifying xenobiotics, preventing the oxidation of –SH groups and scavenging free radicals.

Detoxification of xenobiotics or their metabolites is one of the major functions of GSH. These compounds are electrophiles, and form conjugates with GSH either spontaneously or enzimatically catalyzed by GST. Later, these conjugates are metabolized to mercapturic acid.

GSH is a scavenger of various ROS including $OH^{\cdot}$, HOCl, and singlet oxygen. Scavenging of these radicals results in the formation of thiyl radical.

Although GSH does not react directly with hydroperoxides, it acts as a substrate for SeGPx. As mentioned before, excessive H_2O_2 is reduced by GSH in the presence of SeGPx. As a result GSH is oxidized to GSSG. GR reconverts GSSG to GSH, consuming NADPH (115,116).

It is also capable of chelating copper ions, resulting in a decrease in their ability to form $OH^{\cdot}$ from H_2O_2 (117).

2.4.6.2.2. Uric acid

Uric acid is the final product from the purine catabolism catalyzed by xanthine oxidase or xanthine dehydrogenase. In most species, **urate oxidase** converts uric acid to its more polar metabolites for excretion. However, humans lack a functional urate oxidase gene. Thus, urate oxidase activity is absent and uric acid accumulates in plasma to high concentrations (66).

Uric acid is known for its antioxidant activities (118). Indeed, it is the most abundant metabolite in plasma with antioxidant potential. Most of the total antioxidant potential measured in plasma is due to uric acid (119). It has been demonstrated that uric acid is capable of scavenging singlet O_2, $LOO^{\cdot}$. $OH^{\cdot}$, ozone, HOCl (118) and peroxynitrite (119). Therefore, it is concluded that uric acid protects erythrocyte membranes from peroxidation (118).

Uric acid is also capable of chelating metal ions, since it can bind iron and copper ions and stimulate low density lipoprotein oxidation (66).

2.4.6.2.3. Melatonin

Melatonin is produced by the pineal gland at the base of the brain. It also occurs in some foods, for example walnuts. It participates in the regulation of circadian rhythms, and has been popularly used against “jet lag”. The blood levels of melatonin are low during the day time and increase at night as pineals synthesis accelerates (66).

Melatonin is a powerful scavenger of $OH^{\cdot}$. After it reacts with $OH^{\cdot}$, it is converted to a cationic radical which also reacts with $O_2^{\cdot -}$. Since melatonin is a lipophilic substance, it is capable of penetrating a lot of compartments including blood-brain barrier and exhibits a wide spectrum of antioxidant activity (67).

It is also regarded as scavengers of other ROS and RNS. Furthermore, it has been demonstrated that it has the ability to stimulate important antioxidant enzymes such as GPx (120) and SOD (121). Besides it causes an increase in the total antioxidant capacity of plasma (121).

2.4.6.2.4. Albumin

Albumin is multifunctional protein, which is composed of 585 amino acids and has a molecular weight of 66.000. The normal concentration of it in human plasma is between 35 and 50 g/L. Albumin participates in many important physiological and pharmacological reactions. Its primary function is to transport bile pigments, cholesterol, fatty acids, and metals. It plays a major role in the maintenance of osmotic pressure and distribution of fluid among bodily compartments (122).

Albumin represents the majority of antioxidants present in plasma, where much of oxidative stress occurs. The majority of the total serum antioxidant properties can be referred to albumin (122). It has been suggested that approximately 70% of the free radical trapping activity was due to albumin (123). It binds copper tightly and iron weakly. It takes part in the inhibition of $OH^{\cdot}$ generation. Therefore, it protects important lipoproteins. It contains an open –SH group and this –SH rapidly scavenges HOCl and some RNS (124).

2.4.6.2.5. Ceruloplasmin

Copper is taken by the liver and enters the cell in its reduced form, Cu^{+}. Some of the copper is integrated by the liver into the protein, ceruloplasmin. Its molecular weight is 132.000, and human plasma contains 200 to 400 mg/dm^3 of ceruloplasmin (66).

Ceruloplasmin contains six tightly bound copper ions and it donates copper to cells requiring copper. It transports the majority of copper in plasma, while albumin carries much less (125). It catalyzes the oxidation of Fe^{2+} to Fe^{3+} without any release of oxygen radical intermediates. This process results in the inhibition of Fenton reaction and further formation of free radicals.

Ceruloplasmin binds to myeloperoxidase, causing the inactivation of the enzyme. This results in the inhibition of HOCl formation (66,67).

2.4.6.2.6. Bilirubin

Bilirubin is a bile pigment and arises as a result of catalysis of haem proteins. Over 270 mg of bilirubin is produced daily in adult humans.

It is insoluble in water at physiological pH and binds tightly to albumin. Bilirubin bound to albumin can protect not only the protein itself but also the albumin-bound fatty acids against free radical damage. It has also the capability of scavenging singlet O_2 (66,67).

2.4.6.2.7. Lipoic acid

Lipoic acid (**1,2-dithiolane-3-pentaoic acid)** is present in all kinds of prokaryotic and eukaryotic cells. Dihydrolipoic acid (DHLA) is the reduced form of lipoic acid, and it is a more powerful antioxidant than lipoic acid, itself. They have both metal-chelating capacity and the capability of scavenging ROS. However, only DHLA has the ability to reduce GSSG to GSH, dehydroascrobate to ascorbic acid, and α-tocopheryl radical to α-tocopherol, resulting in the regeneration of important antioxidant molecules. DHLA can also repair oxidative damage (126).

2.4.6.2.8. Mannitol

Mannitol shows modest antioxidant activity as a hydroxyl radical scavenger, however it does not have the capability of scavenging other oxidants including $O_2^{\cdot -}$ and lipid peroxides (127).

2.4.6.2.9. Oxypurinol

Oxypurinol is the metabolite of allopurinol, a well-known inhibitor of xanthine oxidase. It directly scavenges HOCl (128). It also showed antioxidant activity in lenses (128) and in pancreatic tissues of rats (129).

2.4.6.2.10. Ferritin

Ferritin binds the iron and prevents it from participating in the generation of free radicals (67).

2.4.6.2.11. Transferrin

Transferrin is especially present in serum, but it also found at lower concentrations in other body fluids. Its primary function is to transport iron to the proliferating cells; it is also an important growth factor.

Transferrin acts as an antioxidant by reducing the concentration of free ferrous ion that takes part in Fenton reaction, resulting in the formation of free radicals (130).

2.4.6.2.12. Coenzyme Q (Ubiquinone; CoQ)

CoQ is an endogenous, lipophilic compound found in the majority of aerobic organism. The name “ubiquinone” was given as a reference to its ubiquitous presence in every aerobe.

CoQ plays a major role in the mitochondrial electron transport chain as an electron carrier. It is also present in other cells membranes and lipoproteins. Its reduced form “**ubiquinol”** can scavenge $LOO^{\cdot}$ directly and inhibit lipid peroxidation. Furthermore, it participates in the regeneration of α-tocopherol from its radical form in lipoproteins and membranes.

Beside its presence in every aerobic organism, CoQ can be found in dietary sources such as grains, meats and oils (131).

2.4.6.3. Natural Antioxidant Molecules

The human diet comprises of a parade of different compounds with antioxidant potential. The most remarkable representatives of dietary antioxidants are vitamin E, vitamin C, carotenoids and polyphenolic substances. A synergistic effect among these various compounds is likely to occur (132–134).

2.4.6.3.1. Vitamin E

Vitamin E was first discovered in 1922 by Evans and Bishop as a necessary dietary factor for reproduction in rats (135). The term “vitamin E” includes eight naturally–occurring, fat–soluble substances that have vitamin E activity in the rat reproduction test. They are **α-, β-, γ-, δ- tocopherols,** and **α-, β-, γ-, δ- tocotrienols** (66). They have all a chromanol ring, with a hydroxyl group that can donate a hydrogen atom to reduce free radicals and a hydrophobic side chain, which makes penetration to biological membranes easier (136).

The name “tocopherol” originates from the Greek words *tokos* (childbirth) and *phero (*to bring forth). Plants and their oils generally contain a mixture of tocopherols, for example there is more γ-tocopherol than α-tocopherol in soy, walnut, rapeseed, and corn oils. On the contrary, sunflower and almond oil have mostly α-tocopherol (66).

α-tocopherol is the most accumulating form in blood and tissues. It is also synthesized commercially for use in animal nutrition and for human vitamin E supplements (137). The richest sources of vitamin E in the diet are vegetable oils (soybean, maize, cottonseed, and sunflower seed). Besides, wheat germ, nuts, and some green leafy vegetables contribute considerable amounts to the vitamin E depot (134).

α-tocopherol functions as an antioxidant to protect lipids against oxidative damage. It especially protects PUFA within phospholipids of biological membranes and in plasma lipoproteins. Although it has a low reduction

potential, the main mechanism is to inhibit lipid peroxidation because it has the ability to scavenge lipid peroxyl radicals ($LOO^{\cdot}$) much faster than these radicals can react with adjacent fatty acid side chains or with membrane proteins. The phenolic hydroxyl group of tocopherol reacts with an organic peroxyl radical to form the corresponding organic hydroperoxide and the tocopheroxyl radical (α-Toc-$O^{\cdot}$) (66,135,137):

$LOO^{\cdot}$ + α-Toc-OH ⟶ LOOH + α-Toc-$O^{\cdot}$

The resulting α-Toc-$O^{\cdot}$ is stable and does not have adequate reactivity to initiate a lipid peroxidation reaction on its own (67). By this way, the chain reaction of lipid peroxidation is terminated. For this reason vitamin E is called as **chain-breaking** antioxidant (138).

Tocopheroxyl radicals can be reduced to tocopherol by interaction with reductants serving as hydrogen donors. Coenzyme Q, vitamin C (ascorbic acid) and GSH, which are biologically important hydrogen donors, regenerate tocopherol from the tocopheroxyl radical (137):

α-Toc-$O^{\cdot}$ + Ascorbic acid ⟶ α-Toc-OH + Dehydroascorbic acid

2α-Toc-$O^{\cdot}$ + 2 GSH ⟶ 2 α-Toc-OH + GSSG (67)

GPx and vitamin E show a complementary antioxidant effect such as GPx removes the generated peroxides, besides vitamin E inhibits the generation of peroxides.

Vitamin E also participates in the metabolism of Se^{2+}. Se^{2+} is necessary for the absorption of vitamin E and lipids. Furthermore, it assists in keeping vitamin E inside the lipoproteins. Meanwhile, vitamin E decreases the need for Se^{2+} by preventing the loss of it from the organism or keeping it in an active state (67).

α-tocopherol is also capable of inhibiting the protein oxidation. It has been demonstrated that α-tocopherol reduced formation of α-aminoadipic and γ-glutamic semialdehydes from oxidized myofibrillar proteins (139).

Dietary consuming of α-tocopherol increases the incorporation of the antioxidant into the phospholipids membrane region where PUFAs are located (139).

2.4.6.3.2. Vitamin C

Vitamin C (**ascorbic acid**) is water–soluble vitamin. It was first isolated from adrenal glands by Albert Szent-Györgyi in 1928. It is a six-carbon lactone and synthesized from glucose in the liver of most mammalians. Nevertheless, humans cannot carry out the synthesis process since they lack a crucial enzyme (gulonolactone oxidase) required for the terminal step. Therefore, vitamin C needs to be acquired from the diet (140,141). In the case of vitamin C deficiency, scurvy may occur (68).

Vitamin C acts as a cofactor for eight enzymes in humans. Three of them participates in collagen hydroxylation i.e. praline hydroxylase, lysine hydroxylase and asparagine hydroxylase (142). Two other vitamin C dependent enzymes are required for the synthesis of carnitine, which is essential for the transport of fatty acid into mitochondria for ATP production (143). Vitamin C is necessary for the copper-containing enzyme dopamine-β-hydroxylase, which is responsible for the conversion of dopamine to norepinephrine (144).

The levels of vitamin C is 30-90 μM in human plasma; even higher in cerebrospinal fluid, aqueous humor of the eye, gastric juice, seminal fluid and lung lining fluid. It is found in millimolar intracellular concentrations in various cell types (66).

Vitamin C is an electron donor, and therefore it is a powerful reducing agent. By this process it prevents other compounds from oxidation. It is capable of reducing Fe^{3+} to Fe^{2+}, which is required for the hydroxylation reactions in the organism. This process assists the uptake of iron in the duodenum (66,141).

Vitamin C donates two electrons from a double bond between the second and third carbons of the 6-carbon molecule. But this donation process occurs sequentially. The loss of the first electron results in the formation of a free

radical **semidehydroascorbic acid** or **ascorbyl.** Ascorbyl radical participates in the regeneration of α-tocopheroxyl radical to α-tocopherol. It is relatively stable with a half-life of 10^{-5} seconds, besides it is fairly unreactive. In the case of the reaction of a harmful free radical with ascorbic acid, the reactive free radical is reduced, and the ascorbyl radical is much less reactive. Ascorbyl radical is not a stable compound owing to its unpaired electrons and upon the loss of a second electron, **dehydroascorbic acid** is formed (141).

As a powerful reducing agent, vitamin C acts as an important free radical scavenger. It scavenges $O_2^{\cdot -}$, singlet oxygen and $OH^{\cdot}$ directly. It also scavenges water soluble peroxyl radicals. Another mechanism of its antioxidant activity is to prevent the damage by $OH^{\cdot}$ or $LOO^{\cdot}$ upon urate by reacting with urate radicals (145).

In addition to its antioxidant effects, it is known that vitamin C can exert prooxidant effects. Apart from $O_2^{\cdot -}$ vitamin C is the only cellular agent that reduces Fe^{3+} to Fe^{2+}. As already stated, Fe^{2+} takes part in Fenton reactions, therefore it is an important component of free radical generation (146).

2.4.6.3.3. Carotenoids

Carotenoids are a major class of naturally occurring, lipid-soluble pigments (147). They are known as “natural colorants”, and responsible for many of the red, orange, and yellow hues of plant leaves, fruits, and flowers, as well as the colors of some birds, insects, fish, and crustaceans. Only plants, bacteria, fungi, and algae have the capability to synthesize carotenoids, but many animals provide them from their diet (133,148). In plant cells, carotenoids are generally found in cell membranes or stored in plasma vacuoles (147).

Among more than 700 naturally occurring carotenoids, 50 of them are present in the human diet. However, only five of them represent 95% of carotenoids circulating in blood. They are **β-carotene**, **lycopene**, **lutein**, **β-cryptoxanthine**, and **zeaxanthin** (147).

Carotenoids have a unique structure which has a direct effect on their biological actions and functions. They are synthesized from the dimerization of geranylgeranyl pyrophosphate, and composed of eight isoprene units. These isoprene units are joined in “head-to-tail” pattern (149). Most carotenoids are derived from a 40-carbon basal structure, including an extended system of conjugated double bonds (133,150). The central chain may carry cyclic end-groups which can be substituted with oxygen-containing functional groups (148).

The pattern of conjugated double bonds in the polyene backbone of carotenoids is responsible for the antioxidant activity of carotenoids (148). Another factor that contributes to the mentioned activity is their isolated double bonds, open chain, and lack of oxygen substituents (149). Carotenoids are most likely involved in the scavenging of two of the reactive oxygen species, singlet molecular oxygen and peroxyl radicals. Further, they deactivate electronically excited sensitizer molecules, which are involved in the generation of peroxyl radicals and singlet oxygen. The deactivation of singlet oxygen is consequence of physical quenching which involves direct energy transform. The excitation energy is transferred from singlet oxygen to carotenoid molecule, yielding ground state oxygen and triplet excited carotenoid. Since the carotenoids remain intact during the quenching process of singlet oxygen or excited sensitizers, they can be used several times again in these quenching processes. Therefore, carotenoids are considered to act as a “catalyst” for the deactivation of singlet oxygen (133,148,151,152).

As mentioned above, the antioxidant properties of carotenoids with the numbers of conjugated double bonds found in the molecule, determine their lowest triplet energy level. β-carotene and other carotenoids have triplet energy levels close to that of singlet oxygen enabling enery transfer. Other carotenoids like zeaxanthine, β-cryptoxanthine, and lutein have also the capability of scavenging singlet oxygen, and they are detected in plasma and tissues (133,148,151,152).

Another group of radicals which reacts with carotenoids is peroxyl radicals. As already mentioned, they are produced during the lipid peroxidation, and they cause damage in lipophilic compartments. Carotenoids can protect cellular membranes and lipoproteins against oxidative damage due to their lipophilic character, and specific properties to scavenge peroxyl radicals (133,148). Since they inhibit the lipid peroxidation by trapping peroxyl radicals, carotenoids, especially β-carotene, have been suggested as “chain-breaking antioxidants” (153). The peroxyl scavenging activity of carotenoids relies on the generation of radical adducts forming a resonance stabilized carbon-centered radicals (148).

The antioxidant activity of carotenoids depends on the oxygen tension present in the system (153). At relatively lower partial pressures of oxygen such as those found in most tissues under normal physiological conditions, β-carotene was found to inhibit the oxidation. On the contrary, the initial antioxidant activity of β-carotene is followed by a prooxidant action at high oxygen tensions. It has been noted that prooxidant properties of β-carotene can be attributed to adverse effects observed under the supplementation of high doses of β-carotene (148).

Carotenoids act also as a cooperative antioxidant. For instance, β-carotene participates in the regeneration process of α-tocopherol from tocopheroxyl radical. The yielded carotenoid radical cation may subsequently be repaired by ascorbic acid.

2.4.6.3.4. Phenolic compounds

Phenolic compounds are the largest group of plant secondary metabolites ubiquitously found in various herbs and fruits such as grapes, berries, apples, citrus fruits, and vegetables like broccoli, olives, tomatoes, soybeans, grains, cereals, and in addition, green and black teas, coffee beans, propolis and wine (154–156).

The term “phenolics” comprises more than 8000 naturally occurring compounds. They are mostly synthesized via the shikimic acid pathway. They all have a structurally common property, an aromatic ring with at least one hydroxyl group substituent (phenol) (154).

Depending on the number of phenol structures, phenolics are classified in two subgroups; simple phenols and polyphenols. Simple phenols are composed of phenolic acids (157). Polyphenols possess at least two phenol substituents. They include phenylpropanoids, flavonoids, anthocyanins stilbenes. Polymeric polyphenols which are composed of three or more phenol subunits are referred as tannins (158).

Phenolic acids constitute approximately 30% of the dietary phenolics found in plants in free or bound forms. They are hydroxyl derivatives of aromatic carboxylic acids, which arise from two distinguishing constitutive carbon frameworks: benzoic acid or cinnamic acid. Hydroxybenzoic acids include vanillic acid, syringic acid and *p*-hydroxybenzoic acid. some examples of hydroxy cinnamic acid acids are *p*-coumaric acid, caffeic acid, chlorogenic acid, ferulic acid, and sinapic acid (157).

Flavonoids are ubiquitous plant secondary metabolites that are best known as the characteristic red, blue, and purple anthocyanin pigments of plant tissues. The term is derived from the Latin word “*flavus*”, which means yellow (159).

Flavonoids are a large group of low-molecular weight polyphenolic substances. Their basic structural feature is the flavan nucleus (2-phenyl-benzo-γ-pyrane), a system of two benzene rings (**A** and **B**) linked by an oxygen-containing pyrane ring (**C**). Depending on the degree of oxidation of the **C** ring, the hydroxylation pattern of the nucleus, and the substituent at third carbon (C_3), the flavonoids are classified into the subclasses flavones, flavonols, flavanones, flavanols (catechins), isoflavones, and anthocyanins. Flavonols differ from flavanones by the presences of hydroxyl group at the C_3 position, and by a C_2-C_3 double bond. **Anthocyanins** own a charged oxygen atom in the

ring C, and therefore differ from other flavonoids. The ring C is open in the chalcones. Many flavonoids occur as glycosides in nature. The most common sugar moieties are D-glucose, L-rhamnose, D-galactose and D-arabinose (160).

Polymeric phenols, **tannins** are divided into two groups, such as hydrolysable and condensed tannins. Hydrolysable tannins contain gallic acid, or similar compounds, esterified to a carbohydrate (154).

It is a well-known fact that the majority of natural antioxidants are phenolic compounds (132). In addition to their antioxidant activity (161), they have vasodilatory (162), anticarcinogenic, antiinflammatory, immune-stimulating, antiallergenic, antiviral and estrogenic effects and inhibition activities against phospholipase A_2, cyclooxygenase, lipoxygenase, glutathione reductase, and xanthine oxidase enzymes (163).

Antioxidant Activities of Phenols

As already stated, the majority of natural antioxidants are phenols. The antioxidant capacities of many phenolic compounds are stronger than those of vitamins C and E (164,165). For example, the one electron reduction potential of epigallocathechin gallate under standard conditions is 550 mV, which is lower than glutathione (920 mV), under comparable to that of vitamin E (480 mV) (166).

The molecular basis of for the antioxidant properties of phenols is recognized under three main mechanisms (132,154,167–169):

1. **Hydrogen Atom Transfer (HAT)**

As primary antioxidants, phenols inactivate free radicals according to the hydrogen atom transfer. In this reaction, the antioxidant, ArOH, reacts with the free radical, R, by transferring a hydrogen atom to it through homolytic rupture of the O–H bond, as previously described.

$$\mathbf{ArOH + R^{\cdot} \longrightarrow ArO^{\cdot} + RH}$$

The products of this reaction are the harmless RH and the oxidized $ArO^{\cdot}$ radical. Although the latter is a free radical itself, it is relatively less reactive than $R^{\cdot}$.

2. Single Electron Transfer (SET)

It is another mechanism of phenols for free radical scavenging. This mechanism provides an electron to be donated to the $R^{\cdot}$:

$$\mathbf{ArOH + R^{\cdot} \longrightarrow ArOH^{+\cdot} + R^{-}}$$

The anion R^{-} is an energetically stable species with an even number of electrons, while the cation $ArO^{\cdot}$ is also in this case a less reactive radical species. Both $ArO^{\cdot}$ and $ArOH^{+\cdot}$ are structurally aromatic compounds in which the odd electron, originated by the reactions with the free radical, has the possibility of be spread over the whole molecule, causing a radical stabilization.

In HAT mechanism, the **bond dissociation enthalpy** (BDE) of the phenolic O–H bond is an important parameter in evaluating the antioxidant activity; the dissociation of the phenolic O–H bond and the reaction with the free radicals become easier as the BDE value decreases.

In the SET mechanism, the **ionisation potential** is the most significant parameter for the evaluation of scavenging activity. The electron abstraction and the reaction with the free radicals become as the ionization potential value decreases.

3. Transition Metals Chelation

Polyphenols, particularly flavonoids, have the capability to chelate transition metals, leading to stable compounds which entrap metals and avoid them to participate in the Fenton reactions.

The binding sites for metals to polyphenols are the 3-hydroxyl, 4-oxo groups in ring C, 4-oxo and 5-hydroxyl groups of ring A, and the catechol moiety of ring B.

Antioxidant activities of phenolic acids

As previously mentioned, phenolic acids are composed of two major subgroups, hydroxybenzoic and hydroxycinnamic acids. The antioxidant activity of phenolic acids and their derivatives depends on the number of hydroxyl groups in the molecule that would be strengthened by steric hindrance. It also depends on the position of the hydroxyl groups bound to the aromatic ring, the binding site, and the mutual position of the hydroxyl groups in the aromatic ring, and the type of other substituents. The electron-abstracting properties of the carboxylate group in benzoic acids have a negative effect on the hydrogen donating abilities of the hydroxyl benzoates. Hydroxylated cinnamates are more effective than benzoate forms. The presence of the –CH=C–COOH groups in cinnamic acid ensures greater H-donating ability and subsequent radical stabilization than the carboxylate group in benzoic acids (163,170).

The occurrence of different substituents in the phenolic framework enhances the antioxidant potential, particularly their hydrogen-donating capacity. Generally, unsubstituted phenol is inactive as hydrogen donor, and monophenol is a less active in antioxidant than polyphenol. The insertion of electron-donating group such as hydroxyl group in the *ortho* or *para* position elevates the antioxidant potential of phenol or phenolic acid (171). Also, the presence of a carbonyl group, such as aromatic acid, ester, or lactone, enhanced its antioxidant activity. The antioxidant activity of a molecule also increases in the case of separation from the aromatic ring. Cinnamic acid is more efficient as an antioxidant than the corresponding benzoic acid derivative. Steric hindrance of the phenolic hydroxyls by an adjacent inert group, such as methoxyl groups, favors the antioxidant activity (163).

The fact that the antioxidant potentials of phenolic acids have an inverse relationship with the magnitude of their O–H bond dissociation enthalpy values supply evidence in weakly polar organic media. The key mechanism of the chain-breaking action is the hydrogen atom transfer (HAT) from the phenolic –OH to peroxyl radicals, as previously described (132). The incorporation of a

second –OH group in the *ortho* or *para* positions enhances the antioxidant activity (163).

Antioxidant activities of flavonoids

Flavonoids are the most abundant and popular group of dietary polyphenolic compounds, and widely distributed in almost all of the high plants. They are well-known for their antioxidant activities.

Flavonoids have the capability of hydrogen atom transfer (HAT), which is already described. The antioxidant activity of flavonoids is dependent on the arrangement of functional groups on its main framework (169). The main structural features of flavonoids necessary for the ROS scavenging activity can be summarized as below:

a) An *ortho*-dihydoxy (catechol) structure in the ring B, for electron delocalization.

b) 2,3-double bond in conjugation with a 4-oxo function in the ring C provides electron delocalization from the ring B

c) –OH groups at positions 3 and 5 provide hydrogen bonding to the oxo group.

The –OH configuration attached to ring B is the most important determinant of ROS scavenging activity. –OH groups on the ring B transfer hydrogen and an electron to hydroxyl, peroxyl, and peroxynitrite radicals, stabilizing them and giving rise to a stable flavonoid radical. For flavones and flavanones, hydroxyl and peroxyl scavenging activity linearly, according to the total number of OH groups (167,168,172).

Due to their peroxyl radical scavenging activity, flavonoids are effective inhibitors of lipid peroxidation (167). A 3’4’-cathecol structure in the ring B strongly enhances the lipid peroxidation inhibition. The differences in the antioxidant activity between polyhydroxylated flavonoids are most likely because of differences in both hydrophobicity and molecular planarity.

Suppression of antioxidant capacity by *O*-methylation may reflect steric effects that perturb planarity. Although the ratio of methoxy to hydroxyl substituents does not predict the antioxidant potential, the ring B is especially sensitive to the position of the methoxy group. Changing a 6'–OH/4'OMe configuration with 6'–OMe/4'OH entirely abolishes the activity (172). Furhermore, multiple ring A methoxy groups reverse the positive effect of a ring B catechol, as inhibition of formation of the lipid peroxidation product, malondialdehyde by flavones with ring A *ortho*-dimethoxy or trimethoxy structure is not favored (172). Another mechanism for the inhibition of lipid peroxidation by flavonoids is the regeneration of α-tocopheryl radicals. Flavonoids have the capability to act as hydrogen donors to α-tocopheryl radical, resulting in the reformation of α-tocopherol, which is an important chain-breaking antioxidant, and protects low-density lipoproteins (LDL). This interaction causes a great potential in delaying the peroxidation of LDL. Catechins are more effective than ascorbic acid in the regeneration process of α-tocopherol (169).

Special flavonoids are able to chelate iron and cupper. As mentioned before, they participate in the formation of free radicals. The major binding sites for trace metals in the molecule are the catechol moiety in the ring B, the 3-hydroxyl and 4-oxo groups in the heterocyclic ring C, and the 4-oxo and 5-hydroxyl groups between the rings C and A.

The catechol moiety in the ring B plays a significant role for Cu^{2+}-chelate formation and it can be regarded as the major contribution site for the metal chelation. Flavonoids can also form complexes with cadmium (II), and be potential antidotes in cadmium intoxications (173).

In terms of carbohydrate moieties of flavonoids, aglycones are more potent antioxidants than their corresponding glycosides (172). It was also reported that the antioxidant activities of flavonol glycosides decreases as the number of the glycosidic moieties increased (174). In addition to the presence and number of the carbohydrate moieties, the structure and the position of the sugar content also participates in the activity. Luteolin and quercetin aglycones have greater activity than their 3–, 4'– and 7–*O*– glycosides in delaying the

accumulation of hydroperoxides in membrane bilayers; whereas a 4'–sugar was more suppressive than 3– or 7– substitution (172). Another important structural factor that affects the antioxidant activity is the C–glycosylation. The C-glycosylation in the ring A decreases the activity; and this negative effect is probably due to the properties of the sugar itself, rather than the displacement of a free –OH (175). Although most of the dietary flavonoid glycosidic moieties occur at the 3– or 7– position, an A–ring sugar causes greater decrease in the activity than 3–glycosylation in the heterocycle (172).

Another structural difference that affects the activity is the type of the sugar moiety. For instance, a rhamnose moiety on quercetin decreases the free radical scavenging activity, when compared to rutin (176).

In addition to the antioxidant activities mentioned above, flavonoids are also capable of activating particular enzymes like glutathione-S-transferase, UDP-glucuronosyl transferase, which participate in the body's antioxidant defense systems. Furthermore, flavonoids inhibit enzymes like xanthine oxidase and protein kinase C, which are important for free radical formation. They can also inhibit NADH oxidase, cyclooxygenase, microsomal succinoxidase (169). Another contribution to the antioxidant potential of flavonoids is the inhibition of the ability of myeloperoxidase to oxidize low-density lipoproteins (177).

2.4.7. Assays Used to Measure Antioxidant Capacity of Plants

As the interest on natural antioxidants found in medicinal plants and foods grows, the number of the studies reporting their antioxidant potentials increases. The potentials of these antioxidants have been investigated by a vast number of different methods under different conditions. Although all of these methods have been developed for the same purpose, i.e. measuring the antioxidant capacity, generally, there is a lack of correlation between activities determined on the same material by different assays. This situation could be expected, since multiple active species and reaction characteristics and mechanisms are involved in oxidative stress in human body. There is no universal method that measures antioxidant capacity accurately and quantitatively (103).

An ideal method for measuring the antioxidant potential should meet the following criteria (178):

1. Measures chemistry actually occurring in potential applications;
2. Uses a biologically relevant radical source;
3. Simple;
4. Utilizes a method with a defined endpoint and chemical mechanism;
5. Readily available instrumentation;
6. Satisfactory inter- and intraday reproducibility;
7. Adaptable for both hydrophilic and lipophilic substances, and use of different radical sources;
8. Adaptable to “high throughput analysis” for routine quality control analysis.

Generally, the methods for assessing the antioxidant capacity utilize two major reaction mechanisms, which are already mentioned: **HAT** and **SET**. Although these two different mechanisms yields the same end result, their kinetics and potential for side reactions vary.

HAT–based methods measure the ability of an antioxidant to scavenge free radicals by hydrogen donation. They are independent from solvent and pH changes, and very rapid to take place. The presence of reducing agents, including metals, is a complication in HAT assays and can cause high apparent activity (178).

SET–based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and radicals (178).

2.4.7.1. The Methods Using HAT-Mechanisms

2.4.7.1.1. ORAC (Oxygen Radical Absorbance Capacity)

ORAC measures the inhibition of peroxyl radical-induced oxidations by an antioxidant, and reflects the classical chain breaking antioxidant activity by H atom transfer (179). Basically, the peroxyl radical reacts with a fluorescent probe to generate a nonfluorescent product, which can be measured easily by fluorescence. Antioxidant capacity is determined by a decreased rate and amount of product formed over time (178,180). The principal of this assay is particularly based on the intensity of fluorescent molecule such as β-phycoerythrin (a protein isolated from *Pophyridium cruentum*) or fluorescein decrease of the target along time under reproducible and constant flux of peroxyl radicals, generated from the thermal decomposition of 2,2'–azobis(2–amidino–propane) dihydrochloride (AAPH) in aqueous buffer (132).

The ORAC assay provides a controlled source of peroxyl radicals that model reactions of antioxidants with lipids in both food and physiological systems. Another feature of ORAC is that it can be adapted to investigate both hydrophilic and hydrophobic antioxidants by changing the radical source and type of solvent used (181).

2.4.7.1.2. TOSC (Total Antioxidant Scavenging Capacity)

This method was developed by Winston *et al.* (182). It allows the calculation of absorbance capacity of antioxidants particularly toward three potent oxidants, which are hydroxyl radicals, peroxyl radicals, and peroxynitrite. It is possible to evaluate different antioxidants with different biologically relevant radical sources. However, the method cannot be readily adapted for high-throughput analysis necessary for quality control that it requires multiple injections from a single sample into a gas chromatograph to calculate the production of ethylene gas (178).

2.4.7.1.3. CL (Chemiluminescence)

The term means “chemically induced light”. The fundamental chemistry of CL assays depends on the reaction of certain radical oxidants with marker compounds to generate excited state species that emit chemiluminescence. Any molecules that react with the initiating radicals inhibit the formation of light. By altering the initiator, the reaction can be adapted to differentiate scavenging of specific oxidants like $O_2^{\cdot -}$, $OH^{\cdot}$, HOCl, $LOO^{\cdot}$.

CL is characterized by very low emission intensity, tens to a few thousand counts per second, when compared with millions of counts for fluorescence (178).

2.4.7.1.4. β-carotene/linoleic acid bleaching assay

This method was first described by Miler (183). In this assay, β-carotene undergoes rapid discoloration in the absence of an antioxidant due to the coupled oxidation of β-carotene and linoleic acid, which generates free radicals. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly saturated β-carotene molecules. Consequently, β-carotene is oxidized and broken down in part. Later, the system loses its chromophore group and its characteristic orange color. This reaction can be monitored spectrophotometrically (184).

2.4.7.2. The Methods Using SET-Mechanisms

2.4.7.2.1. FRAP (Ferric Reducing Antioxidant Power) Assay

The original method was first described by Benzie and Strain (185). The first method was developed for measuring the reducing power of plasma, but later it was modified, and adapted for the assay of antioxidants in botanicals (178). Basically, the reduction of ferric 2,4,6-tripyridyl-s-triazine (TPTZ) to a colored by-product is measured (185). FRAP is a reasonable screening tool for the capability of maintaining redox status in cells or tissues. Reducing power is associated with the degree of hydroxylation and extent of conjugation in polyphenols (186).

The FRAP assay is performed at acidic pH (3.6) to maintain the stability of iron. Low pH reduces the ionization potential that drives electron transfer and elevates the redox potential, resulting in a shift in the dominant reaction mechanism (178).

A major drawback of the method is that it cannot detect compounds that act by HAT, particularly thiols and proteins like glutathione. FRAP measures only the reducing ability based on the ferric ion (178,180).

FRAP is calculated as the change of absorbance, and related to the absorbance difference (ΔA) of a Fe^{2+} standard solution. ΔA is linearly proportional to the concentration of antioxidant. One FRAP unit is arbitrarily defined as the reduction of 1 mol of Fe^{3+} to Fe^{2+} (180).

2.4.7.2.2. CUPRAC (Cupric Reducing Antioxidant Capacity) Assay

This method is a variant of the FRAP assay using Cu instead of Fe. It is based on the reduction of Cu^{2+} to Cu^{+} by the combined action of all reducing agents in a sample. It was first developed by a group of Turkish scientists (187). The assay uses a specific compound neocuproine (2,9-dimethyl-1,10-

phenanthroline) to generate the chromogenic oxidizing agent, Cu^{2+}-Neocuproine.

Copper has superiorities over iron for antioxidant assays such as all sorts of antioxidant molecules, including glutathione, can be detected with little interference from reactive radicals. Copper has a relatively lower redox potential than iron, therefore its reactions are more selective, and common interferences with FRAP are not observed in CUPRAC. In addition to this, copper has a faster reaction kinetics than that of iron (178).

2.4.7.3. The Methods Using Both HAT and SET

2.4.7.3.1. DPPH (2,2-Diphenyl-1-picrylhydrazyl) Assay

$DPPH^{\cdot}$ scavenging assay is one of the oldest methods used for the purpose of determining antioxidant capacity. The DPPH radical, which bears a deep purple color, is one of the very few stable organic nitrogen radicals; therefore it does not have to be generated before the test. The method was first described by Blois in 1958 (188). This assay is based on the measurement of the reducing ability of antioxidants toward $DPPH^{\cdot}$. The DPPH radical is reduced to the yellow-colored diphenyl-picryl hydrazine in the presence of an antioxidant. This discoloration reaction can be monitored spectrophotometrically at 517 nm, and mainly based on SET reaction, and HAT mechanism can be observed rarely. The percentage of the remaining $DPPH^{\cdot}$ is proportional to the antioxidant concentration, and the concentration which induces a decrease in the initial $DPPH^{\cdot}$ concentration by 50% is defined as EC_{50} (178,180).

2.4.7.3.2. ABTS (2.2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) Assay

The ABTS scavenging method was first described by Miller et al. in 1993 (189) and later modified by Re et al. in 1999 (190). In this assay, ABTS is oxidized by peroxyl radicals or other oxidants to its radical cation, $ABTS^{\cdot+}$, which has an intense color. Antioxidant capacity is measured as the capability of test compounds to decolorize the $ABTS^{\cdot+}$ by reacting directly with it (178).

$ABTS^{\cdot+}$ can be generated by either chemical reaction like manganese oxide (191), potassium persulfate (190) or enzyme reactions like metmyoglobin (189) or horseradish peroxidase (192). Usually, chemical formation of the radical requires long time or high temperature, whereas enzyme formation occurs faster and the reaction conditions are relatively milder (178). An important advantage of this method is that $ABTS^{\cdot+}$ is soluble in both aqueous and organic solvents, and it is resistant to ionic strength. Therefore, it can be used in a diverse array of media to determine both hydrophilic and lipophilic antioxidant capacities of extracts or body fluids (193).

2.4.7.4. Antioxidant Capacity *In Vivo*

The capacity of antioxidants *in vivo* is determined by many factors which are summarized in Figure 2. One of these factors is the bioavailability. The antioxidant molecule should be well absorbed, distributed, and properly retained in biological fluids and tissues. The bioavailability of various well known antioxidant molecules, and the importance of dosage and duration of the treatment have been investigated by analysis of biological fluids and tissues of mostly experimental animals, and sometimes humans, after the consumption of antioxidants (103).

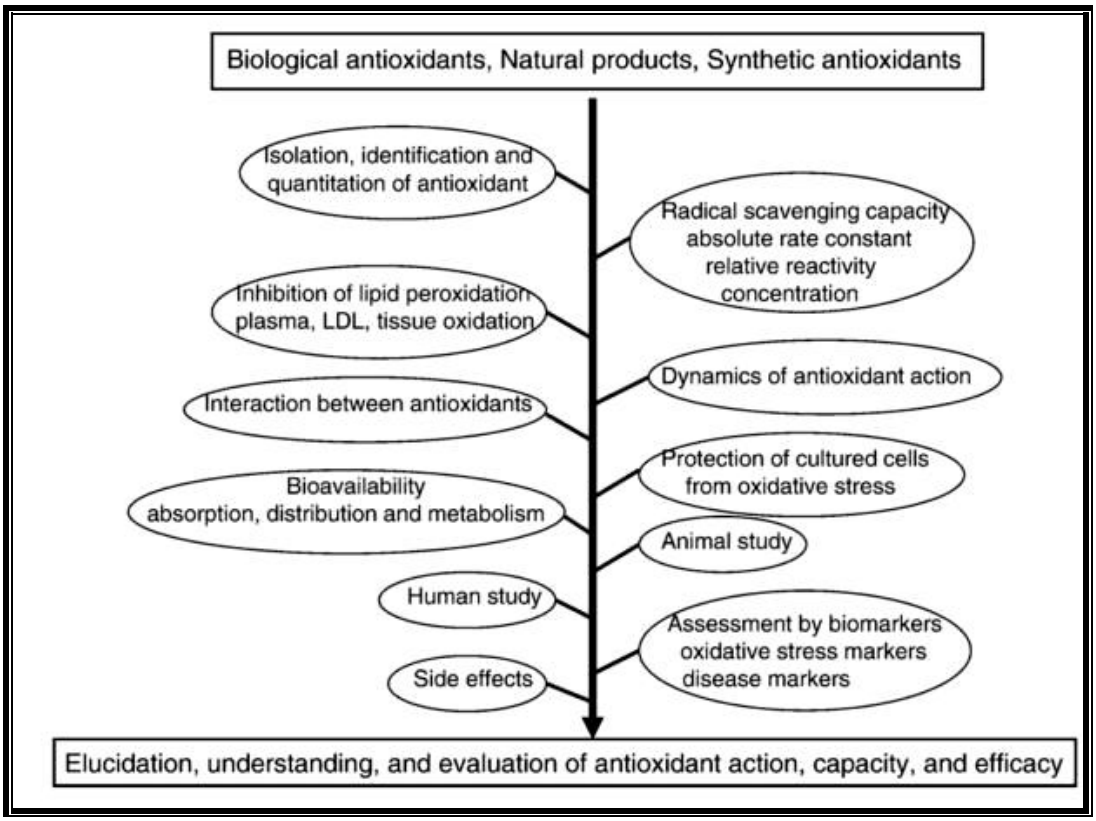


Figure 2: Factors effecting *in* vivo antioxidant studies [Adapted from Niki (103)].

Another factor that should be taken into consideration is the role of antioxidants in gastrointestinal tract. An ordinary diet contains numerous prooxidants including metals such as iron, copper, and haem, nitrite, lipid peroxides, and aldehydes (194). Even the gastric juice, itself may promote lipid peroxidation (103).

The capacity and efficacy of antioxidants *in vivo* might be evaluated most precisely by the effect of antioxidant compounds and materials on the level of oxidation in biological fluids and tissues, such as plasma, serum, erythrocytes, and cerebrospinal fluids from humans and experimental animals. Reliable biomarkers play a major role for this purpose and many biomarkers have been applied to calculate the level of oxidation *in vivo.* These are, as already mentioned, oxidation products of lipids, oxidative-based alteration and expression of proteins and carbohydrates, oxidation products of DNA bases and

strand breaks of DNA, itself. Table 22 summarizes frequently used oxidative stress biomarkers (103).

Table 22: Oxidative stress biomarkers

Lipids	**Proteins**	**DNA**
Ethane and pentane in exhaled gase	Protein carbonyl	Comet Assay
TBARS	Hydroperoxide	8-Hydroxyguanine
Conjugated diene	Disulfide –SS–	5-Hydroxyuracil
Hydroperoxide	Advanced oxidation products	2-, 8-Hydroxyadenine
Aldehydes	Myeloperoxidase	8-bromo-, chloro-, nitro-guanine
Isoprostane	Cleavage products	
Oxidized LDL	Halogenated amino acids	

[Adapted from Niki (103)]

Furthermore, the levels of antioxidants the ratio of their oxidized/reduced forms and their oxidation products are also important in determining oxidative stress. For example, the ratio of oxidized/reduced forms of glutathione increases with the increase of oxidative stress (195).

The effects of numerous antioxidants on the degree of oxidative stress biomarkers have been assessed in many studies of experimental animals and humans under both normal conditions and elevated oxidative stress. The measuring of antioxidant biomarkers of oxidative stress can be performed by using different methods such as (103):

1. Antioxidant compound level: Total antioxidant capacity, antioxidant molecules i.e. glutathione, uric acid, or antioxidant vitamins.
2. Antioxidant enzyme level: SOD, CAT, GPx, GST, etc.
3. Oxidation product of an antioxidant: Tocopheryl quinone
4. Ratio of oxidized to reduced form of an antioxidant: GSSG/GSH

2.4.8. Carbon Tetrachloride (CCl_4)

CCl_4 is a colorless liquid with a sweet smell that can be detected at low levels. It contains four chlorine atoms positioned symmetrically as corners in a tetrahedral configuration joined to a central carbon atom by single covalent bonds. It was formerly widely used in fire extinguishers, as a precursor to refrigerants, and as a cleaning agent (196,197). Although there are some other ways of its synthesis, it is mainly produced from methane (197).

Today CCl_4 is much used as an experimental model in the hepatotoxicity and *in vivo* antioxidant studies. CCl_4 is a hepatotoxic chemical which requires metabolic activation in the liver. In fact, the liver is the major site for CCl_4-induced effects, yet no specific receptor is necessary for the actions of CCl_4. Mitochondria, endoplasmic reticulum, Golgi apparatus and plasma membrane are the main cellular structures of hepatocytes affected by CCl_4 exposure. Following the oral administration, CCl_4 is concentrated in the liver and reaches a maximum degree of about 1 mg per gram of liver tissue within 1 to 2 hours of dosing (196).

CCl_4 induces severe oxidative stress *in vivo*. Sustained exposure to CCl_4 results in increased free radical formation, therefore biochemical alterations are prone to occur and can appear in different cell fractions at different times after the administration. The level of microsomal lipids rises 3 hours after administration. Triglyceride secretion from the endoplasmic reticulum into plasma diminishes within 2 hours. CCl_4 causes uncoupling of oxidative phosphorylation and impairment of Ca^{2+} transport in mitochondria (196,198).

The metabolism of CCl_4 starts with the generation of the trichloromethyl free radical ($CCl_3^{\cdot}$) via the actions of the mixed function CYP450 oxygenase system of the endoplasmic reticulum. This course involves reductive cleavage of a carbon-chlorine bond, and no oxygen is introduced into the molecule during the reaction. The major cytochrome isoenzyme to carry out the biotransformation of CCl_4 is **CYP2E1**, but CYP2B1 and CYP2B2 can also attack CCl_4. Although CCl_4 metabolism in humans is dominated by CYP2E1 at

environmentally relevant concentrations, CYP3A significantly contributes at higher doses (196,198).

The $CCl_3^{\cdot}$ radical reacts with various biologically important substances such as fatty acids, nucleotides and amino acids, and also lipids, nucleic acids and proteins. The rate constants for these reactions are generally lower than 10^5 M^{-1} s^{-1}, but large rises are found when the reaction continues in the presence of oxygen (196).

Figure 3 summarizes the metabolism of CCl_4 involving free radicals. $CCl_3^{\cdot}$ radical is converted to the trichloromethyl peroxy radical, $CCl_3OO^{\cdot}$. This is a more reactive radical, and it is more short-lived than the $CCl_3^{\cdot}$. The lifetime of these radicals is within the milliseconds range, and they are cleared from the target tissues by reacting with appropriate substrates in order to rehabilitate their unpaired electrons. $CCl_3OO^{\cdot}$ is far more likely than $CCl_3^{\cdot}$ to abstract a hydrogen from polyunsaturated fatty acids (PUFA), resulting in the initiation of the chain reaction of lipid peroxidation (196). Metabolism of CCl_4 by rat liver microsomes gives rise to covalently bound lipids, mostly phospholipids.

The abstraction of a hydrogen atom from fatty acid initiates a full series of reactions causing the termination of the complete disintegration of PUFA molecule and the formation aldehydes like malondialdehyde (196,198).

Another complication of the CCl_4 administration is the reduction in the activities of antioxidant enzymes such as SOD, CAT, GPX (199). This decrease in the activities is mainly due to the accumulation of free radicals and the excessive use of these defensive enzymes (200).

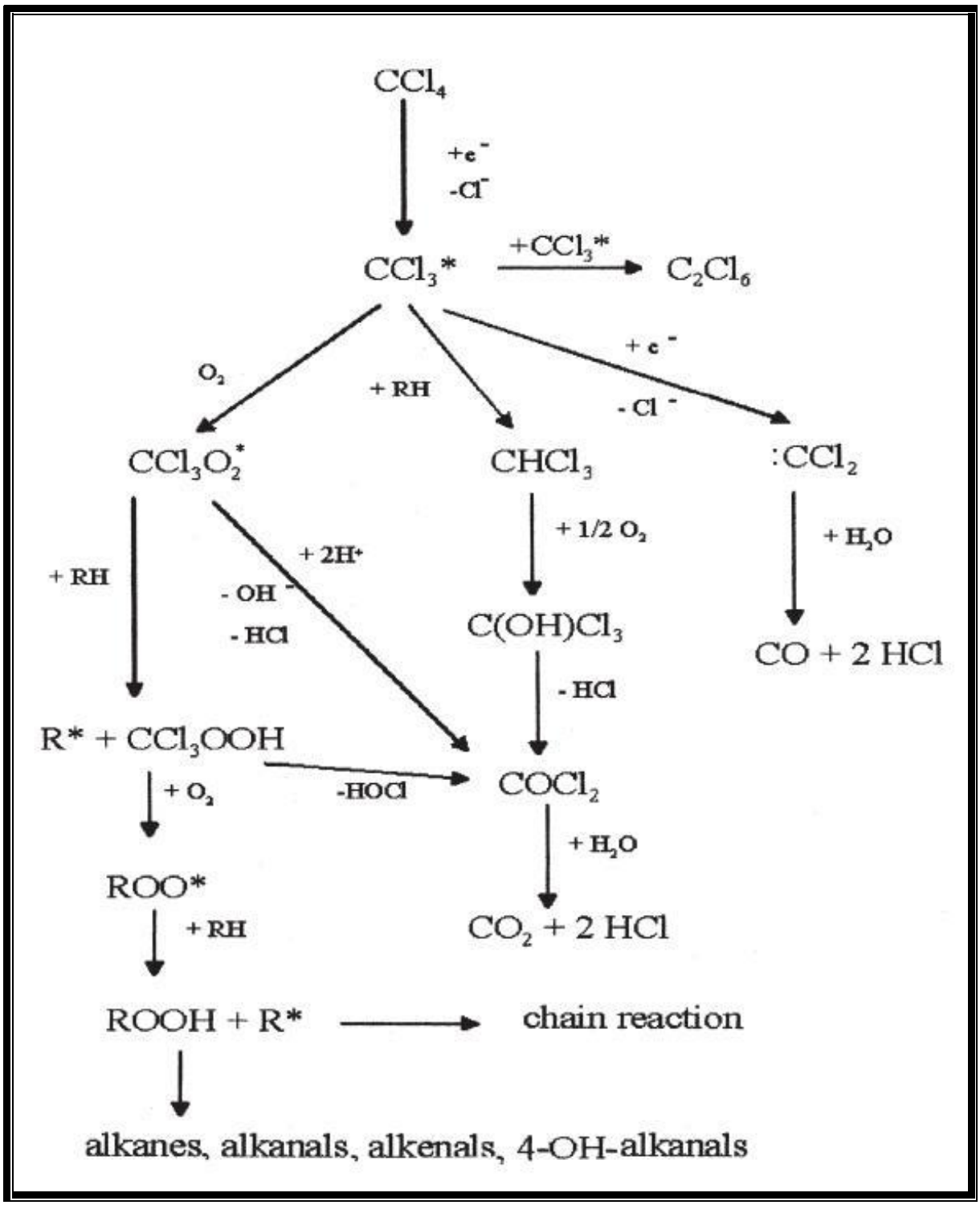


Figure 3: Metabolism of CCl_4 in the presence of oxygen and organic molecules. RH generally represented by PUFA [Transferred from Weber et al. (196)]

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Plant Material

The leaves and fruits of *Cornus mas* L. were collected from the wilderness in Susurluk, Balıkesir province of Turkey, in the second week of June, 2010. The fruits of *Laurocerasus officinalis* Roem. were collected from the campus area of Yeditepe University, Istanbul, Turkey, and those of *Diospyros kaki* L. were purchased from a local market in September, 2010. The fruits were identically selected in terms of shape, color and the ripening stage. The leaves were dried in shade for one week, and later powdered.

The plant materials were authenticated by Prof. Dr. Erdem Yesilada before any process. Voucher specimens for *Cornus mas* L. (YEF 10005), *Diospyros kaki* L. and *Laurocerasus officinalis* Roem. (YEF 10007) were deposited in the herbarium of Faculty of Pharmacy, Yeditepe University, Istanbul, Turkey.

3.1.2. Chemicals & Solvents

1,1,3,3-Tetramethoxypropane	Sigma Aldrich; MKBB0326
2,2'-Azino-bis(3-ethyl benzothiazoline-6-sulfonic acid diammonium acid (ABTS)	Sigma Aldrich; 110M1879V
2,2-diphenyl picryl hydrazil	Sigma Aldrich; 056K1147
2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ)	Fluka; BCBB4473
2-Thiobarbituric acid	Sigma Aldrich; STBB0632
3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS)	Sigma Aldrich; 119K5417
Acetic acid	Riedel de Haen; 30990
Aluminum chloride	Merck; 8.01081.1000
Ammonium acetate	Carlo Erba; 313507
Ammonium molybdate	Riedel de Haen; 30590
Ascorbic acid	Sigma Aldrich; 065K0003
Beta-carotene	Sigma Aldrich; 079K1729
Bovine serum albumin	Sigma Aldrich; 089K1624

Butylated hydroxytoluene	Sigma Aldrich; MKBD8339
Carbon tetrachloride	Merck; 1.02222.2500
Chloroform	Lab-Scan; 0344/6
Copper sulphate	Carlo Erba; 364757
Dichloromethane	Merck; 1.06044.2500
Epigallocatechin gallate	Teavigo; WB00044001
Ethyl acetate	Sigma Aldrich; SZBA113S
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	Sigma Aldrich; BCBC1356
Ferric chloride hexahydrate	Riedel de Haen; 41250
Ferrous chloride tetrahydrate	JT Baker; 0703801013
Ferrous sulphate heptahydrate	JT Baker; 0632701017
Ferrozine	Sigma Aldrich; MKBD0707
Folin-Ciocalteu Reagent	Sigma Aldrich; BCBD5119
Gallic acid	Fluka; 1126284
Glutathione reductase from bakers yeast	Sigma Aldrich; 069K7420
Hydrochloric acid (37%)	Sigma Aldrich; SZBA2250
Hydrogen peroxide (30% solution)	Riedel de Haen; 62340
Kieselgel 60 F_{254} (0.063 – 0.200 mm)	Merck; 1.10757.1000
Iodonitrotetrazolium chloride (INT)	Sigma Aldrich; 0001437943
L-Glutathione (reduced)	Sigma Aldrich; 010M1469
Linoleic acid	Sigma Aldrich; 058K52082
Methanol	Sigma Aldrich; SZE9365S
n-Butanol	Fluka; 52150
n-Hexane	Sigma Aldrich; SZBA0655
Neocuproine	Sigma Aldrich; 120M1890V
Polyamide(for column chromatography)	Merck; 0001447805
Potassium persulfate	Sigma Aldrich; MKBG5868V
Potassium sodium tartarate	Carlo Erba; 363457
Quercetin dehydrate	Sigma Aldrich; 116K1836
Reduced nicotinamide adenine dinucleotide phosphate (NADPH)	Sigma Aldrich; 010M1380
Silicagel 60 F_{254} Aluminium sheet	Merck; 1.107571000
Sodium acetate trihydrate	Riedel de Haen; 33450
Sodium carbonate	Riedel de Haen; 2217A
Sodium dodecyl sulphate	Merck; 8.2205.1000
Sodium hydroxide	Riedel de Haen; 60030
Sodium phosphate monobasic	Riedel de Haen; 62840
Sulfuric acid (98%)	Riedel de Haen; 62260
Trichloroacetic acid	Riedel de Haen; 23100
Trizma base	Sigma Aldrich; 069K5405
TROLOX	Sigma Aldrich; BCBF4547V
Tween 20	Merck; 8.22184.0500

Vanillin	Fluka; 1435805
Xanthine oxidase from bovine milk	Sigma Aldrich; 001M7013V
Xanthine sodium	Sigma Aldrich; 030M1599

3.1.3. Equipments

Balance	Ohaus Explorer
Beaker (50, 100, 250 mL)	
Centrifuge	Sigma 3-16 PK
Eppendorf tubes (1.5 mL)	
Erlenmayer flask (50, 100, 250 mL)	
Hairdryer	Arçelik
Lyophilizator	Christ Alpha 2-4 LD
Micropipette (100-1000 microlt)	Isolab
Micropipette (500-5000 microlt)	Rainin
Micropipette (5-50 microL, 20-200 microlt)	Transferpette
Microplate reader	Thermo Multiskan Ascent
Milli Q water device	Millipore
Oven	Binder
pH meter	Mettler-Toledo MP220
Polypropylene tubes (16 x 100 mm)	
Refrigerator	Arçelik
Rotatory evaporator	Buchi, Heidolph
Spectrophotometer	Spekol 1300
Ultrasonic bath	Sonorex RK156BH
Volumetric flasks (5, 10, 25, 50, 100, 200, 500, 1000 mL)	
Vortex	Heidolph Reax
Waterbath	GFL

3.2. Methods

3.2.1. Chemical Studies

3.2.1.1. Extraction

The plant materials were subjected to subsequent extraction in order to perform phytochemical and activity studies. Primary extraction was carried out using 80% MeOH and the yielded extracts were used for both *in vitro* and *in vivo* activity tests.

2 kg of each fruit were pre-washed to remove contaminants and their seeds were separated. The remaining pulps were blendered and then lyophilized. Dried fruit samples (200 g) were then extracted twice with 80% methanol (500 mL) at 45 °C for 4 h with continuous stirring. The combined extracts were filtered through a filter paper and the filtrates were evaporated to dryness under reduced pressure. The residues were dissolved in water and lyophilized again.

The dried and powedered leaves of *C. mas* (280g) were macerated 24 h with 2200 mL of 80% MeOH. Later, the macerates were extracted twice at 45 °C for 4 h with continuous stirring, then filtered. After removing MeOH under vacuum, the extract was lyophilized.

Table 23: The extraction yields with 80% MeOH (g/g)

Plant name	Part used	Yield (%)
Cornus mas	Leaf	19.86
	Fruit	19.69
Diospyros kaki	Fruit	17.41
Laurocerasus officinalis	Fruit	13.72

3.2.1.2. Activity Guided–Fractionation of Extracts

80% methanolic extract prepared from the leaves of *C. mas* was found to show the highest activity in both *in vitro* and *in vivo* tests. Therefore, it was subjected to further fractionation in order to isolate active compound(s).

45 g of 80% methanolic extract was dissolved in 100 mL of 90% MeOH in H_2O and then partitioned 4 times with 100 mL of ***n*-hexane**, and then MeOH in the remaining extract was removed and diluted with H_2O to 100 mL. The aqueous extract was partitioned 4 times successively with **chloroform, ethyl acetate, *n*-butanol** (saturated with water). The organic layers were dried vacuum while the remaining H_2O extract was lyophilized (Figure 4). Percent yields of each subextract were as follows (g/g):

Table 24: The extraction yields of subfractions

Subextract	**Yield (%)**
n-Hexane	1.8
Chloroform	11.2
Ethyl acetate	5.58
n-Butanol	22.07
Remaining H_2O	59.36

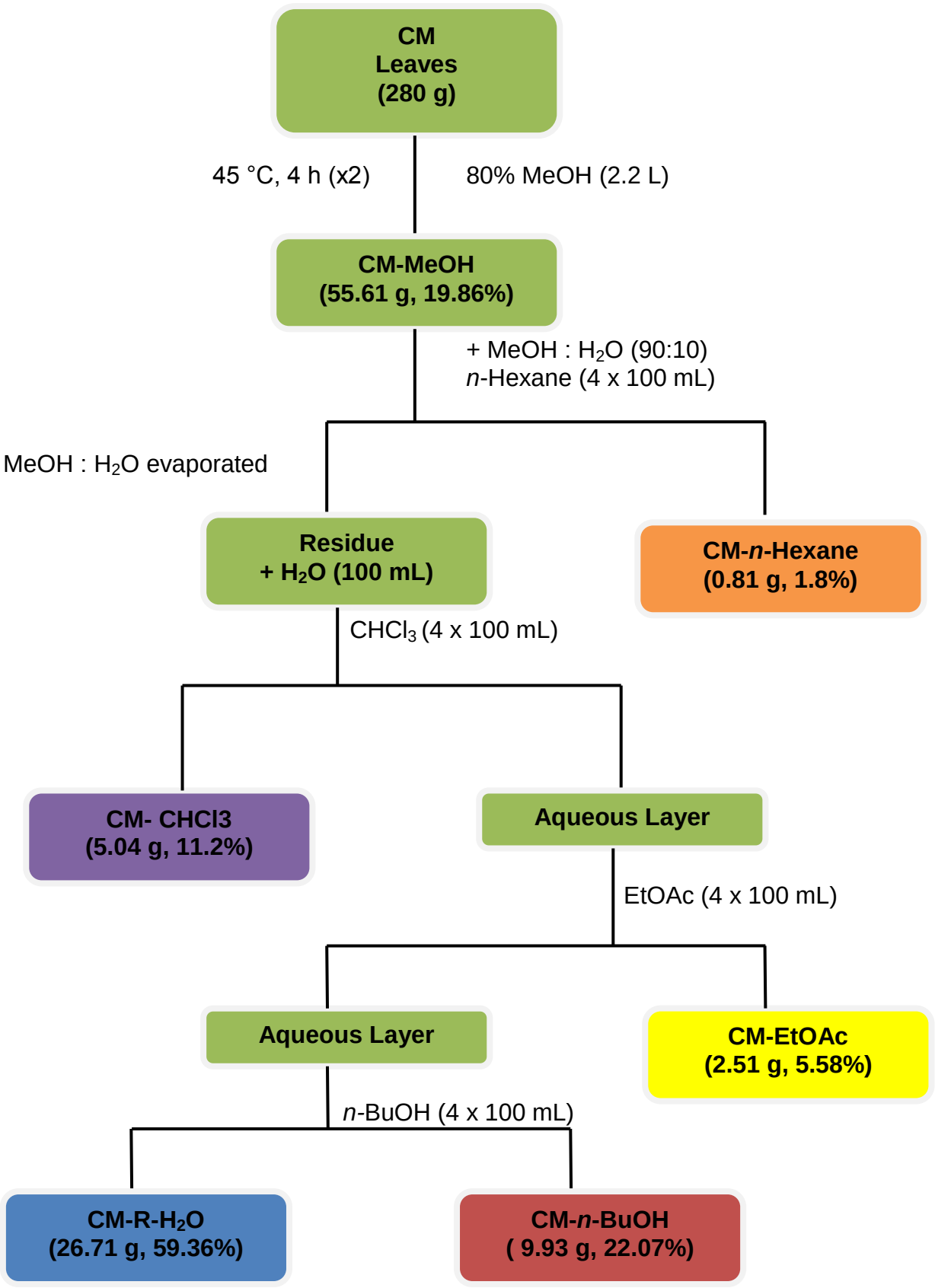


Figure 4: Liquid-liquid extraction of the crude MeOH extract of the leaves of *Cornus mas* L. (CM)

3.2.1.3. Chromatographic Methods

Thin Layer Chromatography (TLC)

TLC was used in order to monitor the isolation studies in this research. Silica gel – coated aluminum plates were used for this purpose:

Stationary phase: Kieselgel 60 F_{254} 0.200 mm (Merck)

Mobile phases:

1. Chloroform : Methanol (95 : 5)
2. Chloroform : Methanol : Water (90: 10: 1)
3. Chloroform : Methanol : Water (80 : 20 : 2)
4. Chloroform : Methanol : Water (70 : 30 : 3)
5. Chloroform : Methanol : Water (61 : 32 : 7)

Visualization: 5% H_2SO_4 (Δ)

1% Vanillin/H_2SO_4 (Δ)

The plates were observed under UV light with the wavelengths of 254 and 360 nm.

Polyamide Columnn Chromatography

According to *in vitro* antioxidant activity tests, EtOAc extract of *C. mas* showed the highest activity. Therefore, EtOAc extract was chosen for further studies.

Polyamide column chromatography was used in fractionation process of the bioactive extract. 20 g of polyamide was mixed with H_2O and kept in room temperature for a certain period of time. Then, this suspension was filled inside a suitable size of glass column and left still for allowing the adsorbent to settle properly in the column. The mobile phase was ranged from MeOH : H_2O (0 : 100) to (100 : 0). 10 different fractions were obtained from this column. The details of experimental process were given in Figure 5.

Sephadex Column Chromatography (Gel Filtration)

Sephadex column chromatography was used in the purification process of the fraction obtained after polyamide column. 12 g of Sephadex LH-20 was mixed with MeOH and kept in room temperature for a certain period of time with stirring. Then, this suspension was filled inside a proper glass column and left still for allowing the adsorbent to settle properly in the column. 100 mL of MeOH was used as eluent. The details of experimental process were given in Figure 5.

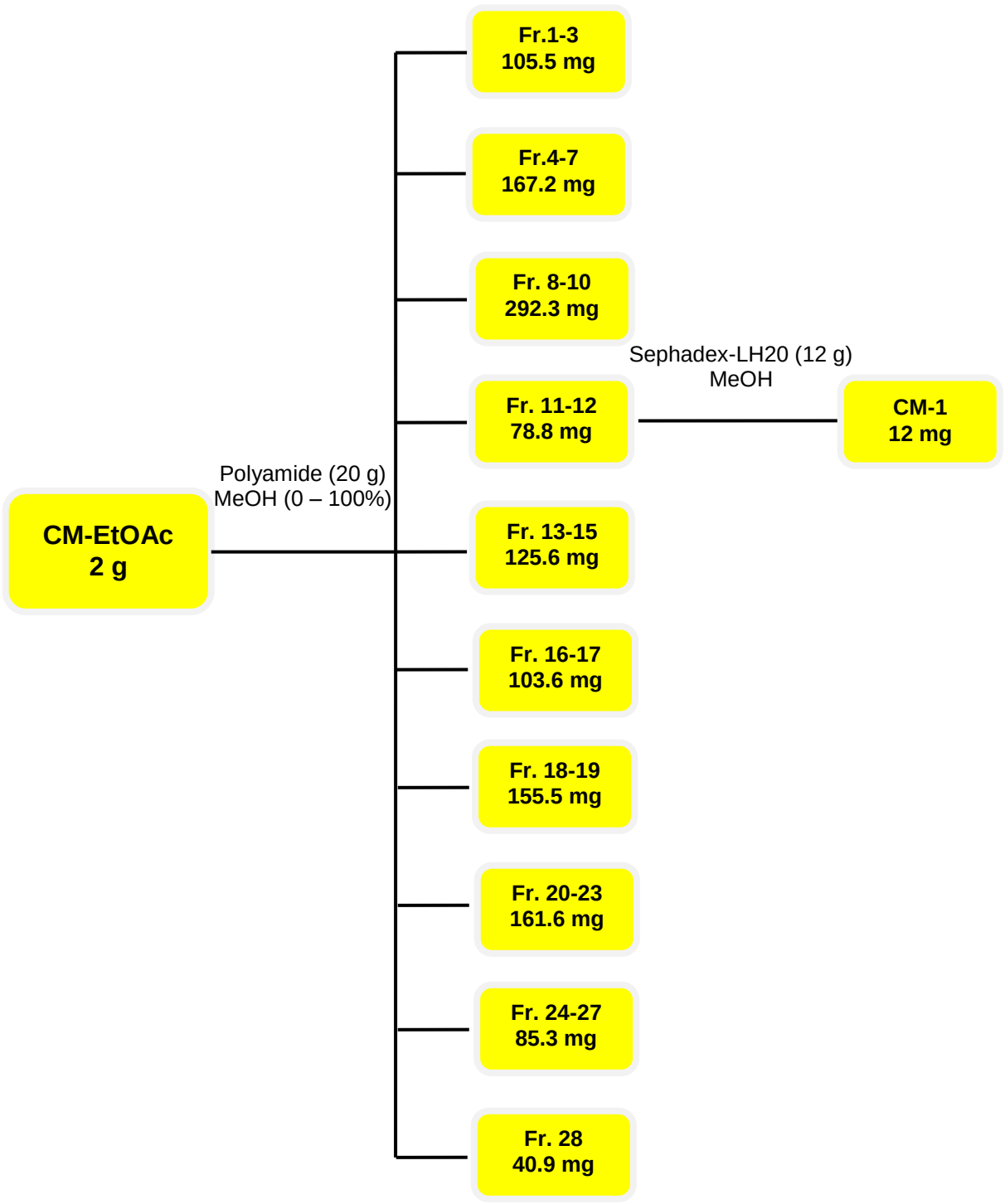


Figure 5: Isolation of CM-1

3.2.2. Antioxidant Activity Studies

3.2.2.1. *In vitro* activity studies

3.2.2.1.1. Determination of Total Phenolic Content

The total phenolic content of the samples was calculated by using the method described by Singleton and Rossi (201). This is a colorimetric assay, involving a blue molybdenum-tungsten complex in the presence of phenolics by using Folin-Ciocalteu Reagent (FCR). This complex can be detected spectrophotometrically at 765 nm. The chemistry behind this assay relies on SET in alkaline medium.

The Chemicals and Reagents

- Na_2CO_3 20% in H_2O
- Folin-Ciocalteu Reagent
- Gallic acid

The Procedure

100 µL of FCR and 300 µL of Na_2CO_3 solution (20%) were added to 20 µL of sample in a closed-cap polypropylene tubes. Then, the volume was completed to 2000 µL with doubly-distilled water. After a 30 minute-incubation period at 45 °C, the absorbance was read at 765 nm. Gallic acid was used as standard subtance, and a calibration curve was plotted in the concentrations of 50, 100, 150, 250, 500 µg/mL. All experiments were performed triplicate. The results were given as mg gallic acid equivalents per g material (mg GAE/g sample).

3.2.2.1.2. Determination of Total Flavonoid Content

The total flavonoid content of the samples were measured according to the aluminum chloride method described by Woisky and Salatino (202). This method depends on the formation of acid stable complexes by $AlCl_3$ with the C-

4 keto group and either C-3 or C-5 hydroxyl groups of flavones or flavonols. Besides, $AlCl_3$ also forms acid labile complexes with *ortho*-dihydroxyl groups in the A- or B- ring of flavonoids (203).

The Chemicals and Reagents

- Ethanol 95%
- Aluminum chloride 10% in H_2O
- Potassium acetate 1 M
- Quercetin

The Procedure

500 µL of samples dissolved in d.d. water were mixed with 1500 µL of 95% ethanol, 100 µL of 10% aluminum chloride solution, 100 µL of 1M potassium acetate. Later, the final volume was adjusted to 5000 µL with doubly-distilled water. Following the incubation period at room temperature for 30 min, the absorbance was read at 415 nm. Quercetin, dissolved in methanol, was used as the standard substance, and a calibration curve was plotted in the concentrations of 15.625, 31.25, 62.5, 125, 250, 500 µg/mL. All of the measurements were done triplicate. The total flavonoid content of the samples was expressed as mg quercetin equivalents per g material.

3.2.2.1.3. Determination of Total Proanthocyanidin Content

The total proanthocyanidin content of each sample was measured by using vanillin-HCl method described by Ariffin et al. (204). The principle of this assay is as follows: Vanillin is protonated in an acid solution, giving a weak electrophilic carbocation that reacts with the flavonoid ring at the C-6 or C-8 position. This intermediate compound is dehydrated to give a red colored compound (205).

The Chemicals and Reagents

- Vanillin 1% in MeOH
- HCl 9 M

- Epigallocathecin gallate (EGCG)

The Procedure

2500 µL of 1% vanillin dissolved in methanol and the same volume of 9 M HCl in methanol were added to 1000 µL of sample in a closed-cap polypropylene tube. The mixture was incubated for 20 min at 30 ^{0}C. The absorbance was measured at 500 nm. Epigallocatechin gallate was used as the standard substance, and a calibration curve was plotted in the concentrations of 10, 20, 50, 100, 200, 500, 1000 µg/mL. The assays were run in triplicate. The total proanthocyanidin content of the samples was expressed as mg epigallocathecin gallate equivalent (EGCG-E) per g material.

3.2.2.1.4. Determination of DPPH Radical–Scavenging Activity

The scavenging activity of the samples against DPPH radical by using the method described by Akter et al. (206). The basic principle of the assay is as described in the section 2.4.7.3.1.

The Chemicals and Reagents

- DPPH 0.1 mM in MeOH

The Procedure

1000 µL of properly diluted samples were separately added to 5000 µL 0.1 mM DPPH solution prepared in methanol just before use. The mixture was vortexed and incubated in the dark for 50 min at room temperature. The absorbance was measured at 517 nm. Milli Q water was used in the control group instead of samples, and the same procedure was applied. Butylated hydroxy toluene was used as the reference substance. DPPH scavenging activity of the control group, the reference substance and the samples were calculated as follows:

$$\text{DPPH scavenging activity} \left(\frac{(ABS_{Control} - ABS_{Sample})}{ABS_{Control}} \right) \times 100$$

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

3.2.2.1.5. Determination of Superoxide Radical Scavenging Activity

Superoxide radical scavenging activity was performed following the method described by Aydin et al. (1) after adaptation for plant material. In this assay, the superoxide radical was generated through xanthine/xanthine oxidase system. The generated radical reacts with iodonitrotetrazolium (INT) and forms violet-colored formazan dye. The absorbance of the color can be measured spectrophotometrically at 505 nm. The reaction is inhibited in the presence of a superoxide radical scavenger, and the inhibition percentage is calculated (1).

The Chemicals and Reagents

- Xanthine sodium 0.05 mM
- INT 0.025 mM
- CAPS 50 mM
- EDTA 0.94 mM
- Xanthine oxidase 80 U/L

The Procedure

The substrate mixture was prepared in a volume of 60 mL containing 0.05 mmol/L xanthine sodium, 50 mmol/L CAPS and 0.94 mmol/L EDTA. The pH was adjusted to 10.2 by adding NaOH pellets. 0.025 mmol/L INT was added to this mixture.

25 µL of properly diluted samples were mixed with 1700 µL of the substrate mixture in a 3 mL-glass UV cuvette. Finally, 250 µL of 80 U/L xanthine oxidase was added to the mixture, and the absorbances in 30^{th} (A_1) and 210^{th} (A_2) seconds at 505 nm were recorded. Water was used instead of sample solution in the control group. Gallic acid was used as the reference substances. The same procedure was applied to both control and reference groups. The

absorbance change of control, sample and reference groups per minute was calculated as:

$$\Delta A/min = \frac{A_2 - A_1}{3}$$

The inhibition percentage of both samples and the reference was calculated against the control group as follows:

$$\text{Inhibition (\%)} = \frac{\Delta A_{sample}/min \times 100}{\Delta A_{control}/min}$$

The activity was expressed as EC_{50} values corresponding to the concentration which shows 50% activity.

3.2.2.1.6. Determination of Ferric Reducing Antioxidant Power (FRAP)

FRAP activity was measured by the assay of Benzie and Strain (185). This assay uses antioxidants as reductants in a redox-linked colorimetric method. Ferric tripyridyl triazine complex (Fe^{3+} TPTZ) is reduced to ferrous form at low pH. The reduced form has an intense blue color and the change in absorption at 593 nm is measured. The reaction is non-specific, in that any half reaction that has lower redox potential than that of ferric ferrous half reaction will cause the formation of ferrous ion. The change in absorbance is thus, directly related to the reducing power of the electron donating antioxidants.

The Chemicals and Reagents

- FRAP Reagent:
  - **A.** Acetate buffer — 300 mM, pH 3.6
  - **B.** TPTZ — 10 mM in 40 mM HCl
  - **C.** $FeCl_3 . 6H_2O$ — 20 mM

  The working FRAP reagent was freshly prepared by mixing A, B and C in the ratio of 10:1:1.
- $FeSO_4 . 7H_2O$

The Procedure

10 µL of properly diluted samples and 30 µL of Milli Q water were mixed with 260 µL of working FRAP reagent in a microplate. The mixture was incubated at 37^{o}C for 30 min. After the incubation period, the absorbance was read at 593 nm using a 96-well microplate reader. Water was used for the control group instead of sample. A standard curve was prepared using different concentrations of $FeSO_4.7H_2O$ (0.25, 0.5, 1, 2 mM). Butylated hydroxy toluene was used as the reference substance. The results were expressed as mM $FeSO_4$ per g material.

3.2.2.1.7. Determination of Cupric Reduced Antioxidant Capacity (CUPRAC)

CUPRAC was measured according to the method developed by Apak et al. (187) with slight modifications. This method was developed as an antioxidant capacity index for dietary polyphenols and plant materials. The method is based on the measurement of absorbance at 450 nm of a stable complex between neocuproine and copper (I), the latter is formed by the reduction of copper (II) in the presence of an antioxidant molecule.

The chemicals and reagents

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

The Procedure

1000 µL of each of 10 mM $CuSO_4$, 7.5 mM neocuproine, and 1 M ammonium acetate buffer (pH 7.0) solutions were mixed in a test tube. Later, 500 µL of sample solution was added, and the final volume was completed to 4100 µL with Milli Q water. Following the 1 h incubation at room temperature, the absorbance was recorded at 450 nm. Ascorbic acid was used as the standard substance, and a calibration curve was plotted in the concentrations of

10, 20, 50, 100, 200 µg/mL. The results were given as mg ascorbic acid equivalent (AAE) per g material.

3.2.2.1.8. Determination of Ferrous Ion-Chelating Capacity

The ferrous ion-chelating capacity was calculated according to the method described by Guo et al. (207). In this method, ferrozine forms complexes with ferrous ion quantitatively. In the presence of a chelating agent, the formation of this complex is disrupted, and the formation of color caused by this complex is inhibited.

The Chemicals and Reagents

- Ferrozine 5 mM
- $FeCl_2.4H_2O$ 2 mM

The Procedure

1 mL of each sample was mixed with 50 µL of $FeCl_2.4H_2O$ (2 mM) and 3.7 mL of distilled water. The reaction was initiated by the addition of 200 µL of ferrozine (5 mM). The mixture was allowed to incubate for 10 min at room temperature, and then the absorbance was measured at 562 nm. EDTA was used as a reference compound. The activity was calculated by using the same formula which was used for DPPH radical scavenging activity.

3.2.2.1.9. Determination of Total Antioxidant Capacity

The total antioxidant capacities of the samples were measured by the phosphomolybdenum method developed by Prieto et al. (208) with slight modifications. The assay is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and subsequent formation of a green phosphate/ Mo (V) complex at acidic pH.

The Chemicals and Reagents

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

The Procedure

3000 µL of the reagent solution was mixed with 300 µL of properly diluted samples. The tubes containing the mixture were tightly capped and incubated at 95^{o}C for 90 min. After the incubation period, the samples were cooled to room temperature, and the absorbance was read at 695 nm. Ascorbic acid was used as the standard substance and a calibration curve was plotted in the concentrations of 10, 20, 50, 100, 200 µg/mL. The results were given as mg ascorbic acid equivalent (AAE) per g material.

3.2.2.1.10. Bleaching assay of β-carotene in a linoleic acid system

This assay was performed according to a method described by Zovko Koncic et al (209). It is based on the ability of antioxidants to decrease the heat-induced oxidative bleaching of β-carotene in β-carotene/linoleic acid emulsified aqueous solution. The basic principle of this method was expressed in detail in the section 2.4.7.1.4.

The Chemicals and Reagents

- β-carotene (in $CHCl_3$) 200 µg/mL
- Linoleic acid
- Tween 20

The Procedure

20 µL of linoleic acid and 200 µL of Tween 20 were pipetted to a 100 mL-round bottom flask. 1000 µL of β-carotene solution in chloroform (0.2 mg/mL) was added. After chloroform was removed in a rotatory evaporator, 50 mL of distilled water was added to the oily residue with shaking vigorously to

form an emulsion. 5000 µL aliquots of the emulsion were pipetted into test tubes containing 200 µL sample or distilled water or reference and left for incubation at 50 ^{o}C for 1 h. The absorbance was read at 470 nm. BHT was used as reference substance.

The antioxidant activity (A_A) was expressed as inhibition percentage relative to control, using the formula:

$$A_A = [(R_{control} – R_{sample\ or\ reference}) / R_{control}] \times 100,$$

where $R_{control}$ and R_{sample} represent the bleaching rates of β-carotene without and with the addition of antioxidant, respectively. Degradation rates (R_D) were calculated according to first order kinetics:

$$R_D = \ln (A_0 / A_t) \times (1/t),$$

where (ln) is natural log, (A_0) is the initial absorbance at 470 nm at t = o and (A_t) is the absorbance at 470 nm at t = 60 min.

3.2.2.1.11. Determination of Trolox Equivalent Antioxidant Capacity (TEAC)

TEAC was determined as previously described by Re et al. (190). The basic principal of this method was explained in the section 2.4.7.3.2.

The Chemicals and Reagents

- ABTS 7 mM
- Potassium persulfate 140 mM
- Trolox

The Procedure

Briefly, 5000 µL of 7 mM ABTS was reacted with 88 µL of 140 mM potassium persulfate solution (final concentration equals to 2.45 mM) overnight in the dark to generate $ABTS^{\cdot+}$ radical cation. Later, $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.7 ± 0.05 at 734 nm. 20 µl of properly diluted samples was mixed with 2000 µL of diluted $ABTS^{\cdot+}$ solution. The absorbance

was measured 6 min after the initial mixing at 734 nm. A standard curve was prepared using different concentrations of Trolox (125, 250, 500, 750, 1000 µM). TEAC was expressed as µM Trolox equivalent per g dry extract

3.2.2.2. *In vivo* Activity Studies

3.2.2.2.1. *In vivo* Experimental Protocol

The experimental protocol was approved by the Ethical Committee of Yeditepe University Experimental Medicine Research Institute (Decision number: 2012/246), and the use of animals was in compliance with US National Institute of Health Guide for Care and Use of Laboratory Animals.

Sprague – Dawley rats, weighing 200 – 250 g, were used in the course of study. The animals were provided by the Yeditepe University Experimental Research Center (YUDETAM). They were kept in Plexiglas cages in a room of which temperature and humidity were controlled with 12 h light and dark cycle, and they had free access to food and water.

3.2.2.2.2. Experimental Protocol on Healthy Animals

Rats were divided into 6 groups with 6 animals in each group with an average weight of 180 – 200 g. Control group received distilled water containing 0.5% carboxymethylcellulose (CMC) p.o. for 21 days. Reference group received 50 mg/kg bodyweight silymarin p.o. The test groups received 80% methanolic extracts of fruits of cornelian cherry, Japanese persimmon and cherry laurel, and also 80% methanolic extract of cornelian cherry leaves for 21 days. The animals were decapitated on day 22. Their blood samples were collected and their livers were dissected for further analysis.

3.2.2.2.3. Experimental Protocol on CCl_4 – Treated Animals

This experimental protocol was accomplished according to the method described by Huang et al. (210) with slight changes.

Rats in this protocol were divided into 6 groups. Control group received distilled water containing 0.5% carboxymethylcellulose (CMC) p.o. for 5 days and olive oil (1 mL/kg body weight, s.c.) CCl_4 – treated group received 0.5% CMC for 5 days, and a 1:1 mixture of CCl_4 and olive oil (2 mL/kg bodyweight, s.c.) on days 2 and 3. Test group animals were administered 80% methanolic extract of cornelian cherry leaves at a dose of 100, 200 and 500 mg/kg

bodyweight for 5 days. Additionally, they received a dose of 1:1 CCl_4 and olive oil (2 mL/kg bodyweight, s.c.) on days 2 and 3, 30 min after the administration of extracts. Reference group received 50 mg/kg bodyweight silymarin p.o. for 5 days, and also received 1:1 CCl_4 and olive oil (2 mL/kg bodyweight, s.c.) on days 2 and 3, 30 min after the administration of silymarin. Animals were decapitated on day 6, blood was collected and their livers were dissected for further analysis.

3.2.2.2.4. Preparation of Total Blood Samples

Total blood samples were prepared according to the method described previously (1).

After the decapitation of animals, fresh blood was collected into the tubes containing EDTA. Each blood sample was centrifuged for 10 min at 4000 *g* and 4 °C. After removal of plasma and buffy coats, erythrocytes were washed three times with two volumes of isotonic saline. Then, erythrocytes were lysed with cold distilled water (1:4), stored in refrigerator at 4 °C for 15 min and the cell debris were removed by centrifugation (2000 *g* for 10 min).

3.2.2.2.5. Preparation of Liver Homogenates

After the decapitation process, liver of each rat was removed and frozen immediately on dry ice. The frozen tissues were stored at –80 °C until further use. 10% (w/v) homogenates were prepared by mincing and homogenizing 1 g of liver in 9 mL ice cold KCl solution (1.15%) with a glass homogenizer. The homogenate was centrifuged at 4400 *g* and 4 °C for 15 min. The resultant supernatant was used for the determination of enzyme activities and lipid peroxidation.

3.2.2.2.6. Determination of Protein Content in Total Blood Samples or Liver Tissues

The protein content of the samples was determined according to the method developed Lowry et al. (211), also known as “Lowry Method”. It is a copper-based assay depending on biuret reaction. In this reaction, peptides containing three or more amino acid residues form a colored chelate complex

with cupric ions (Cu^{2+}) in an alkaline environment containing sodium potassium tartrate. This complex transfers electrons and therefore, reduces the Folin Reagent, producing an intense blue color, which can be observed at 750 nm.

The Chemicals and Reagents

- Alkaline Copper Reagent
  - A. Na_2CO_3 (in 0.1 N NaOH) 2 %
  - B. $CuSO_4$. $5H_2O$ 0.5 %
  - C. Sodium potassium tartrate 1 %

  Alkaline Copper Reagent is prepared by mixing 50 mL A, 1 mL B and 1 mL C immediately before use.
- Folin-Ciocalteu Reagent 1:1 diluted
- Bovine Serum albumin

The Procedure

200 µL of total blood or liver tissue homogenate was mixed with 1000 µL of Alkaline Copper Reagent. The mixture was vortexed and kept at room temperature for 10 min. Later, 100 µL of diluted Folin Reagent was added to the mixture and incubated at room temperature for another 30 min. At the end of the period, the absorbance was read at 750 nm. A standard curve was prepared using different concentrations of bovine serum albumin solutions (100, 200, 500, 750, 1000 µg/mL).

3.2.2.2.7. Determination of Total Antioxidant Capacity of Total Blood Samples and Tissue Homogenate

The same method described in the section 3.2.2.1.11 was used here with the only exception of using erythrocyte lysates or tissue homogenates instead of plant samples. The results were expressed as µM Trolox equivalent per mg protein.

3.2.2.2.8. Antioxidant Enzyme Activities

3.2.2.2.8.1. Determination of CuZnSOD Activity

CuZnSOD activity was measured by the same method described in the section 3.2.2.1.5. The only difference was total blood or tissue homogenate was used instead of plant material or reference substance.

The total blood was diluted 400 times, and the tissue homogenate was diluted 40 times before the assay. CuZnSOD containing standard solutions in the concentrations of 0.217, 0.650, 1.30, 2.60, 5.20 U/mL was used for the calibration curve.

The calculations were also performed as mentioned in the previous section.

3.2.2.2.8.2. Determination of GSH-Px Activity

GSH-Px activity was determined according to the method described by Pleban et al. (212). Basically, reduced glutathione (GSH) is converted to oxidized glutathione (GSSG) by GSH-Px in the presence of *tert*-butyl hydroperoxide. The generated GSSG is re-converted to GSH by glutathione reductase. During this process NADPH is consumed; therefore decrease in the NADPH concentration is expected.

The Chemicals and Reagents

- The reaction mixture
  - Trizma buffer — 50 mM, pH 7.6
  - EDTA — 1 mM
  - GSH — 2 mM
  - NADPH — 0.2 mM
  - Sodium azide — 4 mM
  - Glutathione reductase — 1000 U/L
- *tert*-Butyl hydroperoxide — 1:1000 diluted

The Procedure

Total blood was diluted 15 times, and tissue homogenate was diluted 17 times before the assay. 990 µL of the reaction mixture was pipetted into a 1 mL-cuvette. Then, 10 µL total blood or tissue homogenate is added and the mixture was incubated for 5 min at room temperature. The reaction was initiated with the addition of 10 µL of *tert*-butyl hydroperoxide and the decrease in NADPH absorbance was followed at 340 nm for 3 min. The difference in absorbance per minute is calculated. The calibration curve was plotted with standard GSH-Px solutions in the concentration of 0.1, 0.2, 0.3, 0.4, 0.5 U/mL.

3.2.2.2.8.3. Determination of CAT Activity

As mentioned earlier, catalase converts H_2O_2 to H_2O and molecular oxygen. Catalase activity was determined according to the method developed by Aebi (213). The principal of this method relies on the conversion of H_2O_2 by CAT in a time-dependent manner. The decrease in the absorbance of H_2O_2 can be monitored at 240 nm.

The Chemicals and Reagents

- Phosphate buffer 50 mM, pH 7.0
- Hydrogen peroxide solution 30 mM

The Procedure

Prior to the test, the sample (either total blood or tissue homogenate) was diluted 5000 times. 2000 µL of diluted sample was transferred in a glass cuvette and 1000 µL of hydrogen peroxide solution was added. The reduction rate of H_2O_2 was followed at 240 nm for 60 seconds at room temperature. For a blank test, 1000 µL of buffer was added to the same amount of sample and the same procedure was followed. For the calibration curve, standard CAT solution was prepared in a concentration range of 0.01 – 0.035 KU/mL. The inhibition percentage was calculated as follows:

$$\text{Inhibition \%} = 100 - \frac{\Delta A_{samp/std}\ /\text{min} \times 100}{\Delta A_{blank}\ /\text{min}}$$

The calibration curve was plotted with the percent inhibition of standard solutions against the logarithm of their concentrations.

3.2.2.2.9. Determination of Malondialdehyde Level

As already mentioned, malondialdehyde is an end–product and therefore, a reliable sign of lipid peroxidation. The principle of the assay depends on the reaction of MDA with thiobarbituric acid (TBA) and forming pink colored MDA–TBA complex. The formation of this complex can be observed at 532 nm. The MDA levels were determined in total blood according to the method described by Aydin et al. (1) and in tissues by Jamall and Smith (214).

A) Determination of MDA in Total Blood

The Chemicals and Reagents

- Trichloroacetic acid 15%
- Phosphate buffer 330 mM, pH 7.5
- EDTA 100 mM
- Thiobarbituric acid (in 0.05 N NaOH) 1%

The Procedure

250 µL of each of total blood samples, phosphate buffer and trichloroacetic acid were mixed in 1.5 mL–eppendorf tubes. The mixtures were vortexed and kept on ice bags in refrigerator (4 °C) for 2 h. At the end of the period, the tubes were centrifuged at 4400 rpm and 4 °C for 10 min. 500 µL of the supernatant was mixed with 38 µL of EDTA and 125 µL of TBA. The mixtures were vortexed and kept on boiling water bath for 15 min. The absorbance was recorded at 532 nm. The blank test was prepared by using 500 µL of phosphate buffer instead of supernatant under the same conditions. 1,1,3,3-tetramethoxypropane was used as standard for plotting the calibration curve. The standard solutions were prepared in a concentration range of 0.76 – 3.04 nmol/mL. The results were given as nmol/mg protein.

B) Determination of MDA in Tissues

The Chemicals and Reagents

- Sodium dodecyl sulfate (SDS) 8.1 %
- Acetic acid sol. (pH 3.5) 20 %
- Thiobarbituric acid 0.8 %

The Procedure

In a closed-cap polypropylene tube, 200 µL of sample or standard substance, 200 µL of sodium dodecyl sulfate, 1500 µL of acetic acid solution and 1500 µL of thiobarbituric acid solution were mixed. The volume was completed to 4000 µL with Milli Q water. The tubes were kept at 95 °C for 1 h. At the end of this period, they were cooled under tap water. Later, 2000 µL of the mixture was added to 2000 µL of trichloroacetic acid. They were centrifuged at 1000 *g* for 10 min. The absorbance of the supernatant was read at 532 nm. The same calibration curve that was used for total blood MDA assay was also used here. The results were given as nmol/mg protein.

3.2.2.3. Quantitative Analysis of Gallic Acid in *C. mas* leaves by HPTLC

The amount of gallic acid in the 80% methanolic extract of *C. mas* leaves was quantitatively measured by high performance thin layer chromatography according to the validated method of Sonawane et al. (215). Chromatographic separation was performed on Merck glass TLC plates precoated withsilica gel 60 F_{254}. The HPTLC system (Camag, Hamilton, Switzerland) consisted of TLC scanner 3 connected to a PC running WinCATS software and Linomat V sample applicator using 100 µL syringes and connected to a nitrogen tank. After the application process, the plates were developed to 8 cm in a twin through glass chamber (20 x 10 cm) presaturated with the mobile phase. Scanning was performed using Camag TLC Scanner 3 at 254 nm and operated by WinCATS software.

Chromatographic Conditions

Mobile phase: Dichloromethane: Ethyl acetate: Formic acid (15: 12: 1)

Band length: 6 mm

Distance between bands: 10 mm

Gas flow: 100 nl/s

Slit dimension: 6.00 mm x 0.30 mm

Data resolution: 100 µm/step

Micro scanning speed: 20 mm/s

Preparation of Standard and Sample Solution

100 mg of 80% methanolic extract of *C. mas* leaves prepared as explained in section 3.2.1.1 was dissolved in 10 mL of methanol to obtain a final concentration of 10 mg/mL. 5 µL of sample solution was applied in 5 different spots.

Calibration Curve of Gallic Acid

2 stock solutions gallic acid were prepared in methanol. The concentration of the first stock solution was 0.02 mg/mL and that of second stock solution was 0.1 mg/mL. 5 and 10 µL of stock solution 1 and 4, 6, 9, 12 µL of stock solution 2 were spotted on TLC plates to obtain amounts of 100, 200, 400, 600, 900 and 1200 ng per spot of gallic acid.

Derivatization Agents

After the development process, the plates were dipped into two different derivatization agents, subsequently:

a) Naturschtoff Reagenz A (NA): 1 g of 2-aminoethyl diphenyl borinate is dissolved in 200 mL EtOAc.

b) Polyethyleneglycol 400 (PEG 400): 10 g of PEG 400 was dissolved in 200 mL CH_2Cl_2.

3.2.2.4. Statistics

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

4. RESULTS

4.1. Results of *In vitro* Activity Studies

4.1.1. Total Phenolic Content of Fruit Extracts

The total phenolic contents of 80% methanolic extracts prepared as described previously are given in Table 25, Figure 6. Among the three fruits analyzed, Japanese persimmon showed the highest total phenolic content. However, among the analyzed samples, the highest total phenolic content was found in the leaves of Cornelian cherry.

Table 25: The total phenolic content of 80% methanolic extracts of the examined plants

	Cornelian cherry	Japanese persimmon	Cherry laurel	Cornelian cherry leaves
Total phenolic content[A]	31.25 ± 1.79[B,a]	66.97 ± 2.41[b]	23.64 ± 0.84[c]	342.6 ± 10.71[d]

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

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

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

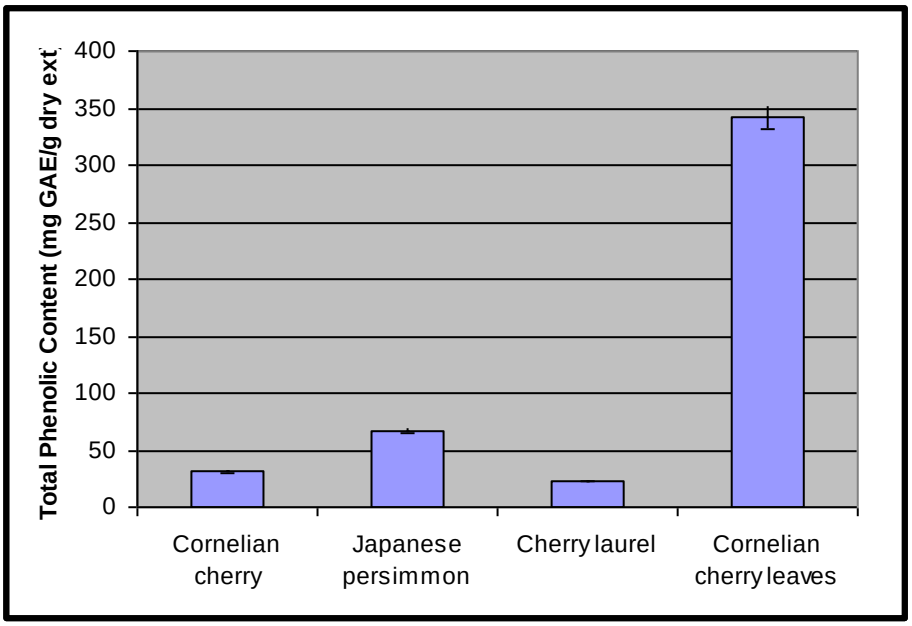


Figure 6: Total phenolic content of 80% methanolic extracts. The results are expressed as mg gallic acid equivalent per g dry extract.

4.1.2. Total Flavonoid Content of Fruit Extracts

The total flavonoid contents of 80% methanolic extracts are given in Table 26, Figure 7. The results indicated that Japanese persimmon had the highest total flavonoid content among fruits; however it was lower than the content of cornelian cherry leaves.

Table 26: The total flavonoid content of 80% methanolic extracts of the examined plants

	Cornelian cherry	Japanese persimmon	Cherry laurel	Cornelian cherry leaves
Total flavonoid content[A]	20.5 ± 1.62[B,a]	32.39 ± 0.79[b]	16.87 ± 0.38[c]	72.83 ± 3.12[d]

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

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

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

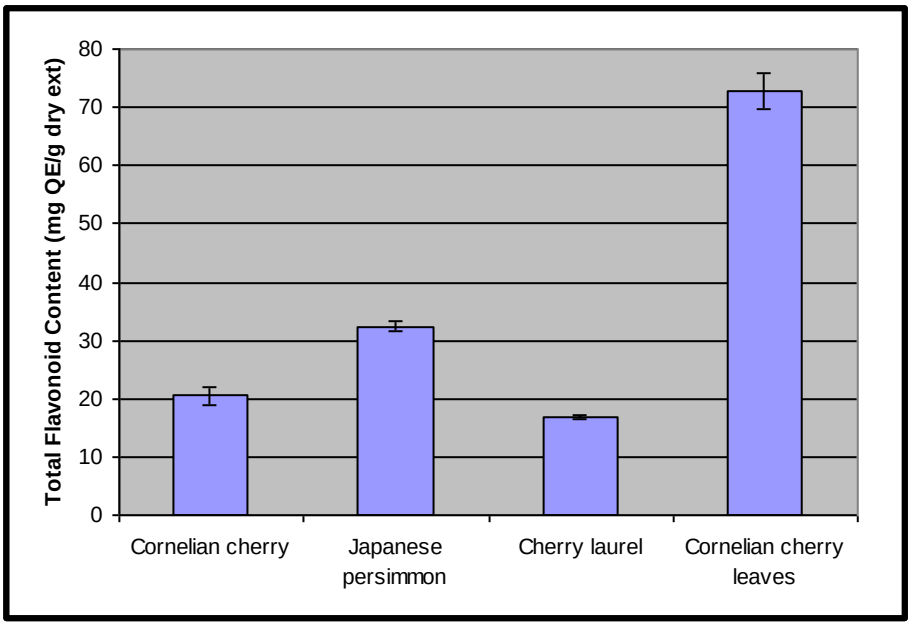


Figure 7: Total flavonoid content of 80% methanolic extracts. The results are expressed as mg quercetin equivalent per g dry extract.

4.1.3. The Total Proanthocyanidin Content of Fruit Extracts

The total proanthocyanidin contents of 80% methanolic extracts are given in Table 27, Figure 8. The results indicated Japanese persimmon had the highest total proanthocyanidin content. However, the total proanthocyanidin content of cornelian cherry was lower than cherry laurel, which is in contrast with both total phenolic and flavonoid contents. The results were expressed as epigallocathecin gallate equivalents (EGCG-E) per g dry extract.

Table 27: The total proanthocyanidin content of 80% methanolic extracts of the examined plants.

	Cornelian cherry	Japanese persimmon	Cherry laurel	Cornelian cherry leaves
Total proanthocyanidin content[A]	229 ± 16.9[B,a]	646 ± 24.1[b]	342 ± 16.3[c]	298 ± 11.83[d]

[A] Total proanthocyanidin content was expressed as mg epigallocathecin gallate equivalents (EGCG-E) in 1 g dry extract.

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

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

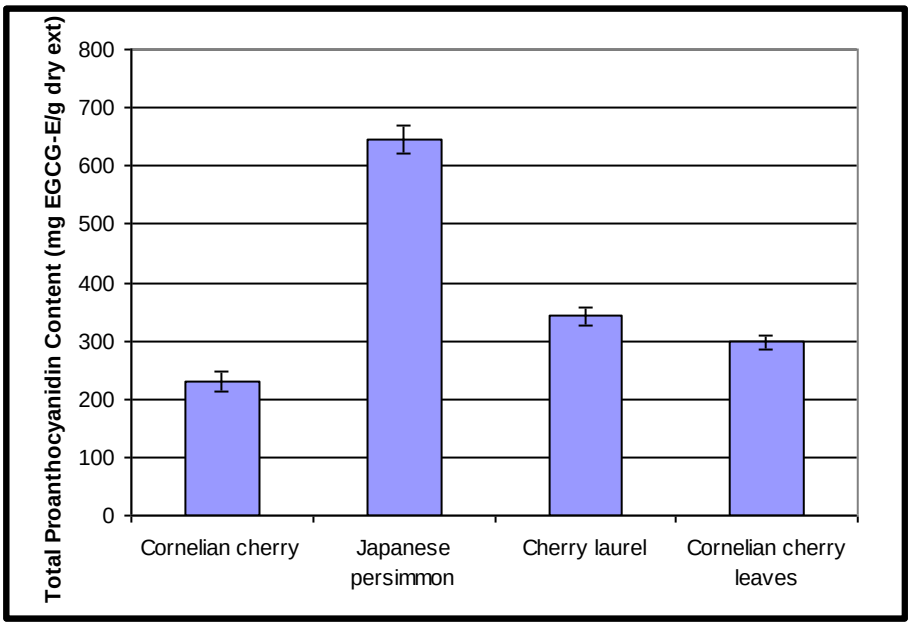


Figure 8: Total proanthocyanidin content of 80% methanolic extracts. The results are expressed as mg epigalocathecin gallate equivalents (EGCG-E) per g dry extract.

4.1.4. DPPH Radical Scavenging Activity

DPPH radical scavenging activities of 80% methanolic extracts of fruits are given in Table 28, Figure 9. The results are expressed as “half maximal effective concentration” (EC_{50}). The lowest EC_{50} value, therefore the highest activity, belonged to Japanese persimmon. However, none of the fruits

analyzed showed as much activity as the reference compound, BHT. But cornelian cherry leaves showed nearly as much activity as BHT did.

Table 28: DPPH radical scavenging activity of 80% methanolic extracts of the examined plants.

	Cornelian cherry	Japanese persimmon	Cherry laurel	Cornelian cherry leaves	BHT*
DPPH radical scavenging activity[A]	725 ± 17.5[B,a]	440 ± 12.1[b]	795 ± 15.8[c]	165 ± 10.2[d]	133 ± 6.4[e]

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

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

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

* Butylated hydroxytoluene

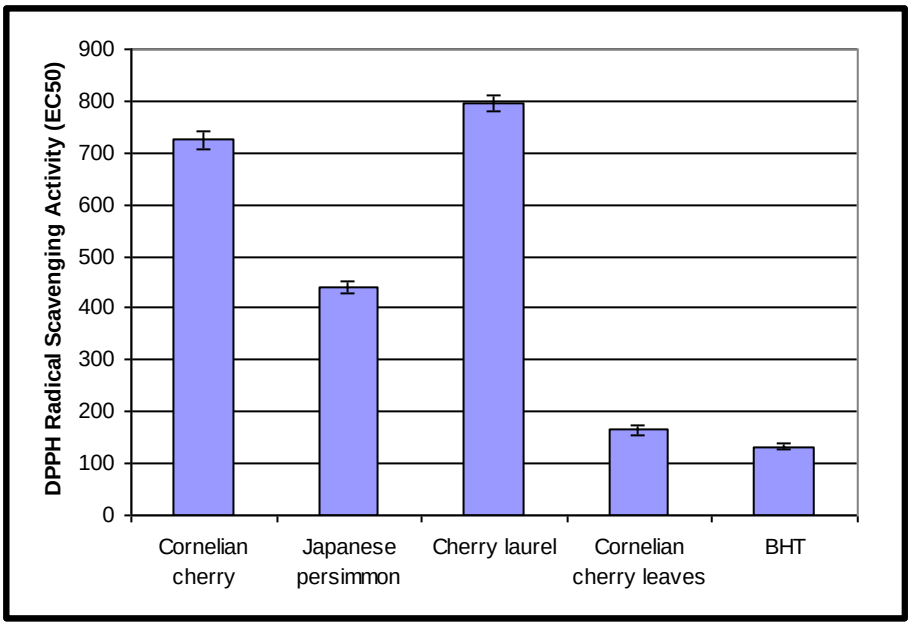


Figure 9: DPPH radical scavenging activity of 80% methanolic extracts. The results are expressed as EC_{50} in µg/mL.

4.1.5. Superoxide Radical Scavenging Activity

Superoxide radical scavenging activities of 80% methanolic extracts of fruits are given in Table 29, Figure 10. The results were expressed as “half maximal effective concentration” (EC_{50}). The lowest EC_{50} value, therefore the highest activity, belonged to Japanese persimmon. However, none of the fruits analyzed showed as much activity as the reference compound, gallic acid. The activity of cornelian cherry leaves was much higher than those of fruits. But it was, again, lower than gallic acid.

Table 29: Superoxide radical scavenging activity of 80% methanolic extracts of the examined plants.

	Cornelian cherry	Japanese persimmon	Cherry laurel	Cornelian cherry leaves	Gallic acid
Superoxide radical scavenging activity[A]	11.61 ± 0.48[B,a]	7.24 ± 0.29[b]	13.8 ± 0.52[c]	1.59 ± 0.27[d]	0.18 ± 0.01[e]

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

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

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

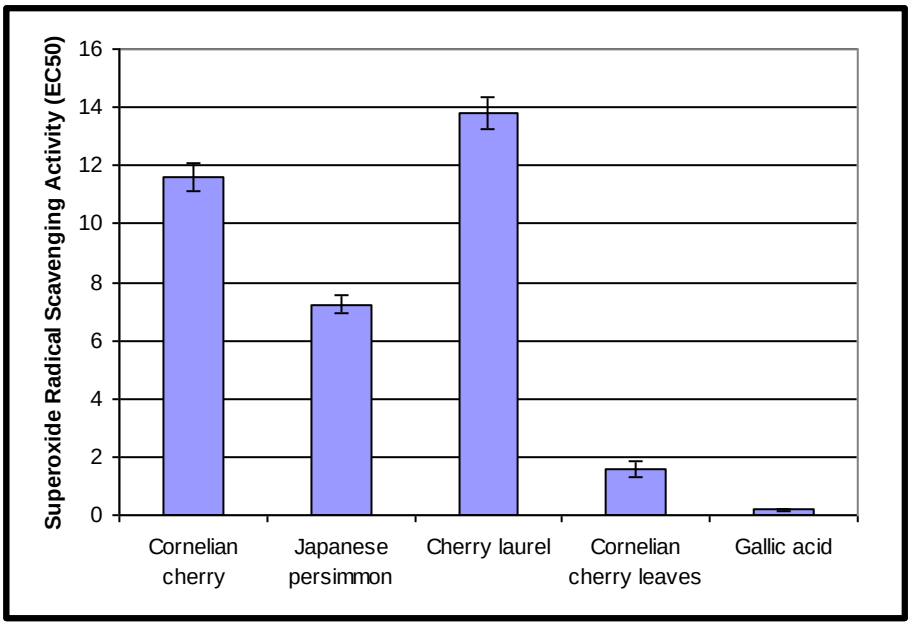


Figure 10: Superoxide radical scavenging activity of 80% methanolic extracts. The results are expressed as EC_{50} in mg/mL.

4.1.6. Ferric Reducing Antioxidant Power Assay (FRAP)

The FRAP results are given in Table 30, Figure 11 and expressed as mM $FeSO_4$ equivalents per g dry extract. All of the 80% methanolic extracts had an appreciable ferric reducing antioxidant power in comparison with the reference compound, BHT. Japanese persimmon had the highest reducing power, while cornelian cherry showed the least. Surprisingly, 80% methanolic extract of cornelian cherry leaves was higher than the reference compound, itself.

Table 30: FRAP of 80% methanolic extracts of the examined plants.

	Cornelian cherry	Japanese persimmon	Cherry laurel	Cornelian cherry leaves	BHT*
Ferric reducing antioxidant power[A]	0.42 ± 0.01[B,a]	1.25 ± 0.04[b]	0.57 ± 0.02[c]	3.44 ± 0.09[d]	3.02 ± 0.07[e]

[A] FRAP activity was expressed as mM $FeSO_4$ equivalents in 1 g dry extract.

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

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

* Butylated hydroxytoluene

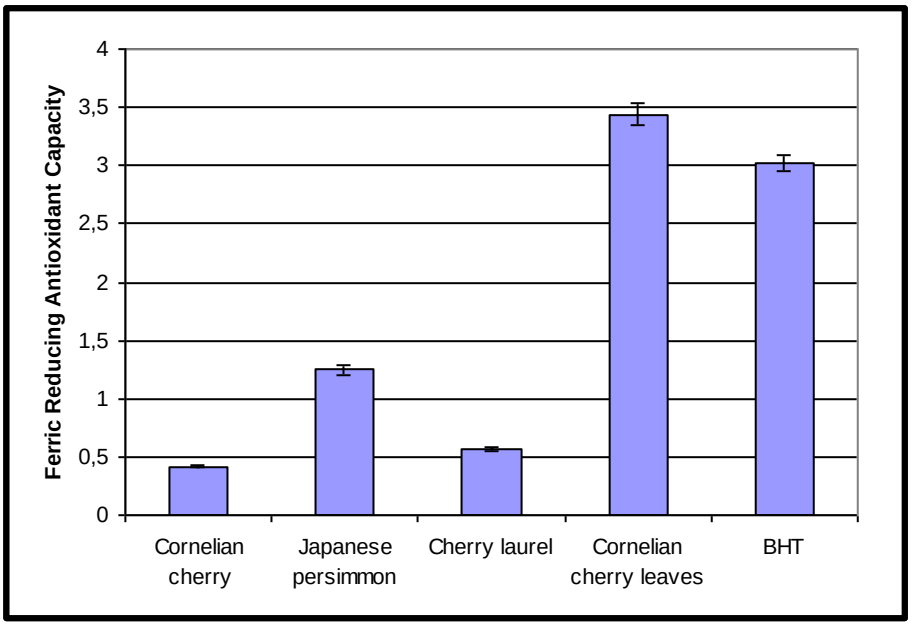


Figure 11: FRAP of 80% methanolic extract. The results are expressed as mM $FeSO_4$ equivalents in 1 g dry extract or compound.

4.1.7. Cupric Reducing Antioxidant Capacity (CUPRAC)

Cupric reducing antioxidant capacities of 80% methanolic extracts of fruits are given in Table 31, Figure 12. The results were expressed as mg ascorbic acid equivalents per g dry extract. According to these results, Japanese persimmon had the highest capacity, while cornelian cherry had the least among fruits. But the capacity of cornelian cherry leaves was much higher.

Table 31: CUPRAC of 80% methanolic extracts of the examined plants.

	Cornelian cherry	Japanese persimmon	Cherry laurel	Cornelian cherry leaves
Cupric reducing antioxidant capacity[A]	20.9 ± 0.1[B,a]	71.0 ± 0.14[b]	23.7 ± 0.11[c]	214.45 ± 2.15[d]

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

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

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

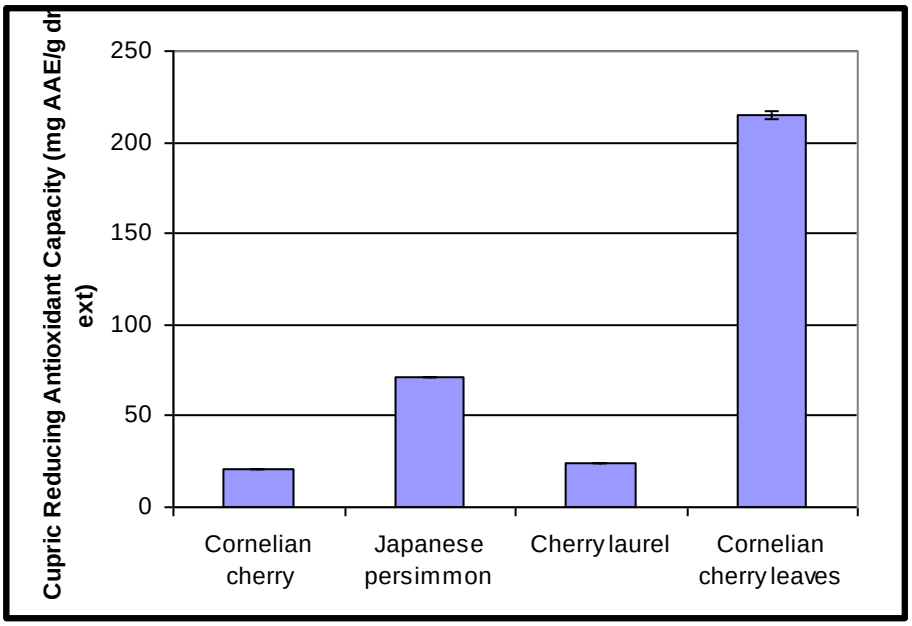


Figure 12: CUPRAC of 80% methanolic extracts. The results are expressed as mg ascorbic acid in 1 g dry extract.

4.1.8. Ferrous Ion Chelating Capacity

The metal chelating activity of the extracts was investigated using three different concentrations, such as 1, 2, 5 mg/mL. We found no activity of either extracts at the mentioned concentrations. EDTA, a well known chelating agent, was used as reference compound. Its EC_{50} value was measured as 9.1 ± 0.4 µg/mL.

4.1.9. Bleaching assay of β-carotene in a linoleic acid system

β-carotene bleaching activity of the extracts are given in Table 32, Figure 13. The results were given as "% activity" in 1 mg/mL extract or reference compound. According to these results, Japanese persimmon showed the highest activity, while cherry laurel showed the least among fruits, while cornelian cherry leaves as much activity as the reference compound, BHT, did.

Table 32: β-carotene bleaching activity of 80% methanolic extracts of the examined plants.

	Cornelian cherry	Japanese persimmon	Cherry laurel	Cornelian cherry leaves	BHT*
β-carotene bleaching activity [A]	65 ± 1.6[B,a]	75 ± 3.5[b]	61 ± 0.9[c]	93 ± 2.4[d]	96 ± 2.6[d]

[A] β-carotene bleaching activity was expressed as "% activity" in 1 mg/mL extract or compound.

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

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

* Butylated hydroxytoluene

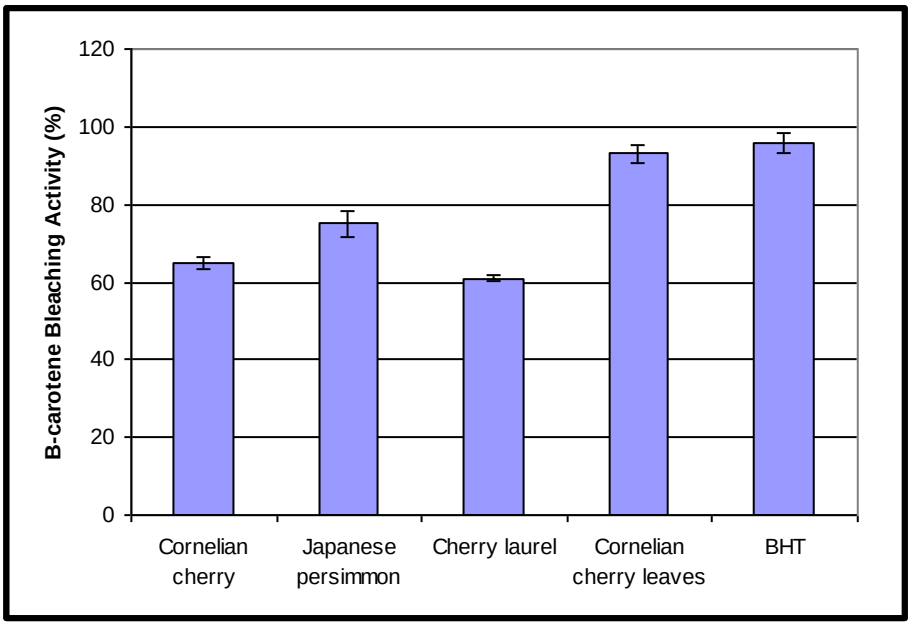


Figure 13: β-carotene bleaching activity of 80% methanolic extracts. The results were expressed as "% activity" in 1 mg/mL extract or compound.

4.1.10. Total Antioxidant Capacity

Total antioxidant capacities of 80% methanolic extracts are given in Table 33, Figure 14. The results were expressed as mg ascorbic acid equivalents per g dry extract. According to these results, Japanese persimmon

had the highest capacity, while cornelian cherry had the least among fruits. But the capacity of cornelian cherry was much higher.

Table 33: Total antioxidant capacities of 80% methanolic extracts of the examined plants.

	Cornelian cherry	Japanese persimmon	Cherry laurel	Cornelian cherry leaves
Total antioxidant capacity[A]	66.06 ± 3.19[B,a]	94.61 ± 4.83[b]	50.74 ± 1.49[c]	320.17 ± 10.10[d]

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

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

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

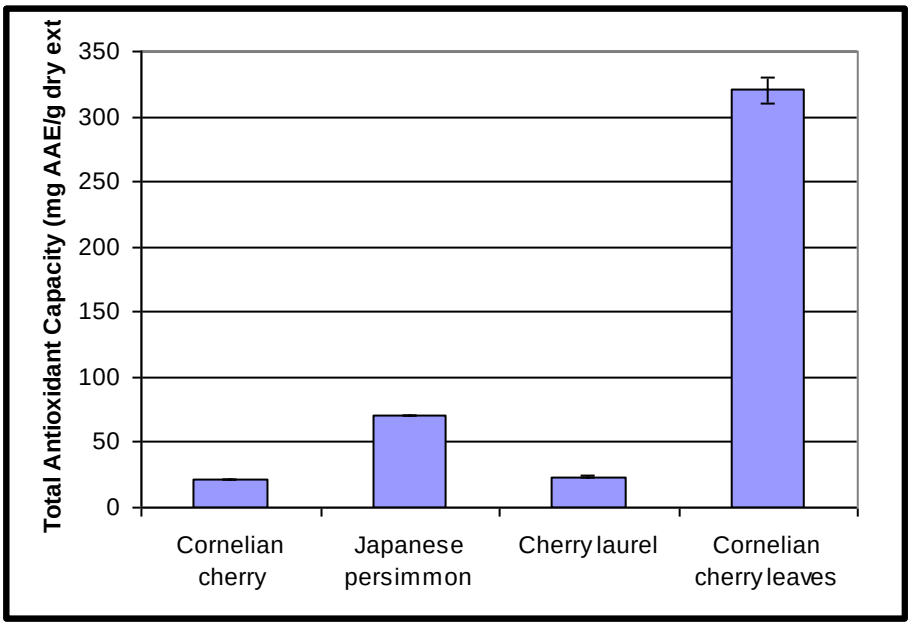


Figure 14: Total antioxidant capacities of 80% methanolic extracts. The results are expressed as mg ascorbic acid in 1 g dry extract.

4.1.11. Trolox Equivalent Antioxidant Capacity (TEAC)

Trolox equivalent antioxidant capacities of 80% methanolic extracts are given in Table 34, Figure 15. The results were expressed as μM Trolox equivalents per g dry extract.

Table 34: Trolox equivalent antioxidant capacities of 80% methanolic extracts of the examined plants.

	Cornelian cherry	Japanese persimmon	Cherry laurel	Cornelian cherry leaves
Trolox equivalent antioxidant capacity[A]	103 ± 8.9[B,a]	267 ± 8.9[b]	147 ± 3.4[c]	869 ± 20.52[d]

[A] Total antioxidant activity was expressed as μM Trolox equivalents (TE) in 1 g dry extract.

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

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

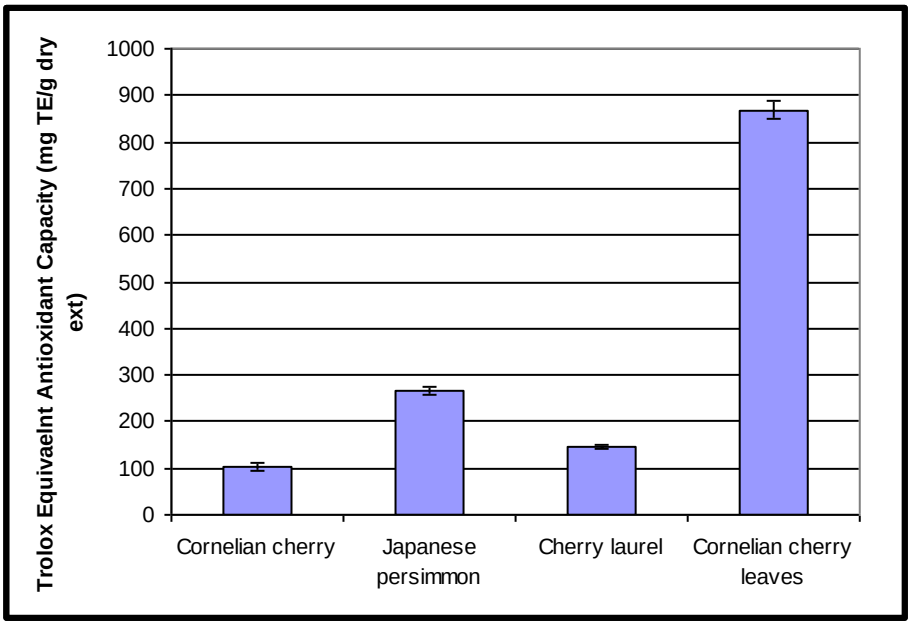


Figure 15: Trolox equivalent antioxidant capacities of 80% methanolic extracts. The results are expressed as μM Trolox equivalents (TE) in 1 g dry extract.

4.1.12. Correlation Between Total Phenolic/Flavonoid/ Proanthocyanidin Contents and Antioxidant Activity

The correlation between phenolic contents and *in vitro* antioxidant properties by using Pearson correlation coefficient, and the results are given in Table 35.

Table 35: Pearson's correlation coefficients (*r*) between antioxidant parameter and total phenolic, flavonoid and proanthocyanidin content.

	Total Phenol Content	Total Flavonoid Content	Total Proanthocyanidin Content
DPPH radical scavenging activity	0.9998*	0.9993*	0.8907
Superoxide radical scavenging activity	0.9992	0.9995	0.8916
FRAP	0.9273*	0.8714	0.9943*
CUPRAC	0.9729*	0.9362	0.9769*
β-Carotene bleaching activity	0.9204	0.7534	0.7597
TOAC	0.9248	0.9912*	0.6647
TEAC	0.8825	0.8631	0.8903

* $p < 0.05$

4.2. Results of *In vivo* Activity Studies

4.2.1. Results on Total Blood of Healthy Rats

4.2.1.1. Total Blood CuZnSOD Activities of Healthy Rats

CuZnSOD activities of healthy rats after 21-day treatment are given in Table 36, Figure 16. No statistically significant change was observed in either animals administered 80% methanolic plant extracts or the reference compound silymarin ($p > 0.05$).

Table 36: Total Blood CuZnSOD activities in healthy rats

	Control	Cornelian cherry	Japanese Persimmon	Cherry laurel	Cornelian cherry leaves	Silymarin
Total blood CuZnSOD activities[A]	120.9 ± 11.1[B]	119.63 ± 14.29	123.35 ± 8.9	119.6 ± 11.41	126.5 ± 9.84	125.9 ± 13.76

[A] CuZnSOD activities were expressed as U/mg protein.

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

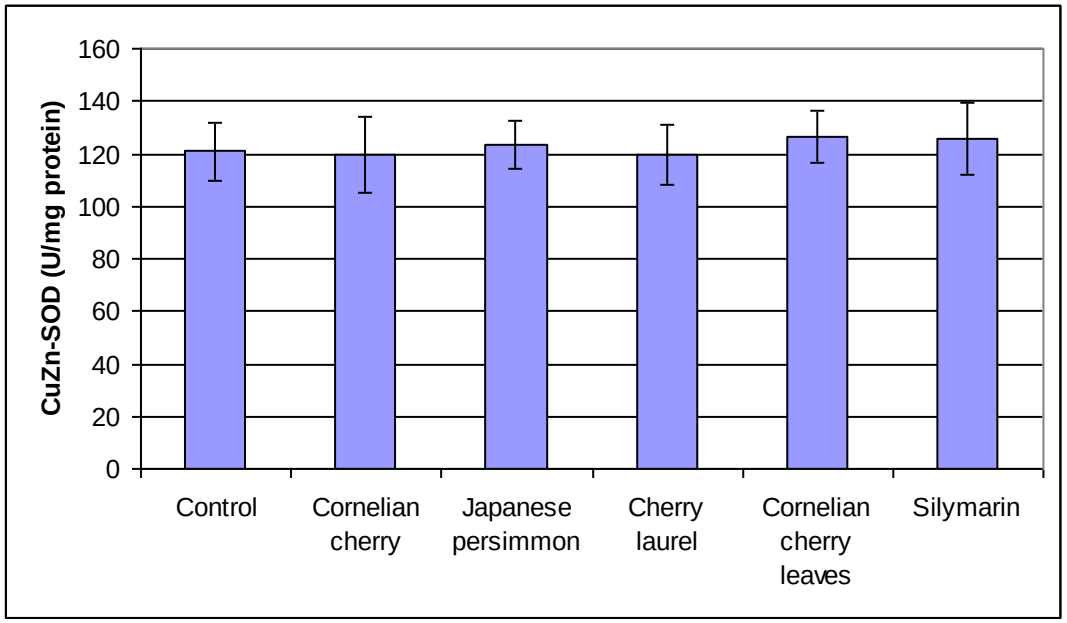


Figure 16: Total blood CuZnSOD activities of healthy rats treated with 80% methanolic extracts of plants. The results were expressed as the mean of triplicates ± standard deviation.

4.2.1.2. Total Blood GSH-Px Activities of Healthy Rats

GSH-Px activities of healthy rats after 21-day treatment are given in Table 37, Figure 17. No statistically significant change was observed in either animals administered 80% methanolic extracts of plants or the reference compound silymarin ($p > 0.05$).

Table 37: Total blood GSH-Px activities in healthy rats

	Control	Cornelian cherry	Japanese Persimmon	Cherry laurel	Cornelian cherry leaves	Silymarin
Total blood GSH-Px activities[A]	76.2 ± 9.4[B]	75.32 ± 5.54	75.15 ± 8.9	75.14 ± 4.73	78.27 ± 8.15	79.2 ± 7.5

[A] GSH-Px activities were expressed as U/mg protein.

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

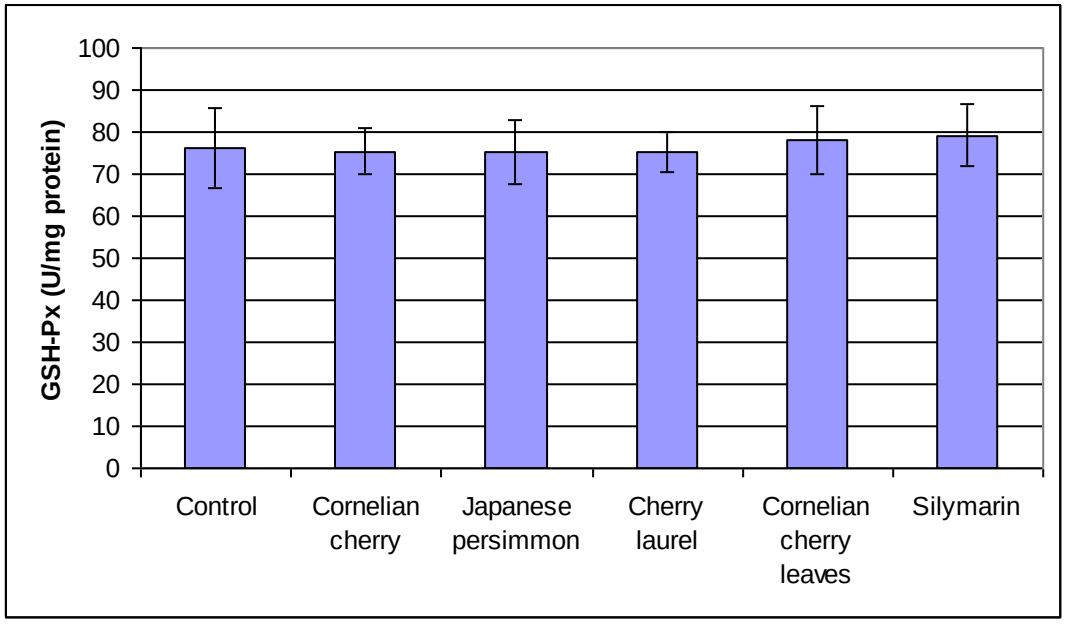


Figure 17: Total blood GSH-Px activities of healthy rats treated with 80% methanolic extracts of plants. The results were expressed as the mean of triplicates ± standard deviation.

4.2.1.3. Total Blood CAT Activities in Healthy Rats

CAT activities in healthy rats after 21-day treatment are given in Table 38, Figure 18. No statistically significant change was observed in either of the animals administered 80% methanolic extracts of plants or the reference compound silymarin ($p > 0.05$).

Table 38: Total blood CAT activities in healthy rats

	Control	Cornelian cherry	Japanese Persimmon	Cherry laurel	Cornelian cherry leaves	Silymarin
Total blood CAT activities[A]	434.13 ± 24.21[B]	431.25 ± 24.59	442.34 ± 45.2	436.71 ± 24.3	430.81 ± 30,81	440.03 ± 36.2

[A] CAT activities were expressed as U/mg protein.

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

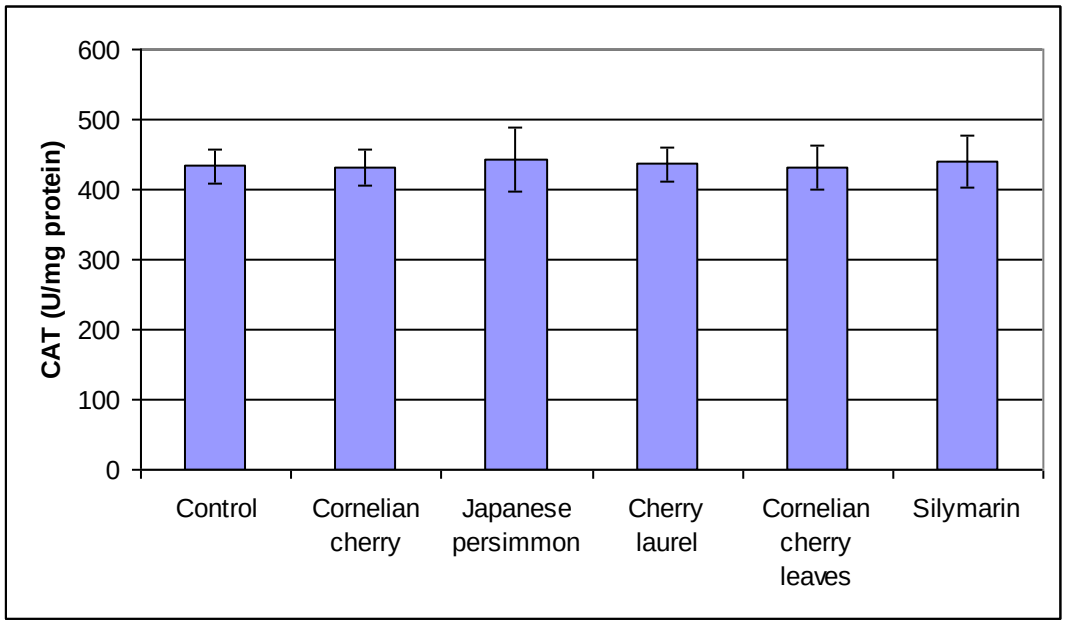


Figure 18: Total blood CAT activities of healthy rats treated with 80% methanolic extracts of plants. The results were expressed as the mean of triplicates ± standard deviation.

4.2.1.4. Total Blood MDA Levels in Healthy Rats

MDA levels in healthy rats after 21-day treatment are given in Table 39, Figure 19. No statistically significant change was observed in animals administered 80% methanolic fruit or leaf extracts or silymarin ($p > 0.05$).

Table 39: Total blood MDA levels in healthy rats

	Control	Cornelian cherry	Japanese Persimmon	Cherry laurel	Cornelian cherry leaves	Silymarin
Total blood MDA levels[A]	3.53 ± 0.34[B]	3.49 ± 0.31	3.40 ± 0.20	3.54 ± 0.47	3.32 ± 0.33	3.39 ± 0.36

[A] MDA levels were expressed as nmol/mg protein.

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

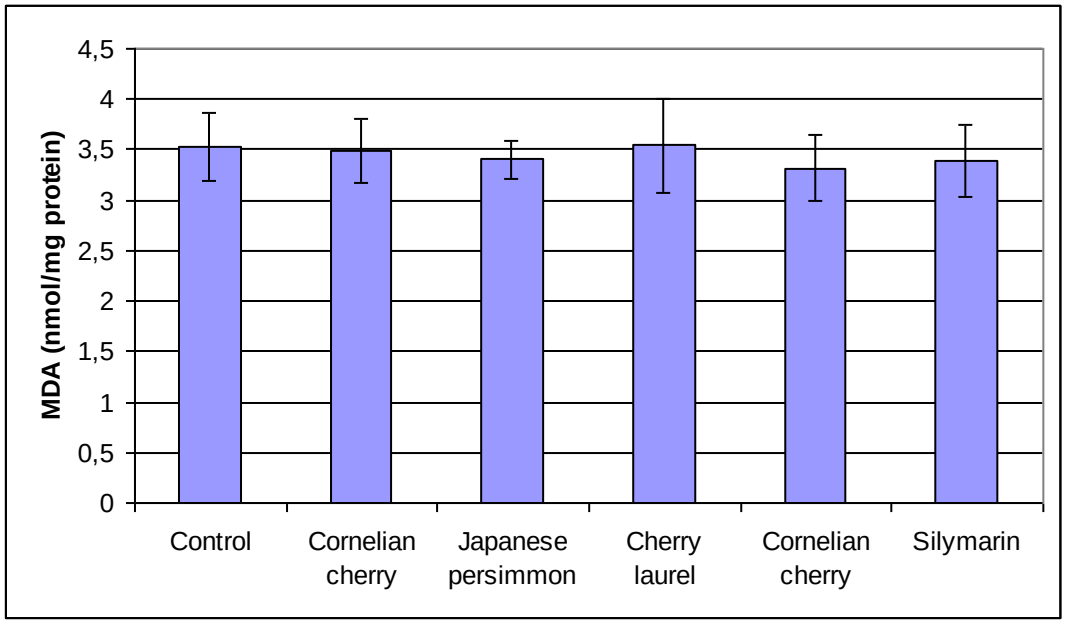


Figure 19: Total blood MDA levels of healthy rats treated with 80% methanolic extracts of plants. The results were expressed as the mean of triplicates ± standard deviation.

4.2.1.5. Total Blood TEAC Levels in Healthy Rats

TEAC levels of healthy rats after 21-day treatment are given in Table 40, Figure 20. Among the groups, cornelian cherry leaves ($p < 0.01$) and silymarin ($p < 0.001$) groups showed statistically significant increases in TEAC levels compared to the control group. In fact, the increase in cornelian cherry leaves

group (20.51%) was very close to the increase in silymarin group (25.38%). Although the fruits of Japanese persimmon, cornelian cherry and cherry laurel showed numerical increase, the differences were not statistically significant ($p > 0.05$).

Table 40: Total blood TEAC levels in healthy rats

	Control	Cornelian cherry	Japanese Persimmon	Cherry laurel	Cornelian cherry leaves	Silymarin
Total blood TEAC levels[A]	30.38 ± 2.45[B]	30.24 ± 3.52	33.04 ± 1.32	31.25 ± 3.44	36.61 ± 2.15 (20.51%)**	38.09 ± 3.21 (25.38%)***

[A] TEAC levels were expressed as µM Trolox equivalent (TE)/mg protein.

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

** $p < 0.01$ (Compared to the control group)

*** $p < 0.001$ (Compared to the control group)

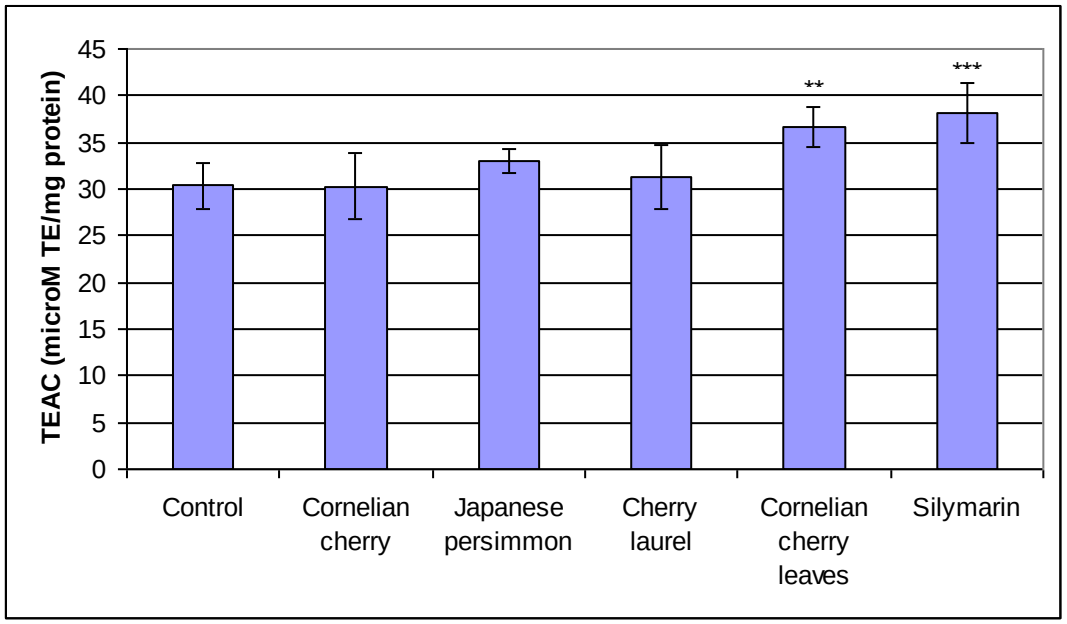


Figure 20: Total blood TEAC levels of healthy rats treated with 80% methanolic extracts. The results were expressed as the mean of triplicates ± standard deviation.

** $p < 0.01$ (Compared to the control group)

*** $p < 0.001$ (Compared to the control group)

4.2.2. Results on Liver Homogenates of Healthy Rats

4.2.2.1. CuZnSOD Activities of Liver Homogenates in Healthy Rats

CuZnSOD activities of liver homogenates in healthy rats after 21-day treatment are given in Table 41, Figure 21. No statistically significant change was observed in either animals administered 80% methanolic plant extracts or the reference compound silymarin ($p > 0.05$).

Table 41: CuZnSOD activities of liver homogenate in healthy rats

	Control	Cornelian cherry	Japanese Persimmon	Cherry laurel	Cornelian cherry leaves	Silymarin
CuZnSOD activities[A]	151.5 ± 17.9[B]	150.8 ± 22.7	151.0 ± 27.3	152.9 ± 11.8	155 ± 21.1	158.4 ± 17.20

[A] CuZnSOD activities were expressed as U/mg protein.

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

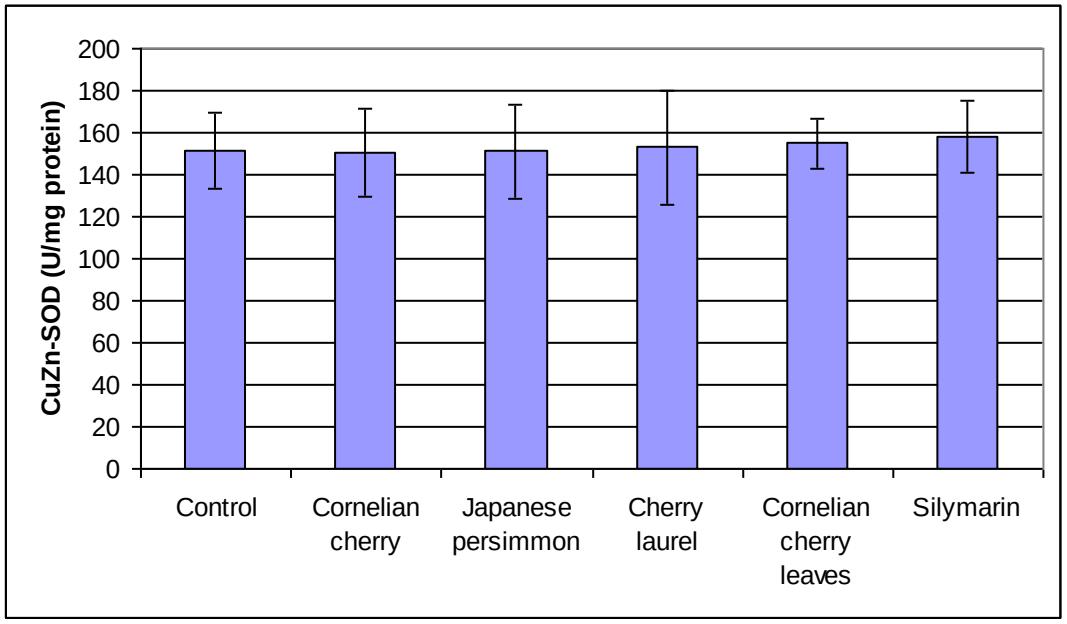


Figure 21: CuZn-SOD activities of liver homogenates in healthy rats treated with 80% methanolic extracts of plants. The results were expressed as the mean of triplicates ± standard deviation.

4.2.2.2. GSH-Px Activities of Liver Homogenates in Healthy Rats

GSH-Px activities of liver homogenates in healthy rats after 21-day treatment are given in Table 42, Figure 22. No statistically significant change was observed in either animals administered 80% methanolic plant extracts or the reference compound silymarin ($p > 0.05$).

Table 42: GSH-Px activities of liver homogenate in healthy rats

	Control	Cornelian cherry	Japanese Persimmon	Cherry laurel	Cornelian cherry leaves	Silymarin
GSH-Px activities[A]	81.5 ± 5.3[B]	78.03 ± 4.40	83.30 ± 4.8	82.37 ± 6.0	84.31 ± 4.73	84.8 ± 5.10

[A] GSH-Px activities were expressed as U/mg protein.

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

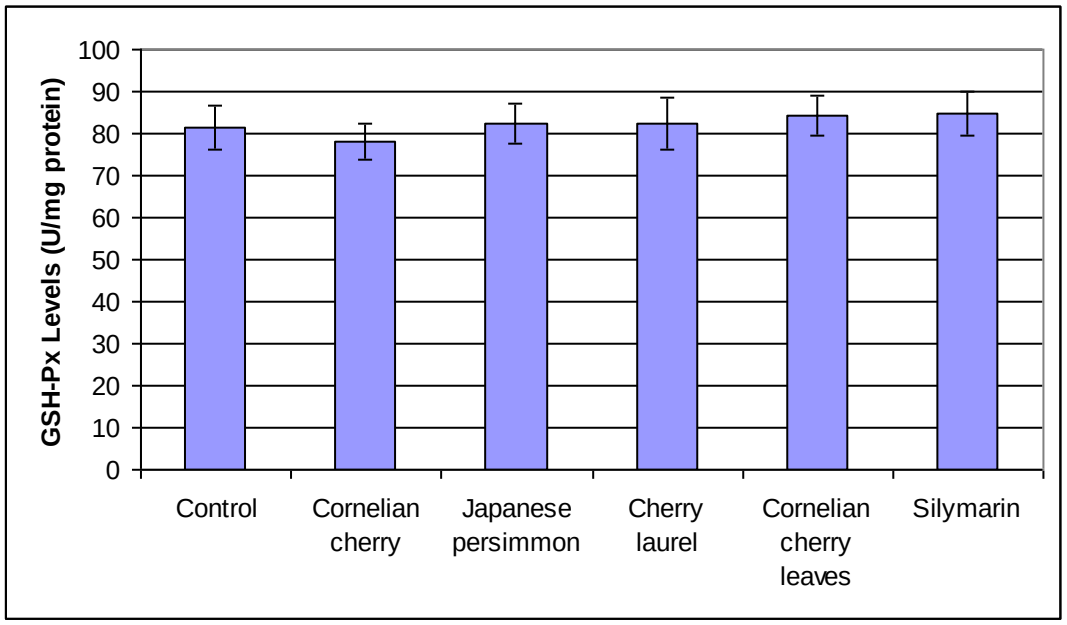


Figure 22: GSH-Px levels of liver homogenates in healthy rats treated with 80% methanolic extracts of plants. The results were expressed as the mean of triplicates ± standard deviation.

4.2.2.3. CAT Activities of Liver Homogenates in Healthy Rats

CAT activities of liver homogenates in healthy rats after 21-day treatment are given in Table 43, Figure 23. No statistically significant change was observed in either animals administered 80% methanolic plant extracts or the reference compound silymarin ($p > 0.05$).

Table 43: CAT activities of liver homogenate in healthy rats

	Control	Cornelian cherry	Japanese Persimmon	Cherry laurel	Cornelian cherry leaves	Silymarin
CAT activities[A]	854.15 ± 42.5[B]	820.31 ± 51.4	835 ± 68.9	799.30 ± 54.8	837 ± 14.3	829.15 ± 26.3

[A] CAT activities were expressed as U/mg protein.

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

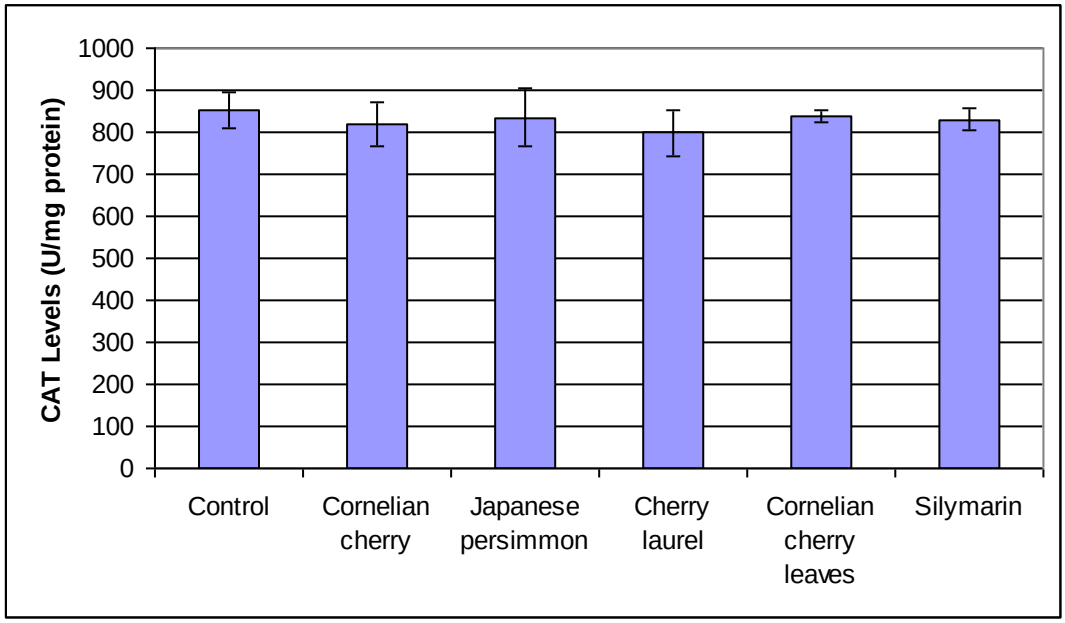


Figure 23: CAT activities of liver homogenates in healthy rats treated with 80% methanolic extracts of plants. The results were expressed as the mean of triplicates ± standard deviation.

4.2.2.4. MDA Levels of Liver Homogenates in Healthy Rats

MDA levels of liver homogenates in healthy rats after 21-day treatment are given in Table 44, 24. No statistically significant change was observed in animals administered 80% methanolic extracts of fruits of leaf ($p > 0.05$). The same result was reached in silymarin group.

Table 44: MDA levels of liver homogenate in healthy rats

	Control	Cornelian cherry	Japanese Persimmon	Cherry laurel	Cornelian cherry leaves	Silymarin
MDA levels[A]	3.89 ± 0.20B	3.75 ± 0.14	3.56 ± 0.32	3.71 ± 0.19	3.60 ± 0.40	3.48 ± 0.28

[A] MDA levels were expressed as nmol/mg protein.

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

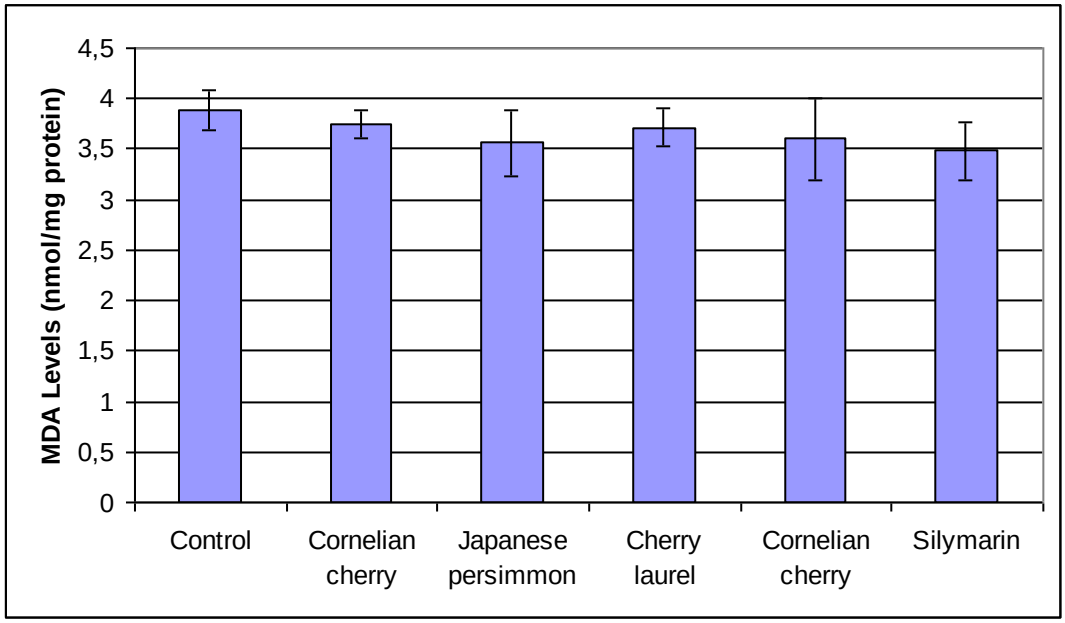


Figure 24: MDA levels of liver homogenates in healthy rats treated with 80% methanolic extracts of plants. The results were expressed as the mean of triplicates ± standard deviation.

4.2.2.5. TEAC Levels of Liver Homogenates in Healthy Rats

TEAC levels of liver homogenates in healthy rats after 21-day treatment are given in Table 45, Figure 25. All of the groups showed significant increase in the levels of TEAC levels.

Table 45: TEAC levels of liver homogenate in healthy rats

	Control	Cornelian cherry	Japanese Persimmon	Cherry laurel	Cornelian cherry leaves	Silymarin
TEAC levels[A]	39.02 ± 3.75[B]	44.04 ± 1.48 (12.87%)*	47.49 ± 3.10 (21.71%)**	43.25 ± 0.89 (10.84%)*	50.41 ± 3.10 (29.19%)***	51.33 ± 6.58 (31.55%)***

[A] TEAC levels were expressed as µM Trolox equivalent (TE)/mg protein.

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

* $p < 0.05$ (Compared to the control group)

** $p < 0.01$ (Compared to the control group)

*** $p < 0.001$ (Compared to the control group)

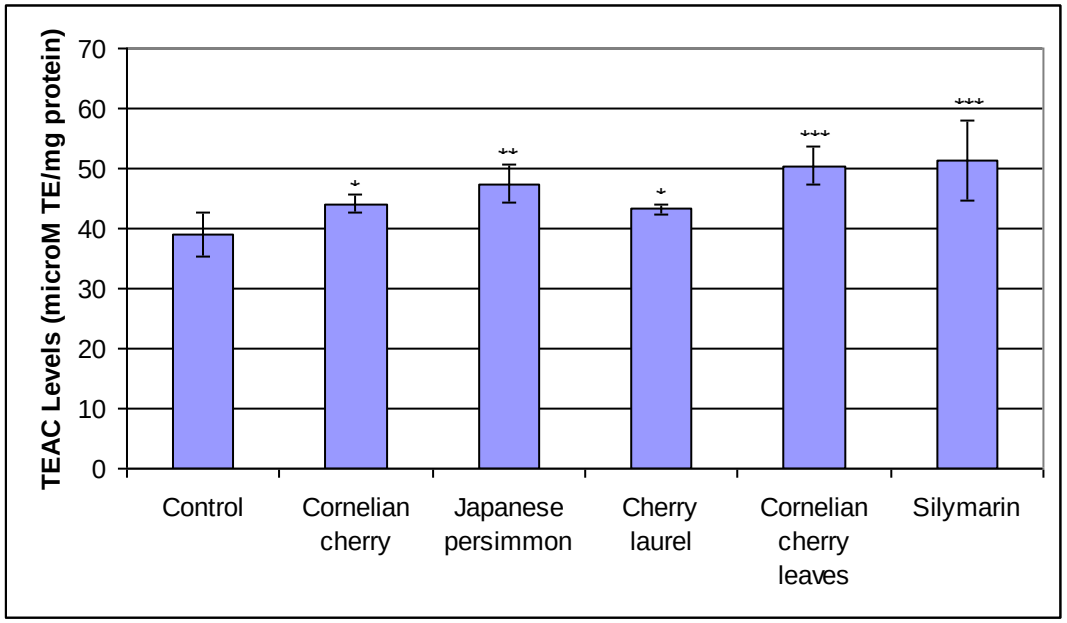


Figure 25: TEAC levels of liver homogenates in healthy rats treated with 80% methanolic extracts of plants. The results were expressed as the mean of triplicates ± standard deviation.

4.2.3. Results on Total Blood of CCl_4 – treated Rats

4.2.3.1. Total Blood CuZnSOD Activities of CCl_4 – treated Rats

CuZnSOD activities of CCl_4–treated rats after 5-day treatment are given in Table 46, Figure 26. In CCl_4–treated group, CuZnSOD levels were significantly decreased ($p < 0.01$). There were significant changes in animals administered 80% methanolic extract of cornelian cherry leaves at doses of 100 ($p < 0.05$),_200 ($p < 0.05$) and 500 mg/kg ($p < 0.01$). Besides, the reference group, silymarin, showed significant increase ($p < 0.001$).

Table 46: CuZnSOD activities of total blood in CCl_4 – treated rats

	Control	CCl_4	100 mg/kg	200 mg/kg	500 mg/kg	Silymarin
CuZnSOD activities[A]	139,6 ± 13.6[B]	90.13 ± 6.20^{++}	97.11 ± 9.40 (7.74%)*	99.53 ± 3.21 (10.42%)*	102.3 ± 3.87 (13.50%)**	107.34 ± 5.60 (19.09%)***

[A] CuZnSOD activities were expressed as U/mg protein.

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

$^{++}$ $p < 0.01$ (Compared to the control group)

* $p < 0.05$ (Compared to CCl_4 group)

** $p < 0.01$ (Compared to CCl_4 group)

*** $p < 0.001$ (Compared to CCl_4 group)

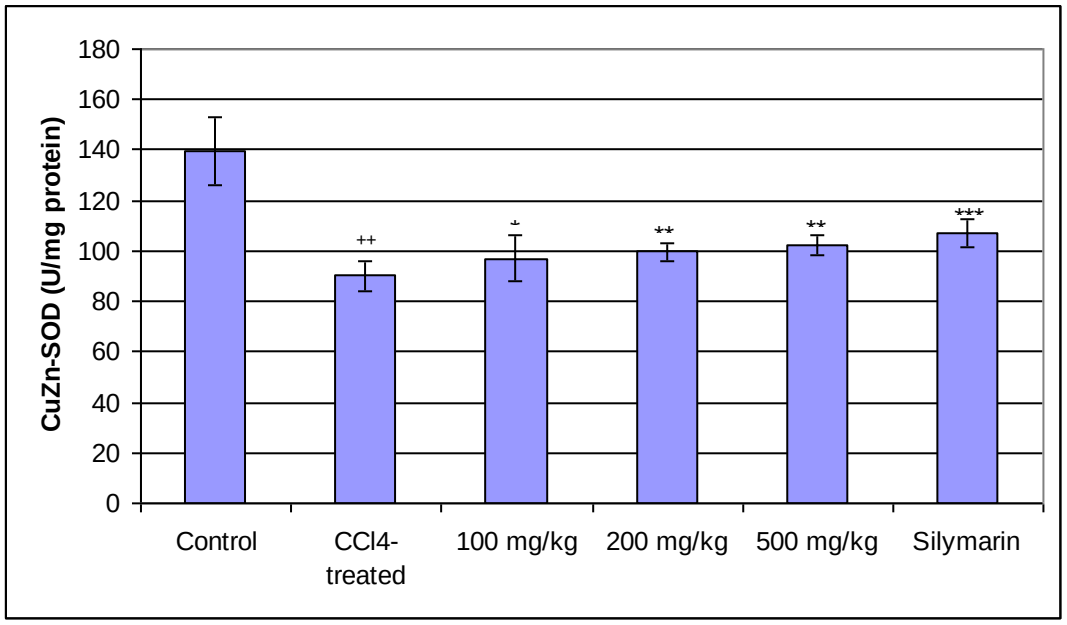


Figure 26: Total blood CuZnSOD activities of CCl_4–treated rats treated with 80% methanolic extract of cornelian cherry leaves. The results were expressed as the mean of triplicates ± standard deviation.

4.2.3.2. Total Blood GSH-Px Activities of CCl_4 – treated Rats

GSH-Px activities of CCl_4–treated rats after 5-day treatment are given in Table 47, Figure 27. In CCl_4–treated group, GSH-Px activities were significantly decreased ($p < 0.001$). Although some increases were observed in animals administered 100 and 200 mg/kg of 80% methanolic extract of cornelian cherry leaves, they are not statistically significant. There were significant changes at the dose of 500 mg/kg ($p < 0.05$) and in the reference group, silymarin ($p < 0.01$).

Table 47: GSH-Px activities of total blood in CCl_4–treated rats

	Control	CCl_4	100 mg/kg	200 mg/kg	500 mg/kg	Silymarin
GSH-Px activities [A]	89.61 ± 4.2[B]	67.54 ± 4.9^{+++}	70.31 ± 6.4	74.40 ± 7.1	81.30 ± 7.3 (20.37%)*	84.83 ± 11 (25.60%)**

[A] GSH-Px activities were expressed as U/mg protein.

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

$^{+++}$ $p < 0.001$ (Compared to the control group)

* $p < 0.05$ (Compared to CCl_4 group)

** $p < 0.01$ (Compared to CCl_4 group)

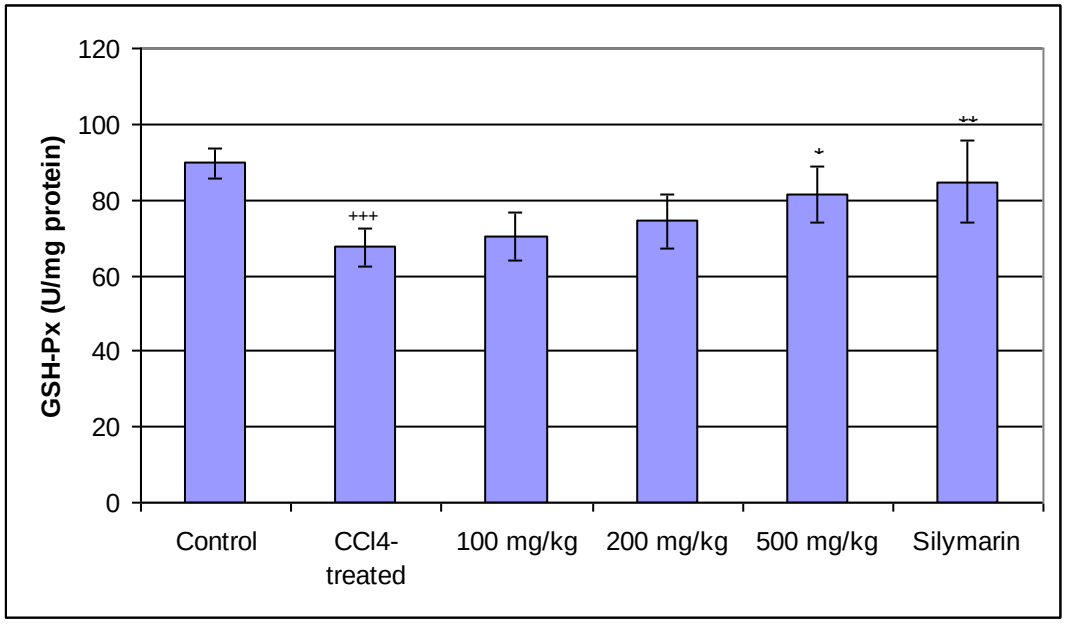


Figure 27: Total blood GSH-Px activities of CCl_4 – treated rats treated with 80% methanolic extract of cornelian cherry leaves. The results were expressed as the mean of triplicates ± standard deviation.

4.2.3.3. Total Blood CAT Activities of CCl_4 – treated Rats

CAT activities of CCl_4–treated rats after 5-day treatment are given in Table 48, Figure 28. In CCl_4–treated group, CAT activities were significantly decreased ($p < 0.01$). Although some increases were observed in animals administered 100 and 200 mg/kg of 80% methanolic extract of cornelian cherry leaves, they are not statistically significant. There were significant changes at the dose of 500 mg/kg ($p < 0.05$) and in the reference group, silymarin ($p < 0.01$).

Table 48: CAT activities of total blood of CCl_4–treated rats

	Control	CCl_4	100 mg/kg	200 mg/kg	500 mg/kg	Silymarin
CAT activities[A]	630.5 ± 44[B]	517 ± 34.2^{++}	530 ± 53.9	564 ± 47.4	589 ± 35.9 (14.01%)*	608 ± 48.7 (21.56%)**

[A] CAT activities were expressed as U/mg protein.

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

$^{++}p < 0.01$ (Compared to the control group)

* $p < 0.05$ (Compared to CCl_4 group)

** $p < 0.01$ (Compared to CCl_4 group)

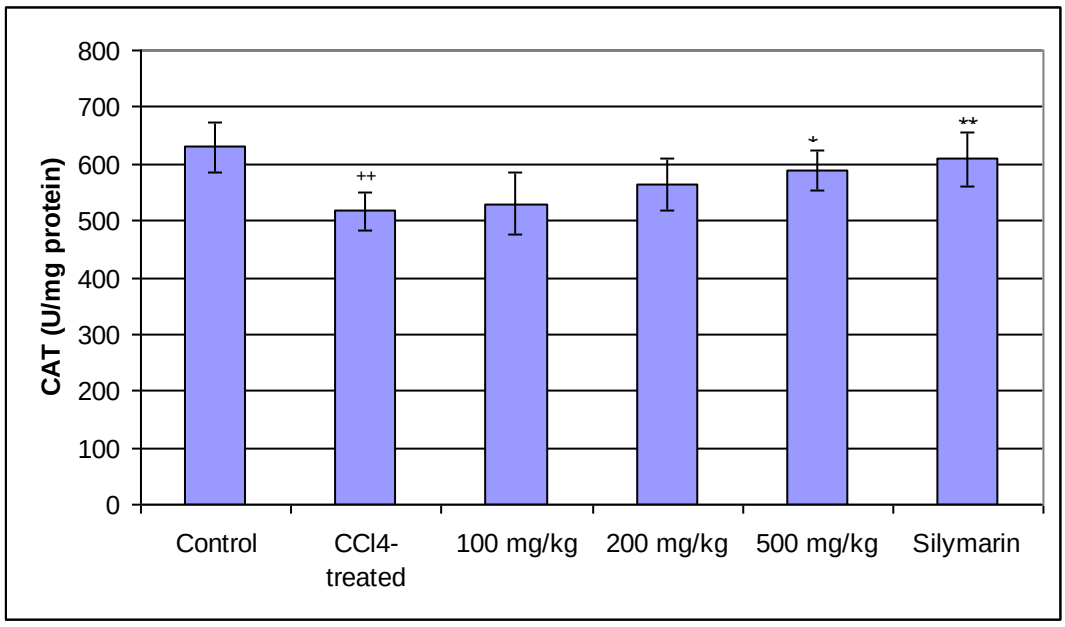


Figure 28: Total blood CAT activities of CCl_4 – treated rats treated with 80% methanolic extract of cornelian cherry leaves. The results were expressed as the mean of triplicates ± standard deviation.

4.2.3.4. Total Blood MDA Levels of CCl_4 – treated Rats

MDA levels of CCl_4–treated rats after 5-day treatment are given in Table 49, Figure 29. In CCl_4–treated group, MDA levels were significantly increased ($p < 0.001$). Although some decrease was observed in animals administered 100 mg/kg of 80% methanolic extract of cornelian cherry leaves, it was not statistically significant. There were significant changes at the doses of 200 and 500 mg/kg ($p < 0.05$) and in the reference group, silymarin ($p < 0.01$).

Table 49: MDA levels of total blood of CCl_4–treated rats

	Control	CCl_4	100 mg/kg	200 mg/kg	500 mg/kg	Silymarin
MDA levels[A]	3.71 ± 0.37[B]	9.34 ± 2.03^{+++}	8.46 ± 1.10	7.44 ± 1.02	6.89 ± 1.46 (-26.23%)*	608 ± 48.7 (-34.80%)**

[A] MDA levels were expressed as nmol/mg protein.

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

$^{+++}$ $p < 0.001$ (Compared to the control group)

* $p < 0.05$ (Compared to CCl_4 group)

** $p < 0.01$ (Compared to CCl_4 group)

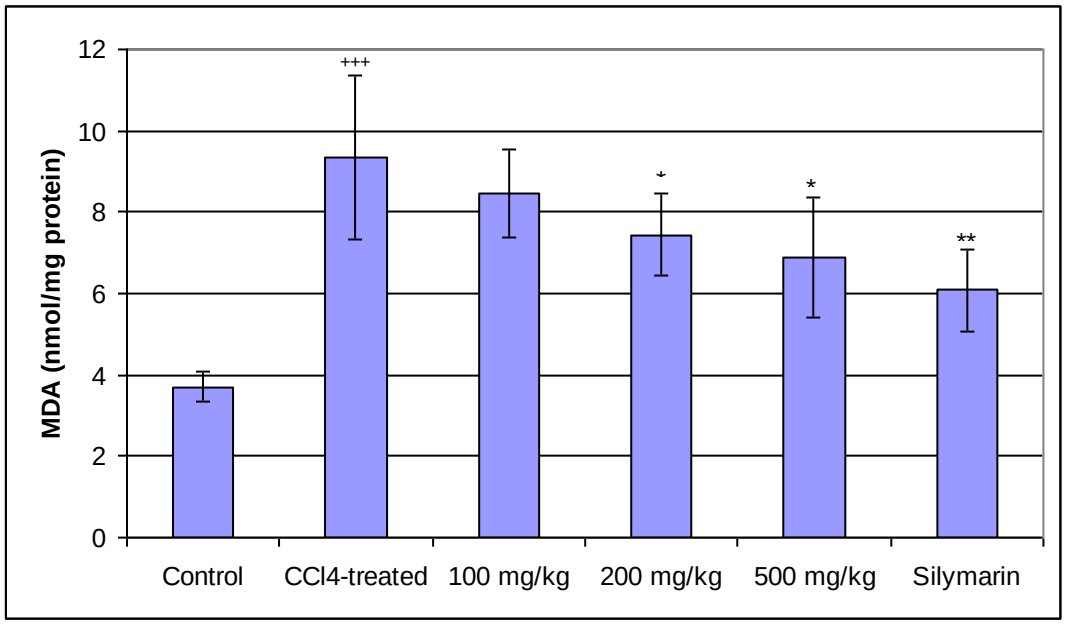


Figure 29: Total blood MDA levels of CCl_4–treated rats treated with 80% methanolic extract of cornelian cherry leaves. The results were expressed as the mean of triplicates ± standard deviation.

4.2.3.5. Total Blood TEAC Levels of CCl_4–treated Rats

TEAC levels of CCl_4–treated rats after 5-day treatment are given in Table 50. In CCl_4–treated group, TEAC levels were significantly decreased ($p < 0.001$). Although some increase was observed in animals administered 100 mg/kg of 80% methanolic extract of cornelian cherry leaves, it was not statistically significant. There were significant changes at the doses of 200 and 500 mg/kg ($p < 0.01$) and in the reference group, silymarin ($p < 0.001$).

Table 50: TEAC levels of total blood of in CCl_4–treated rats

	Control	CCl_4	100 mg/kg	200 mg/kg	500 mg/kg	Silymarin
TEAC levels[A]	34.16 ± 3.72[B]	19.79 ± 1.77^{+++}	22.19 ± 1.36	23.94 ± 1.00 (17.33%)**	25.34 ± 1.43 (28.49%)**	28.83 ± 2.42 (31.35%)***

[A] TEAC levels were expressed as nmol/mg protein.

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

$^{+++}$ $p < 0.001$ (Compared to the control group)

** $p < 0.01$ (Compared to CCl_4 group)

*** $p < 0.001$ (Compared to CCl_4 group)

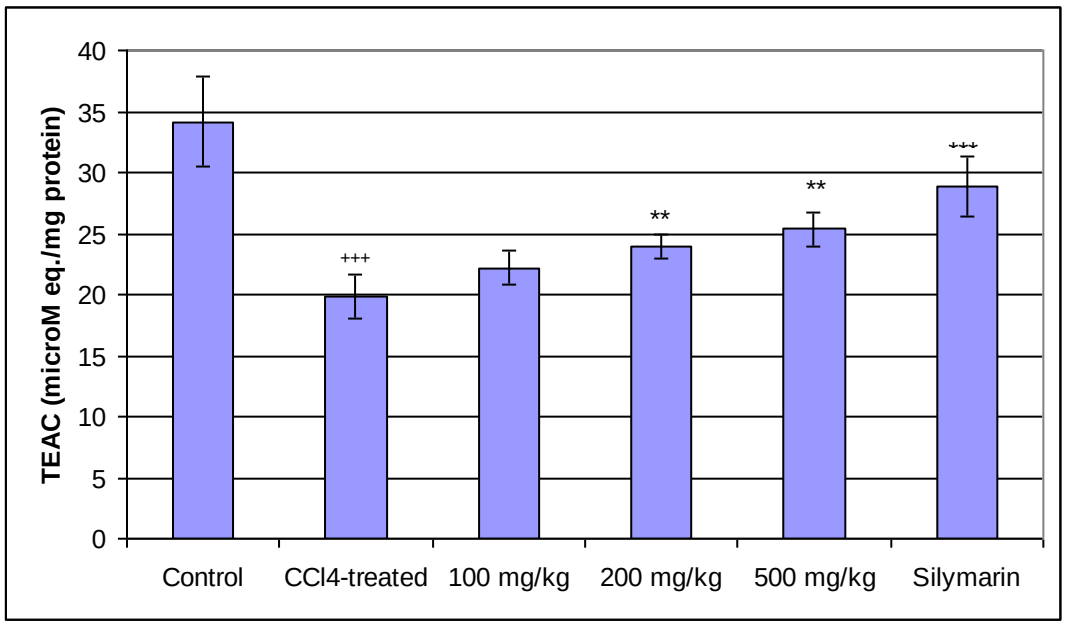


Figure 30: Total blood TEAC levels of CCl_4–treated rats treated with 80% methanolic extract of cornelian cherry leaves. The results were expressed as the mean of triplicates ± standard deviation.

4.2.4. Results on Liver Homogenates of CCl_4–treated Rats

4.2.4.1. CuZnSOD Levels of Liver Homogenates in CCl_4–treated Rats

CuZnSOD activities of liver homogenates in CCl_4–treated rats after 5-day treatment are given in Table 51, Figure 31. In CCl_4–treated group, CuZnSOD activities were significantly decreased ($p < 0.01$). Although some increase was observed in animals administered 100 mg/kg of 80% methanolic extract of cornelian cherry leaves, it was not statistically significant. There were significant changes at the doses of 200 and 500 mg/kg ($p < 0.05$) and in the reference group, silymarin ($p < 0.01$). It is worthwhile that the levels of the increase at the doses of 200 and 500 mg/kg were very close.

Table 51: CuZnSOD activities of liver homogenates in CCl_4–treated rats

	Control	CCl_4	100 mg/kg	200 mg/kg	500 mg/kg	Silymarin
CuZnSOD activities [A]	175.7 ± 16.7[B]	127.6 ± 12.6^{++}	136,6 ± 6.7	158.6 ± 23.3 (19.51%)*	159.1 ± 19 (19,80%)*	169.7 ± 20 (24.81%)**

[A] CuZn-SOD levels were expressed as U/mg protein.

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

$^{++}$ $p < 0.01$ (Compared to the control group)

* $p < 0.05$ (Compared to CCl_4 group)

** $p < 0.01$ (Compared to CCl_4 group)

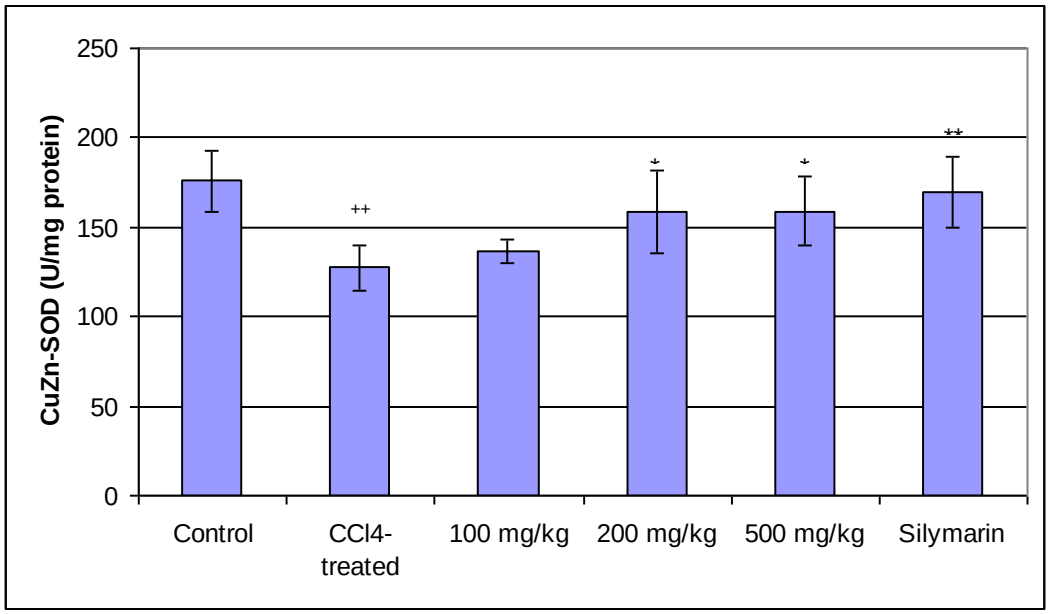


Figure 31: CuZnSOD activities of liver homogenates in CCl_4–treated rats treated with 80% methanolic extracts of cornelian cherry leaves. The results were expressed as the mean of triplicates ± standard deviation.

4.2.4.2. GSH-Px Activities of Liver Homogenates in CCl_4–treated Rats

GSH-Px activities of liver homogenates in CCl_4–treated rats after 5-day treatment are given in Table 52, Figure 32. In CCl_4–treated group, GSH-Px activities were significantly decreased ($p < 0.001$). Although some increases were observed in animals administered 100 and 200 mg/kg of 80% methanolic extract of cornelian cherry leaves, these differences are not statistically significant. There were significant changes at the dose of 500 mg/kg ($p < 0.05$) and in the reference group, silymarin ($p < 0.01$).

Table 52: GSH-Px activities of liver homogenates in CCl_4–treated rats

	Control	CCl_4	100 mg/kg	200 mg/kg	500 mg/kg	Silymarin
GSH-Px activities [A]	88.61 ± 5.73[B]	70.71 ± 4.12^{+++}	77.58 ± 5.55	78.90 ± 7.74	82.91 ± 6.07 (14.71%)*	86.45 ± 6.79 (18.20%)**

[A] GSH-Px activities were expressed as U/mg protein.

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

$^{+++}$ $p < 0.001$ (Compared to the control group)

* $p < 0.05$ (Compared to CCl_4 group)

** $p < 0.01$ (Compared to CCl_4 group)

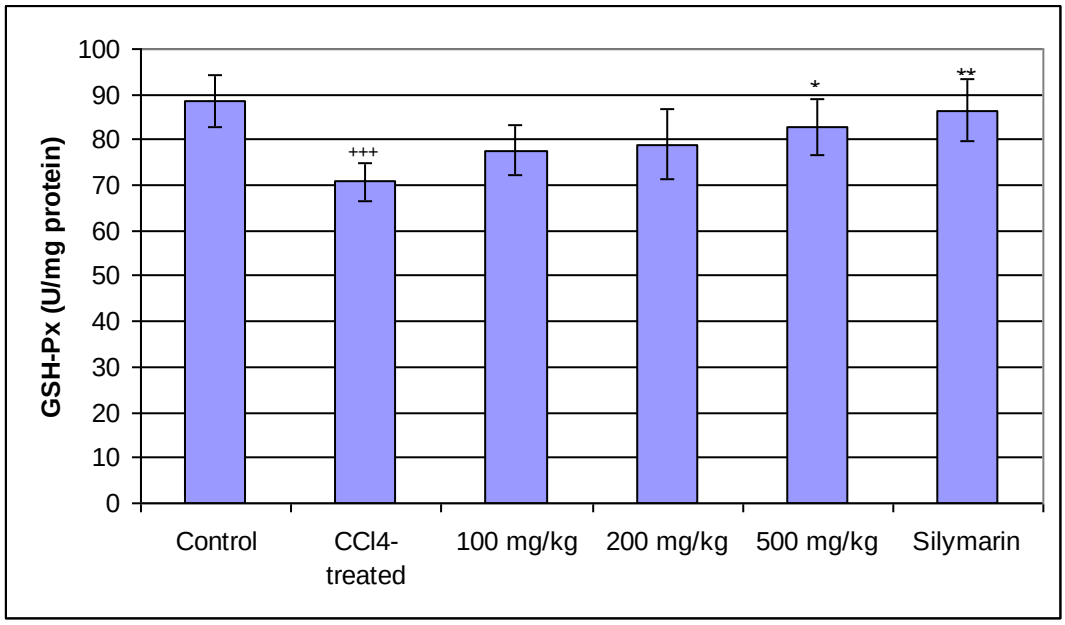


Figure 32: GSH-Px activities of liver homogenates in CCl_4–treated rats treated with 80% methanolic extracts of cornelian cherry leaves. The results were expressed as the mean of triplicates ± standard deviation.

4.2.4.3. CAT Activities of Liver Homogenates in CCl_4–treated Rats

CAT activities of liver homogenates in CCl_4–treated rats after 5-day treatment are given in Table 53, Figure 33. In CCl_4–treated group, CAT activities were significantly decreased ($p < 0.001$). Although some increase was observed in animals administered 100 mg/kg of 80% methanolic extract of cornelian cherry leaves, it was not statistically significant. There were significant

improvements at the doses of 200 mg/kg ($p < 0.05$) and 500 mg/kg ($p < 0.001$) and in the reference group, silymarin ($p < 0.001$).

Table 53: CAT activities of liver homogenates in CCl_4–treated rats

	Control	CCl_4	100 mg/kg	200 mg/kg	500 mg/kg	Silymarin
CAT activities [A]	737.4 ± 76.2[B]	319 ± 28.2^{+++}	370.5 ± 54.6	427.1 ± 35.5 (25.3%)*	587.5 ± 86.7 (37%)***	625.2 ± 21.5 (49%)***

[A] CAT activities were expressed as U/mg protein.

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

$^{+++}p < 0.001$ (Compared to the control group)

* $p < 0.05$ (Compared to CCl_4 group)

*** $p < 0.001$ (Compared to CCl_4 group)

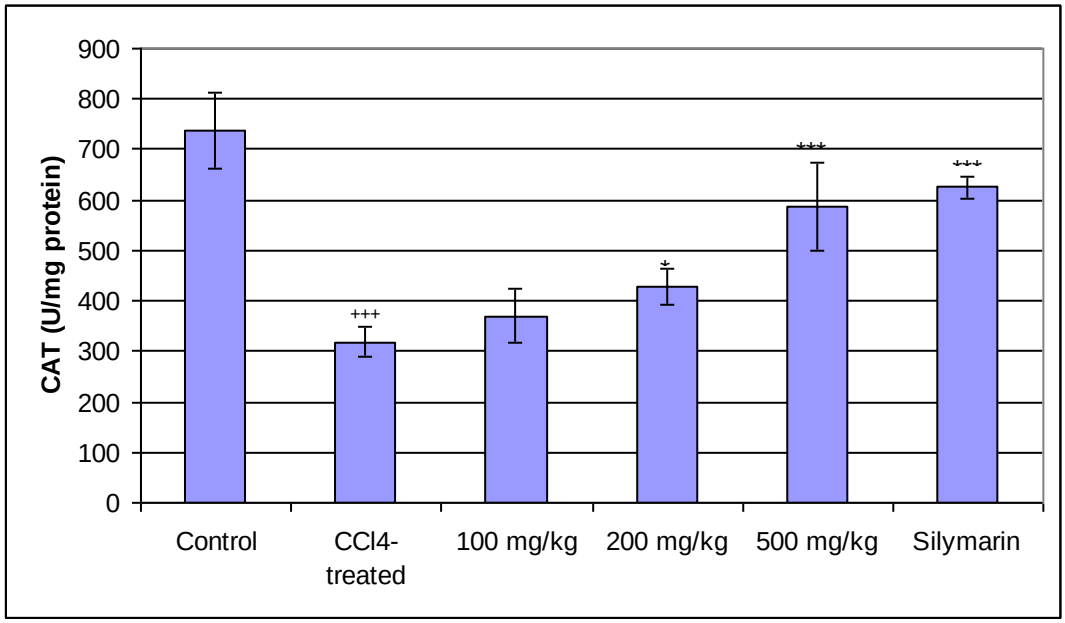


Figure 33: CAT levels of liver homogenates in CCl_4–treated rats treated with 80% methanolic extracts of cornelian cherry leaves. The results were expressed as the mean of triplicates ± standard deviation.

4.2.4.4. MDA Levels of Liver Homogenates in CCl_4–treated Rats

MDA levels of liver homogenates in CCl_4–treated rats after 5-day treatment are given in Table 54. In CCl_4–treated group, MDA levels were significantly increased ($p < 0.001$). Although some decrease was observed in animals administered 100 mg/kg of 80% methanolic extract of cornelian cherry leaves, it was not statistically significant. There were significant changes at the doses of 200 and 500 mg/kg ($p < 0.01$) and in the reference group, silymarin ($p < 0.001$).

Table 54: MDA levels of liver homogenates in CCl_4 – treated rats

	Control	CCl_4	100 mg/kg	200 mg/kg	500 mg/kg	Silymarin
MDA levels[A]	4.26 ± 0.7[B]	13.24 ± 3.7^{+++}	10.98 ± 1.8	9.03 ± 1.6 (31.8%)**	8.04 ± 2.5 (38.46%)**	7.69 ± 0.75 (41.92%)***

[A] MDA levels were expressed as nmo/mg protein.

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

$^{+++}$ $p < 0.001$ (Compared to the control group)

** $p < 0.01$ (Compared to CCl_4 group)

*** $p < 0.001$ (Compared to CCl_4 group)

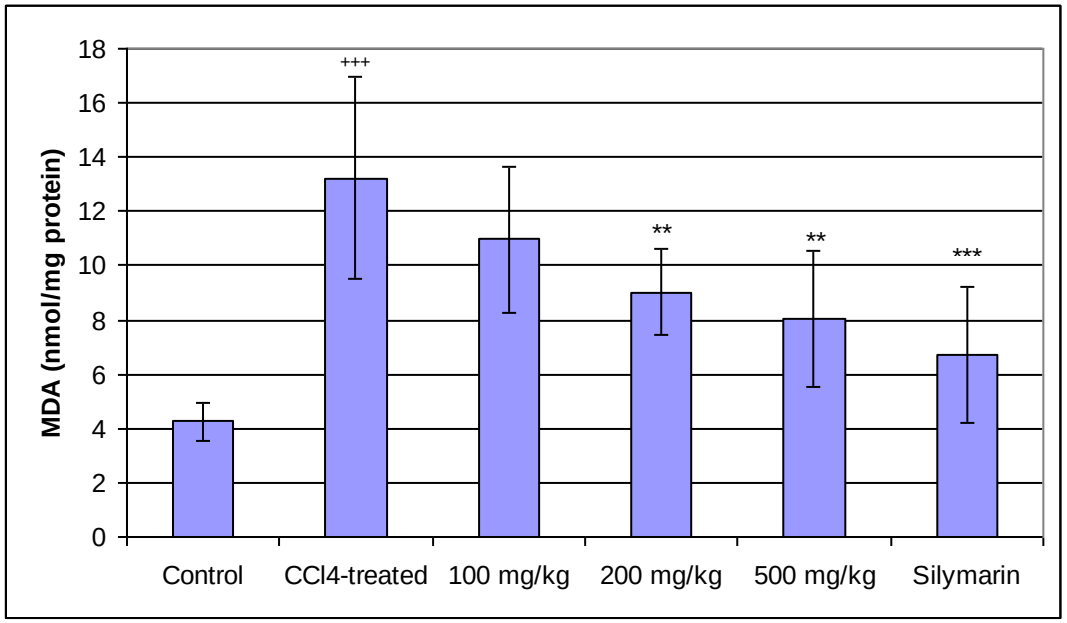


Figure 34: MDA levels of liver homogenates in CCl_4 – treated rats treated with 80% methanolic extracts of cornelian cherry leaves. The results were expressed as the mean of triplicates ± standard deviation.

4.2.4.5. TEAC Levels of Liver Homogenates in CCl_4–treated Rats

TEAC levels of liver homogenates in CCl_4–treated rats after 5-day treatment are given in Table 55, Figure 35. In CCl_4–treated group, TEAC levels were significantly decreased ($p < 0.001$). Although some decrease was observed in animals administered 100 mg/kg of 80% methanolic extract of cornelian cherry leaves, it was not statistically significant. There were significant changes at the doses of 200 mg/kg ($p < 0.05$) and 500 mg/kg ($p < 0.001$) and in the reference group, silymarin ($p < 0.001$).

Table 55: TEAC levels of liver homogenates in CCl_4–treated rats

	Control	CCl_4	100 mg/kg	200 mg/kg	500 mg/kg	Silymarin
TEAC levels[A]	48.32 ± 2.3[B]	29.59 ± 3.1^{+++}	31.08 ± 0.9	33.67 ± 2.2 (13.79%)*	39.17 ± 3.6 (32.04%)***	39.17 ± 3.6 (51.77%)***

[A] TEAC levels were expressed as µM eq./mg protein.

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

$^{+++}$ $p < 0.001$ (Compared to the control group)

** $p < 0.01$ (Compared to CCl_4 group)

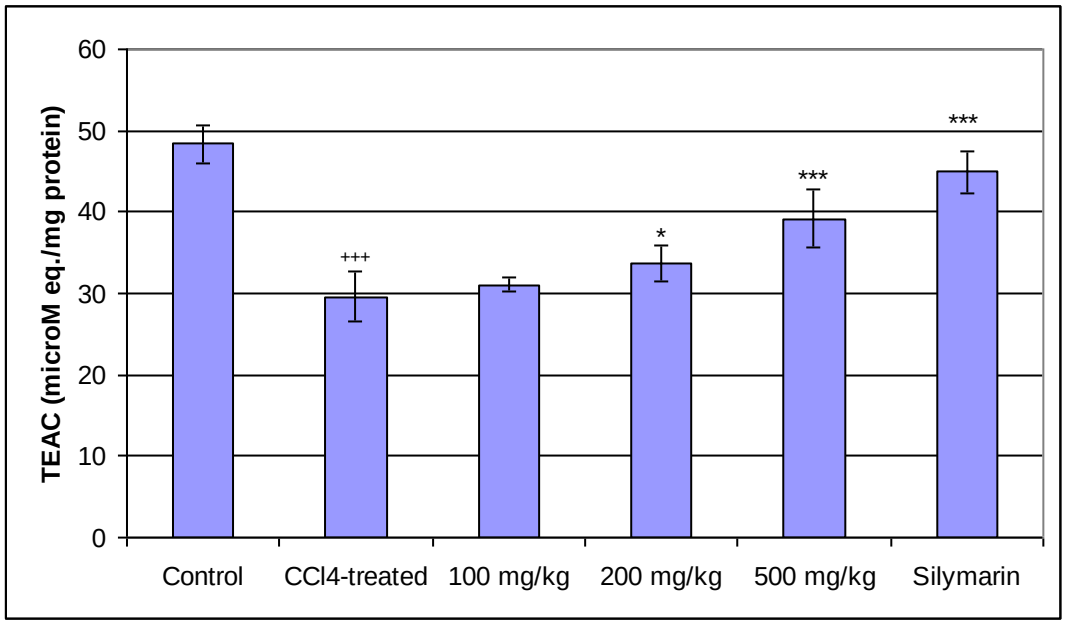


Figure 35: TEAC levels of liver homogenates in CCl_4 – treated rats treated with 80% methanolic extracts of cornelian cherry leaves. The results were expressed as the mean of triplicates ± standard deviation.

4.3. Results of the Bioactivity Guided Fractionation Studies on 80% Methanolic extract of *C. mas* leaves

4.3.1. Results of *In vitro* Activity Studies on Fractions

In vitro antioxidant activity results of fractions obtained from 80% methanolic extract of *C. mas* leaves are given in Table 56. The results indicated that EtOAc fraction of *C. mas* had both the highest radical scavenging activity and ferric reducing antioxidant power. CM-1, isolated from EtOAc fraction, showed better activity than the fraction itself.

Table 56: Antioxidant activities of fractions obtained from *C. mas* leaves

	DPPH[A]	FRAP[B]
CM – *n*-Hexane	466 ± 15.2[C,a]	1.03 ± 0.07[a]
CM – $CHCl_3$	287 ± 10.4[b]	2.80 ± 0.15[b]
CM – EtOAc	**141 ± 6.5**[c]	**4.08 ± 0.18**[c]
CM – *n*-BuOH	372 ± 19.8[d]	2.17 ± 0.14[d]
CM – R-H_2O	518 ± 17.3[e]	0.67 ± 0.07[e]
CM-1	**96 ± 11.3**[f]	**4.47 ± 0.21**[f]
BHT*	133 ± 6.4[g]	3.02 ± 0.10[g]

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

[B] FRAP activity was expressed as mM $FeSO_4$ equivalents in 1 g dry material.

[C] Results were expressed as the mean of triplicates ± standard deviation (S.D.) (n: 3)

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

* Butylated hydroxytoluene

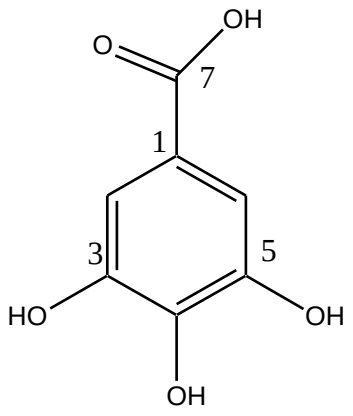
4.3.2. Structure Elucidation

Following the activity studies, the fraction which showed the highest activity was subjected to chromatographic studies. The structure elucidation of the isolated active compound was accomplished by using spectroscopic methods.

Structure elucidation of CM-1 (Gallic Acid)

CM-1 was obtained from CM–EtOAc fraction as an amorphous white powder.

The ^{1}H NMR spectrum (Table 57, Spectrum 1) contained only a singlet at δ 7.15 (2H). The ^{13}C NMR spectrum (Table 57, Spectrum 2) displayed an equivalent aromatic methine resonance at δ 108.9 (2C) and a carbonyl signal at δ 169.2 as well as four quaternary signals in the aromatic region at δ 120.8, 138.1 and 144.9 (2C) which were indicative of a C_6C_1 phenolic acid. Taken all together, the above NMR findings were in good agreement with those of **gallic acid** (216).



^{1}H NMR (600 MHz, MeOD) : Spectrum 1

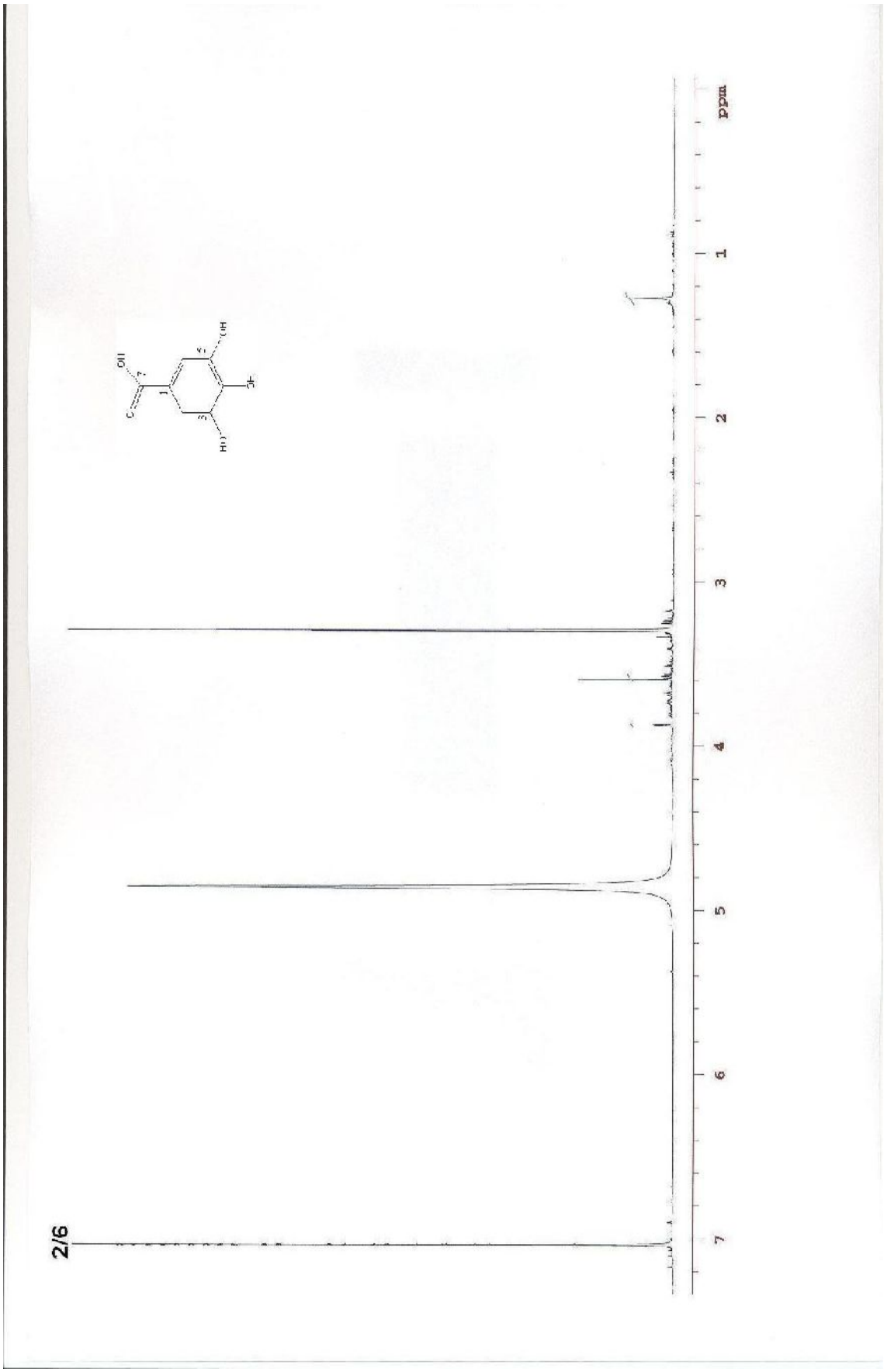
^{13}C NMR (150 MHz, MeOD): Spectrum 2

$C_7H_6O_5$, 170.12 g/mol

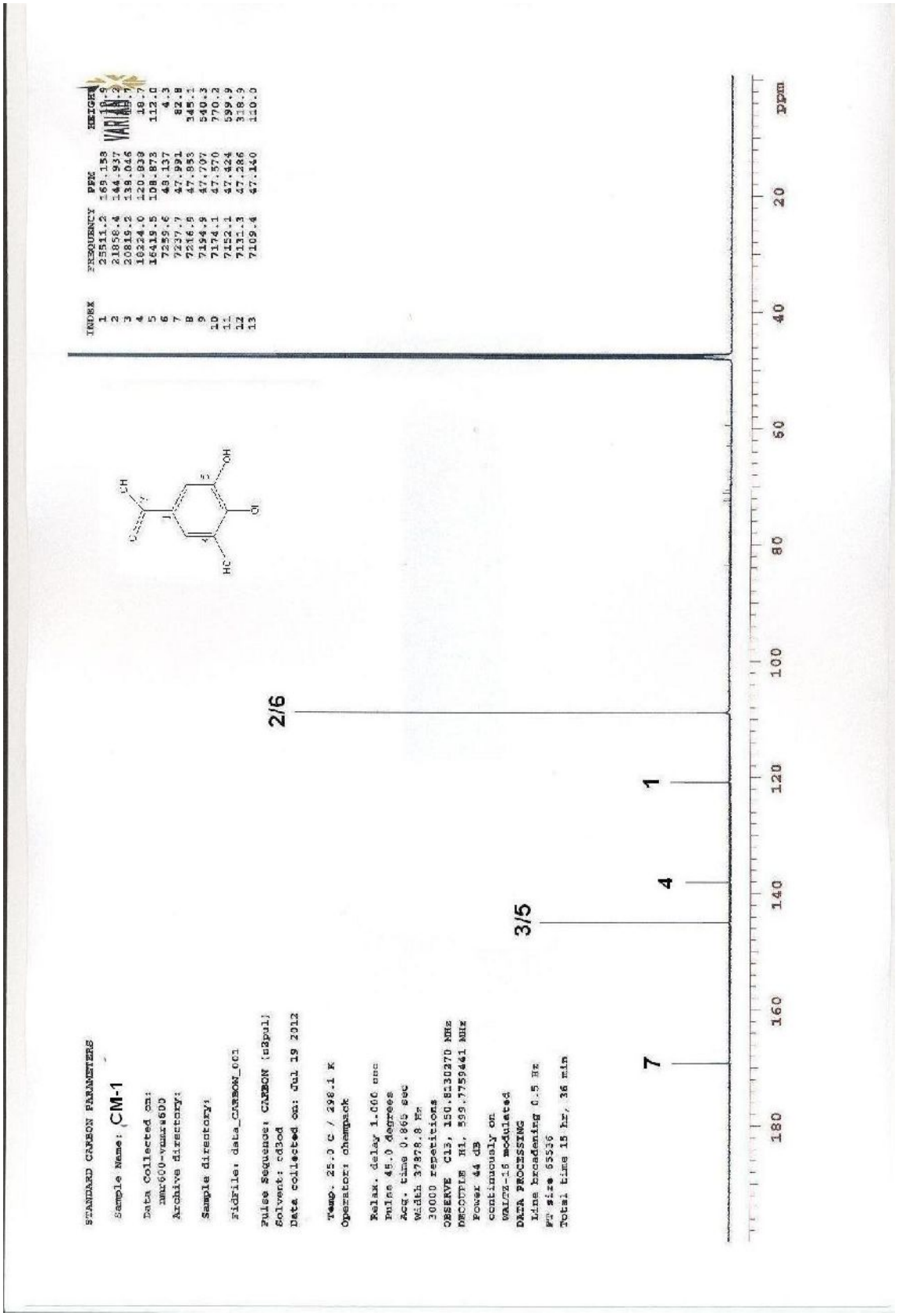
Figure 36: Chemical Structure of CM–1

Table 57: ^{1}H NMR (600 MHz, MeOD) and ^{13}C NMR (150 MHz, MeOD) Data of CM-1

Position	**δ_H, ppm**	**Ref** (216)	**δ_c, ppm**	**Ref** (216)
1	-		120.8	120.8
2	7.15 (1H, *s*)	7.15 (1H, *s*)	108.9	109.1
3	-		144.9	144.9
4	-		138.1	137.8
5	-		144.9	144.9
6	7.15 (1H, *s*)	7.15 (1H, *s*)	108.9	109.1
7	-		169.2	168.39



Spectrum 1: ^{1}H spectrum of CM–1



Spectrum 2: ^{13}C spectrum of CM–1

4.4. Quantitative Analysis of Gallic Acid in 80% Methanolic Extract of *C. mas* leaves by HPTLC

The pictures of the plate under 366 nm of UV light, the spectra comparison of the spots, the peak areas of the spots and the calibration curve are given in Figures 37a, 37b, 37c, 38, 39 and 40, respectively. HPTLC analysis indicates that 80% methanolic extract of *C. mas* leaves contain 0.585 ± 0.007 % (n: 5) gallic acid.

Developed

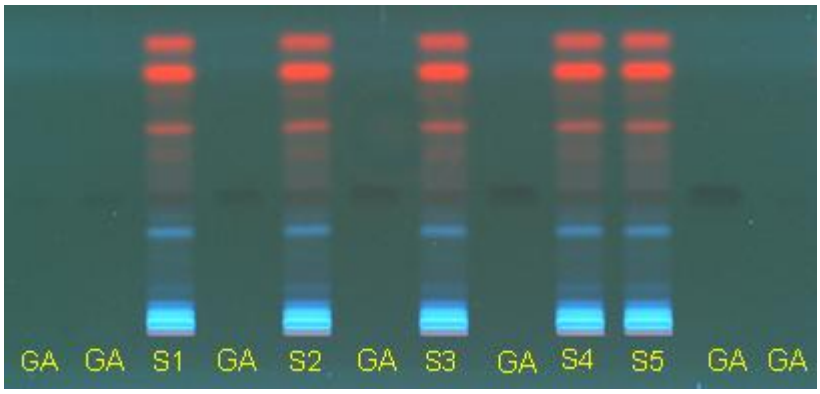


Figure 37a: The pictures of spots under 366 nm of UV light
S1-S5: Sample 1-6

Derivatized by NA

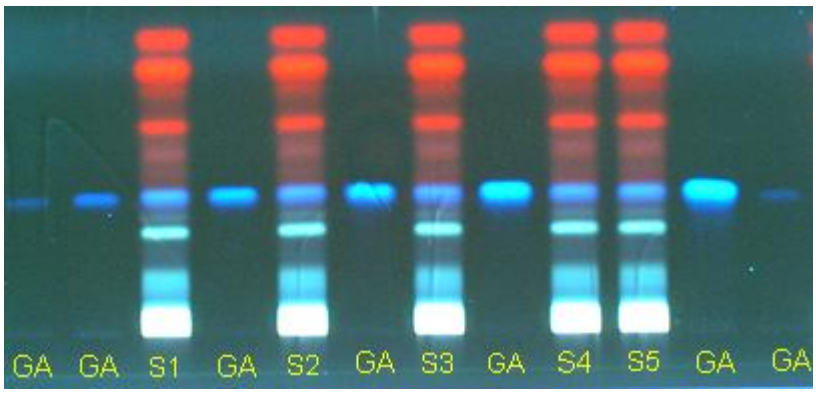


Figure 37b: The pictures of spots under 366 nm of UV light

Derivatized by NA/PEG 400

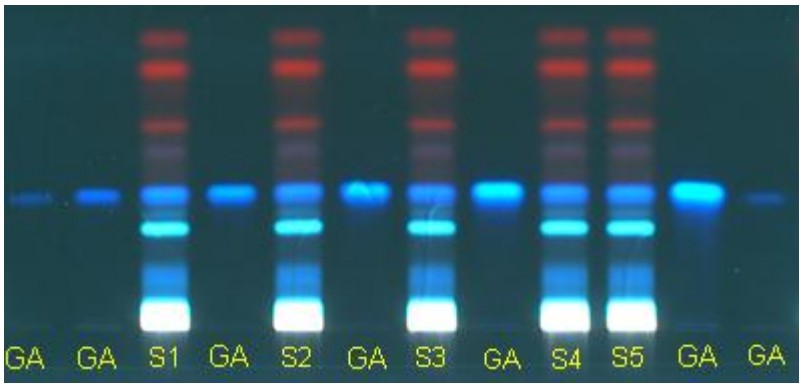


Figure 37c: The pictures of spots under 366 nm of UV light

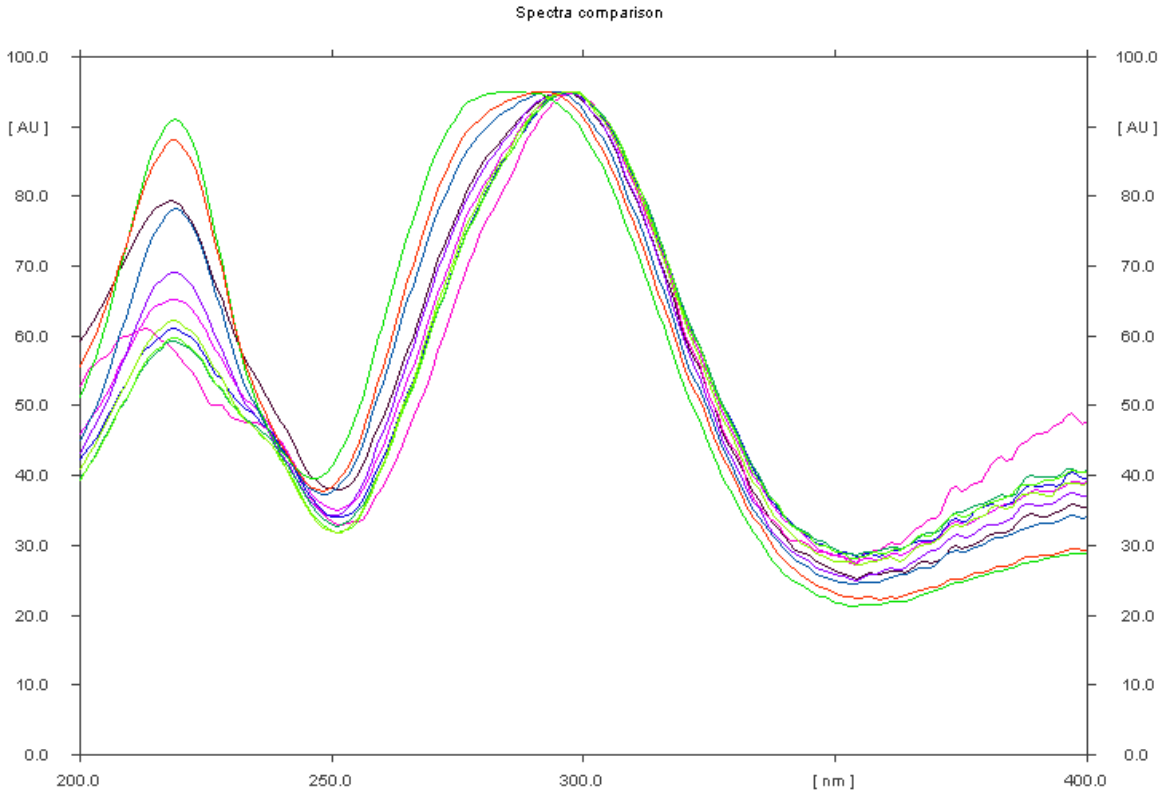


Figure 38: Overlaid UV spectra of gallic acid in all tracks.

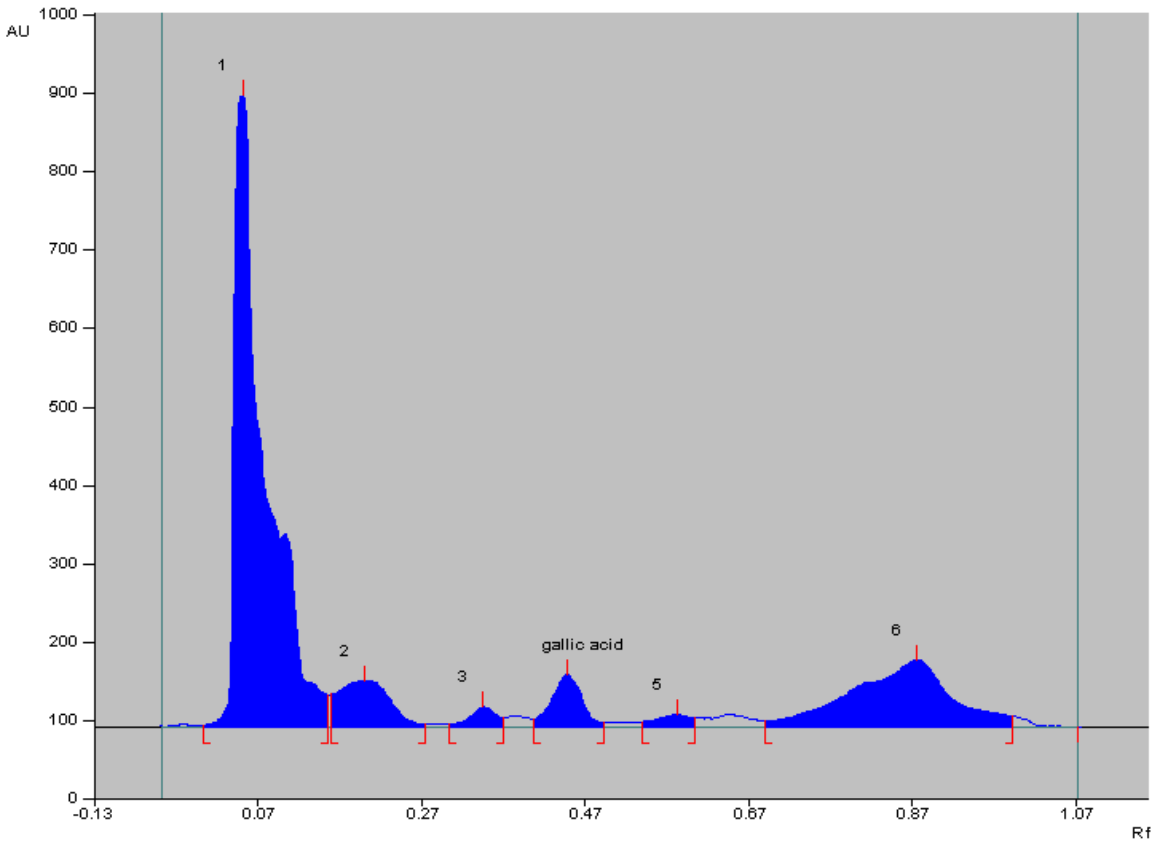


Figure 39: 2D chromatogram of the extract

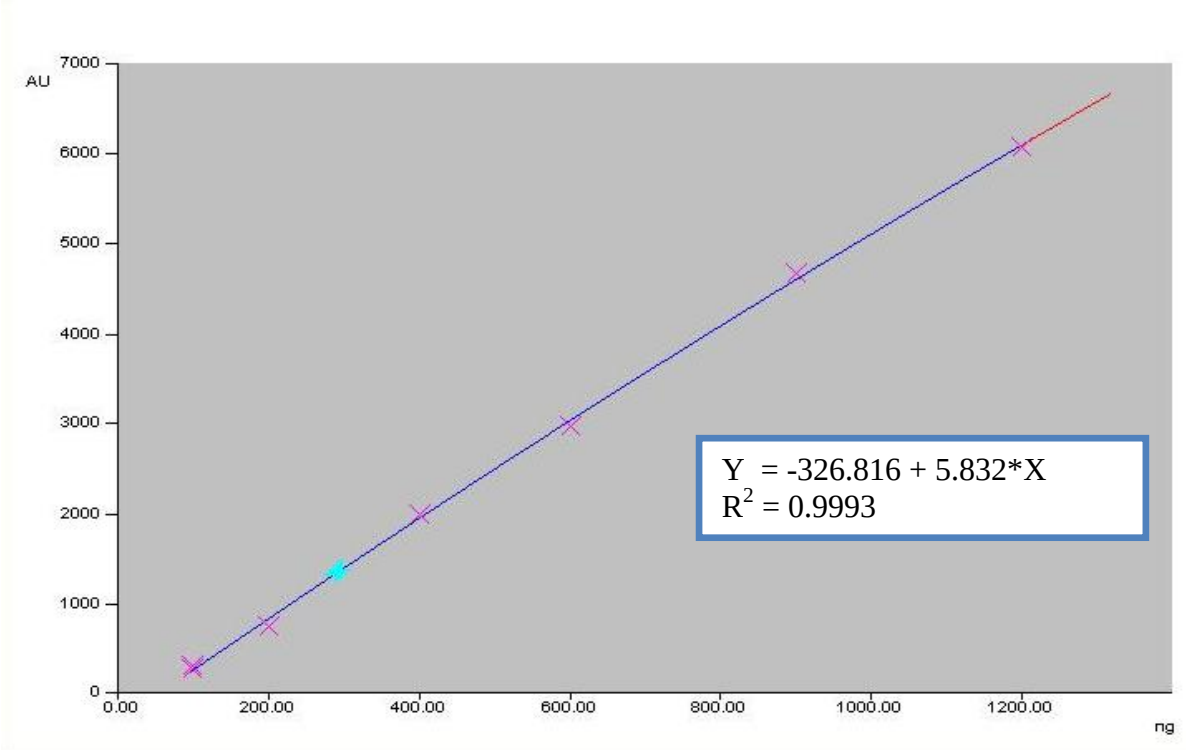


Figure 40: The calibration curve for gallic acid

5. DISCUSSION

Every living organism, with the exception of anaerobic microorganisms, requires molecular oxygen as an electron acceptor for energy production. Since oxygen itself is a powerful oxidant, secondary oxidation reactions in normal physiological metabolism are inevitable (70). These secondary oxidation reactions cause the generation of reactive oxygen species (ROS), including free radicals. The deleterious effect of free radicals resulting in potential damage in biological systems is termed as oxidative stress, causing in damage in DNA, lipids, proteins. Aerobic organisms developed enzymatic and non-enzymatic antioxidant defense system in order to neutralize these harmful effects. Sies defines oxidative stress as "*an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage*" (71).

Oxidative stress is known to play a critical role in several diseases such as cardiovascular disease, cancer, diabetes, inflammatory diseases, ischemia/reperfusion injury, Alzheimer's disease, immune diseases, eye diseases (3). Oxidative stress has also direct relationship with aging process and the degenerative diseases of aging (73).

The enzymatic and non–enzymatic endogenous antioxidant defense systems in the body are not totally efficient to combat oxidative stress. Therefore, dietary antioxidants are important in diminishing the cumulative effect of oxidative damage during the lifespan of humans and also play a substantial role in protection from oxidative stress–related diseases. Interestingly, Halliwell demonstrated the inadequate dietary intake of α-tocopherol or ascorbic acid as a reason for increased oxidative stress (138). Besides, there are a vast number of studies regarding the importance of dietary antioxidants in the prevention of these diseases. For example, Gaziano et al. highlighted the importance of dietary antioxidants in the protection cardiovascular diseases due to atherosclerosis (217). Another study revealed a 39% reduction in major coronary events in the highest intake category for vitamin E as compared to the lowest (218). Various studies prove a very good

relationship between natural antioxidant intake and the reduced risk of several types of cancers including lung, colon, oral, stomach and esophageal (219). In another study, type II diabetic patients were given a quercetin rich diet for two weeks, after a low quercetin diet for the preceding two weeks. It was observed that DNA damage was significantly reduced following the consumption of high quercetin containing diet, when compared to the lower quercetin content (220).

All of these data indicate that regular consumption of fruit and vegetables containing natural antioxidants is associated with reducing the risk of various diseases. To increase the body's antioxidant potential and to protect from oxidative stress, it has been recommended that individuals increase their intake of dietary antioxidants. Therefore, we chose three edible fruits consumed widely in Turkey to comparatively investigate their antioxidant potentials. These are cornelian cherry (*Cornus mas* L.), Japanese persimmon (*Diospyros kaki* L.) and cherry laurel (*Laurocerasus officinalis* Roem.). For a complete evaluation of antioxidant potential of these fruits, both *in vitro* and *in vivo* experimental models were used. The antioxidant effect of the leaves of *Cornus mas* L was also investigated. The main purpose behind choosing the leaf extract of cornelian cherry was to compare the activities of fruit and leaf extracts. Furthermore, cornelian cherry leaves are reported to be used against diabetes in ethnobotanical literature (5,6), and the relationship between diabetes and oxidative stress was already revealed (1). Besides, our literature survey revealed that the number of investigations on the biological efficacy of the cornelian cherry leaves were scarce.

Eight different *in vitro* methods for the purpose of a detailed investigation of the antioxidant activity of 80% methanolic extracts prepared from four test materials have been employed. Since antioxidant molecules exert their activities with different mechanisms, these methods were chosen in order to reveal the antioxidant activity profile of the test materials thoroughly.

DPPH, superoxide radical scavenging tests and Trolox equivalent antioxidant capacity (TEAC) tests were performed in order to measure the

general free radical-scavenging abilities, since free radicals are the direct cause of oxidative stress (72).

Ferric reducing antioxidant power (FRAP) and Cupric reducing antioxidant capacity (CUPRAC) were studied in order to evaluate the ferric and cupric ion reducing capacities of the plants, because these ions play a crucial role in the generation of free radicals (81). Ferrous ion-chelating capacity test was also performed for a similar purpose. Chelating metal ions participating in free radical formation is a well-known mechanism in evaluating the antioxidant activity (154).

Total antioxidant capacity test was carried out in order to measure total reducing power of the samples. Besides, β-carotene bleaching test was performed since it reflects the abilities of samples to inhibit the lipid peroxidation.

As previously mentioned in detail, phenolic compounds are a major class of bioactive components. They were shown to possess the ideal chemistry for antioxidant activity since they have high reactivity as hydrogen or electron donors and also capability of chelating metal ions (154,165,221). A substantial portion of the antioxidant properties of plant extracts have been attributed to the phenolic content. There are over 150.000 research papers related with the beneficial effects of phenolic compounds as natural antioxidants (222). Therefore, we also measured the total phenolic, flavonoid and proanthocyanidin contents of the extracts.

Among the fruit extracts Japanese persimmon showed the highest total phenolic, flavonoid and proanthocyanidin content. However, the total phenolic and flavonoid content of cornelian cherry leaf extract was much higher than fruit extracts. Interestingly, its total proanthocyanidin content (298 ± 11.83 mg EGCG-E/extract) was lower than Japanese persimmon (646 ± 24.1 mg EGCG-E/extract).

Ercisli et al. investigated total phenolic content of different genotypes of Japanese persimmon fruits in Turkey and found lower values than our results (from 19.1 to 42.3 mg GAE/g extract) (223). This might be due to the differences in phenolic contents of fruits, which may vary depending on the type of extraction solvent and method employed as well as source, post-harvesting conditions and maturity level of the fruits (224).

The results of a recent report (50) on total phenolic content of water extract prepared from cherry laurel (32.83 mg GAE/g extract) are consistent with our findings (23.64 ± 0.84 mg GAE/g extract). In addition, Gülçin et al. found similar total phenolic content of water extract of cornelian cherries (26.49 mg GAE/g extract (25). These results indicate that different extraction solvents with similar polarities did not cause important changes on phenolic content.

The results of total flavonoid content assay of 80% methanolic extracts did not differ much from total phenolic content. Japanese persimmon had the highest flavonoid content, but much lower than cornelian cherry leaf extract. Orhan and Akkol reported that total flavonoid content of cherry laurel fruits could not be calculated due to trace amount (50). However, we calculated the total flavonoid content of 80% methanolic extract as 16.87 ± 0.38 mg QE/g extract. In the literature survey, we found no report on the total flavonoid content of cornelian cherry or Japanese persimmon fruits grown in Turkey.

Total proanthocyanidin content results were quite different from both total phenolic and flavonoid contents. The content of cornelian cherry leaf extract (298 ± 11.83 mg EGCG-E/ g extract) was much lower than those of Japanese persimmon (646 ± 24.1 mg EGCG-E/ g extract) and cherry laurel (342 ± 16.3 mg EGCG-E/ g extract). To best of our knowledge, this is the first report on total proanthocyanidin content of all these plants.

The results of *in vitro* antioxidant tests revealed that all four extracts have antioxidant potential to some extent. According to the results of DPPH scavenging tests, Japanese persimmon showed the best activity among the fruits. However, cornelian cherry leaf extract had much higher activity.

DPPH scavenging activity of cherry laurel fruit juice was previously investigated by Liyana-Pathirana et al. (225) and moderate scavenging activity was found, which is in compatible with our results on 80% methanolic extract. The lack of difference in the activity between total fruit juice and 80% methanolic extract indicates that all of the scavenger components were extracted with this method. Besides, Orhan and Akkol found lower activity (EC_{50}> 2000 µg/mL) with methanolic extract of the fruits (50). This difference was most probably due to the differences in polarities of the extraction solvents employed in both studies. Scavenging activity of water extract of cornelian cherry was investigated by Gülçin et al. and the results were similar to the ones we found with 80% methanolic extract (25). Another study was performed on 12 different cornelian cherry cultivars (26). When the results were compared, it was revealed that cultivated forms of cornelian cherry have lower DPPH radical scavenging capacity than wild growing ones, which were used in our study. A variety of studies on DPPH radical scavenging activity of different parts of Japanese persimmon have been conducted so far. Sun et al. (2) and Han et al. (226) studied the mentioned effect of Japanese persimmon leaves, while Sakanaka et al. investigated that of an indigenious Japanese persimmon leaf tea (227). Besides, Akter et al. studied DPPH scavenging activity of Japanese persimmon seeds (206).

Superoxide radical is the most common free radical generated *in vivo*. It plays an important role in the formation of other reactive oxygen species (66). Therefore, scavenging superoxide radical gives important data about antioxidant potential. The results of superoxide radical scavenging tests revealed that three fruit extracts showed very little activity, when compared to the reference compound, gallic acid (Table 29)**.** The EC_{50} levels of the extracts were extremely high. However, cornelian cherry leaf extract showed considerable activity. Orhan and Akkol reported that cherry laurel was found to be inactive against superoxide radical (50). This difference seems to be rooted from the low concentration of the sample in the test procedure. As far as we know, there are no reports of other test plants regarding the superoxide radical scavenging activity.

The TEAC assay is one of the popular indirect methods of determining the antioxidant capacity of compounds or extracts. Besides, the assay is accomplished by generating ABTS radical, therefore it gives information about the free radical scavenging capacity of the samples. Japanese persimmon had the highest activity. Interestingly, cherry laurel showed better activity than cornelian cherry fruit extract, regarding that the trend was different from DPPH and superoxide radical scavenging activities.

Fe^{3+} reduction is commonly used as an indicator of electron donation as well as one of the major mechanisms of actions of antioxidants. The FRAP test is a simple, reproducible and reliable method to measure the ability of antioxidant activity (178). All of the 80% methanolic extracts had an appreciable ferric reducing power in comparison with the reference compound, BHT (Table 30). Moreover, cornelian cherry leaf extract showed better ferric reducing capacity than BHT. The results indicated that Japanese persimmon had the highest activity with a 40% of the capacity of BHT, while cornelian cherry fruit extract had the least. The order of the activity among fruit extracts is different from DPPH and superoxide radical scavenging assays, but the same as that of TEAC assays. Pantelidis et al. (8) and Yılmaz et al. (228) also investigated the FRAP activity of cornelian cherry fruit extract and found similar results to ours, expressed as ascorbic acid equivalents. Although Gülçin et al. used a different methodology to determine the FRAP activity of cornelian cherry fruit extract, they also reported that they found significant activity, but lower than BHT (25).

CUPRAC is a relatively new method on metal ion reducing compared to FRAP. Cu^{2+} also participates in the formation of free radicals just like Fe^{3+} (67). Hence, the reduction of cupric ion is another mechanism that reflects antioxidant potential, and CUPRAC assay is an important parameter in evaluating the activity. According to the results given in Table 31, Japanese persimmon had the highest cupric reducing power, while cornelian cherry fruit had the least among the fruit extracts. The activity of cornelian cherry leaf extract was much higher than the fruit extracts. The order of the fruits in the cupric reducing capacity assay was similar in the ferric reducing power.

Aktümsek et al. recently reported the same correlation between FRAP and CUPRAC assays on different *Centaurea* species (229).

As already mentioned, iron plays substantial role in the generation of free radicals, hence in oxidative stress. Ferrous ions are also commonly found in food systems and considered as prooxidants (207). Therefore, ferrous ion-chelating capacity assay gives important data about antioxidant potential. The metal chelating activity of the extracts was investigated using three different concentrations: 1, 2 and 5 mg/mL. We found no activity of either fruits at the mentioned concentrations. However, as mentioned above, they exerted good reducing activity on both ferric and cupric ions. These data state that an extract with a good rate of metal reducing capacity does not necessarily show good metal chelating activity.

Total antioxidant capacity test is based on the reduction of Mo^{6+} to Mo^{5+}. Consequently, it gives general information about the whole reduction capacity of the samples. The results given in Table 33 indicate that Japanese persimmon possessed the highest capacity but not as higher as cornelian cherry leaf extract did. According to our literature survey, this is the first report on total antioxidant capacity of the fruits determined by the phosphomolybdenum complex.

β-carotene bleaching assay is based on the discoloration of β-carotene owing to its reaction with linoleic acid-generated free radicals in an emulsion system. In the presence of an antioxidant compound, this degradation process is prevented and can be measured spectrophotometrically. It also reflects the ability to inhibit the lipid peroxidation. The results are given in Table 32. All of the investigated extracts inhibited the bleaching of β-carotene with respect to reference compound, BHT. Japanese persimmon had the highest activity among the fruits, but lower than cornelian cherry leaf extract, which is in accordance with other *in vitro* activity tests. However, the difference was much lower than the other tests. Ercisli et al. investigated the same activity of different Japanese persimmon genotypes in Turkey, and found compatible results with ours (223). In addition, Yılmaz et al. studied the β-carotene bleaching activity of

several genotypes of cornelian cherry fruits and found that all of the different genotypes exhibited good activity (228).

All of these eight *in vitro* assays evaluating the antioxidant potential revealed that 80% methanolic extracts prepared from the investigated plants showed significant activity. The results exposed that Japanese persimmon had the highest antioxidant capacity among the fruit extracts. It was observed that cherry laurel had higher ferric and cupric ion reducing capacity and TEAC activity than cornelian cherry fruit. In other tests, however, cornelian cherry fruit showed better activity. On the other hand, in all of the tests cornelian cherry leaf extract had the best activity.

80% methanolic extract of cornelian cherry leaf was found to have by far the highest total phenolic and flavonoid content. As mentioned before, phenolic compounds are important antioxidant compounds. The antioxidant activities of phenolic compounds are mainly due to their ability to act as hydrogen donors, reducing agents and radical scavengers (209). The correlation between phenolic contents and antioxidant properties was determined by using Pearson correlation coefficient, and the results were given in Table 35. According to these results, the scavenging activity of the extracts against both DPPH and superoxide radicals show linear correlation, in relation to their phenolic and flavonoid contents. Whereas, in the case of total proanthocyanidin content, correlation coefficient was much lower. Although total proanthocyanidin content of Japanese persimmon was higher than cornelian cherry leaf extract, the activities were *vice versa*. According to these data, the contribution of proanthocyanidins to antioxidant potential of extract seems to be very low; however flavonoids contribute much to the activity. Besides, there were no statistically significant correlations between total phenolic/flavonoid/proanthocyanidin content and lipid peroxidation inhibitory activity (β-carotene bleaching assay). These findings suggest that the activity of extracts against lipid peroxidation might be attributable to the presence of non-phenolic compounds. An interesting point of view by Huang et al. states that simple phenols, in spite of the fact that some of them have relatively low

antioxidant activity, react with Folin-Ciocalteu reagent. In conclusion, it should be noted that differences in the structure of phenolic compounds as well as the methodology of the antioxidant activity test may cause different results in the assessment of antioxidant activity.

Although *in vitro* antioxidant assays are widely used in the studies of antioxidant activities of plants, the results may sometimes conflict with the results obtained from *in vivo*. The main reason behind this aspect lies on the bioavailability of active compounds.

Most of the health benefits of fruits and vegetables have been attributed to their polyphenol and flavonoid contents. However, their whole mechanism of antioxidant activity remains unclear in spite of indefinite numbers of researches conducted in this area. First, the absorption of polyphenols in organism is rather low, especially that of anthocyanins. Another factor is the short half-life of polyphenols in plasma, which are usually in the range of a couple of hours. In addition of poor absorption, polyphenols and especially flavonoids are extensively metabolized in liver and intestine. Flavonoids are excellent substrates and inducers of phase II enzymes. These factors may cause limitations in the capability of dietary polyphenols in performing antioxidant activity *in vivo.* Therefore, in order to ascertain the role of dietary antioxidants fully, *in vivo* tests are incredibly necessary (167,230–232), though they are so few.

In the light of these data, we studied the *in vivo* antioxidant potentials of 80% methanolic extracts of plants on healthy, male Sprague-Dawley rats. The reason for choosing healthy rats was to observe any possible effects on normal metabolic conditions. By this way, more reliable information might be deducted for making comments about their efficacy in daily life of humans.

The extracts were administered orally for 21 successive days to observe the sub-acute effects. Oral administration was chosen so that the bioavailability of the active components would be observed. After the mentioned period, the rats were decapitated; their fresh blood and liver samples were collected.

For the purpose of evaluating *in vivo* antioxidant activity, we measured the activities of enzymes SOD, CAT and GSH-Px since they form the first line of defense against ROS in the organism by converting the active oxygen molecules into non-toxic compounds (73). They are also referred as primary antioxidant enzymes (67). SOD dismutates superoxide radicals into hydrogen peroxide and molecular oxygen. CAT further detoxifies hydrogen peroxide into water. GSH-Px also participates in the detoxification of hydrogen peroxide. Hence, these enzymes act mutually and compose the enzymatic antioxidant capacity against ROS.

After 21-day treatment we observed no statistically significant changes ($p > 0.05$) in the activities of these enzymes of both total blood and liver homogenates, comparing to the control group. Also, no changes were observed in the positive control group (silymarin), too. This consequence was somehow expected since the consumption of the studied extracts did not induce the synthesis of these enzymes in normal rats. Otherwise this would be an intervention to the homeostasis of the body.

These results are in accordance with the study conducted by Öztürk et al. They investigated the antioxidant capacity of apricot in Wistar rats after 5–months of oral administration. They reported that no changes in the activities of SOD, CAT or GSH-Px in liver were observed in healthy rats (200).

Hsouna et al. investigated the antioxidant activity of *Ceratonia siliqua* leaf extract in rats after 8 days of treatment. They also did not observe any changes in the levels of these enzymes in the liver and kidney of healthy rats, compared to the control group (233). Furthermore, the results of the study performed by Özden et al. also supported our results. They investigated the effects of 28 day–treatment with vitamin E and taurin, which are well-known to be very powerful antioxidant molecules, in the livers and kidneys of normal rats; and they found no changes in the activities of SOD, CAT and GSH-Px (234). Pandanaboina et al. also found no difference in the activities of these enzymes in the liver of vitamin E-administered normal rats (235). In addition, Luczaj et al. studied the effect of sweet grass extract against oxidative stress in rat serum. After 4 weeks

of treatment, they observed no changes in the levels of these enzymes in serum samples (236). They, also, studied the effect of vitamin E administration in normal rats, and concluded the same results. An important study about the antioxidant effects of silymarin was performed by Pradeep et al. After 30 days of oral administration, they observed no changes in the activities of these enzymes in the liver of normal rats (237).

During oxidative stress, membrane lipids are continuously subjected to oxidative reactions. Lipid peroxidation is also one of the most important mechanisms contributing to oxidative stress. Hence, the measurement of lipid peroxidation is an important indicator in the assessment of antioxidant potential. We calculated the amount of MDA, which is one of the most important by-products of lipid peroxidation. For this purpose, we used thiobarbituric acid reactive substances test (TBARS) which is the most widely used test in understanding the levels of lipid peroxidation (1, 238).

We found no statistically significant changes in the levels of MDA in liver tissues or total blood of normal rats ($p > 0.05$). Neither of the extracts nor silymarin managed to induce any decrease in MDA levels, therefore showed no effect on lipid peroxidation in normal metabolic conditions and oxidative stress status. Pradeep et al. stated that 30 day-administration of silymarin caused no alteration in the MDA levels of normal rats (237). Özden et al. (234), Pandanaboina et al. (235) and Luczaj et al. (236) reported that vitamin E did not induce any changes in MDA levels. Hsouna et al. reported the same results for *Ceratonia siliqua* leaf extract (233), and Öztürk et al. for apricot (200).

Another important point about lipid peroxidation that should be highlighted is the correlation between β-carotene bleaching assay and MDA levels. β-carotene bleaching assay is, as previously mentioned, used as a screening tool for the inhibition of lipid peroxidation *in vitro*. However, we observed that although the fruit extracts and cornelian cherry leaf extract showed good activity in β-carotene bleaching assay, they did not induce any decrease in the levels of MDA in normal rats, which is an important precursor of lipid peroxidation.

Since it is well known that the synergic effect of antioxidants in human serum provides better activity against oxidative stress than any antioxidant alone, it is very important to determine the total antioxidant capacity of biological fluids (239). The role of the non–enzymatic antioxidant molecules like glutathione, uric acid, albumin etc. should also be taken into account. They also play significant roles in the defense mechanism of the body against oxidative stress. By this way, it is possible to determine the capacity of the system to fight against oxidative stress. There are diverse methods which are widely used to measure the antioxidant activity of plants and foods in biological fluids. Among them, the oxygen radical absorbance capacity (ORAC) assay, FRAP and TEAC assays (adapted to be effective with *in vivo* tests) are commonly used for this purpose. These techniques are based on different mechanisms with different sources of free radicals or oxidants. Consequently, the results of these assays may not always be correlated with each other (240). For example, Leite et al. investigated the antioxidant potential of *Myrciaria jaboticaba* in healthy rats with both ORAC and TEAC assays. They found the increase in plasma antioxidant capacity 1.7 times by TEAC method, and 1.3 times by ORAC (232). In another study, Gorinstein et al. studied the antioxidant properties of some rare Thai fruits in cholesterol-fed rats with TEAC, DPPH and FRAP, and found 16.9%, 21.9% and 11.8% activity, respectively (241). Interestingly, Tulipani et al. conducted a study about the plasma antioxidant capacity in humans after 4 days of strawberry consumption. They reported an 8.5% increase with FRAP test. However, they found no significant activity with TEAC (242).

As it can be clearly understood from previous studies, different tests for antioxidant capacity may give conflicting results. Among these tests, we chose TEAC assay for evaluating the total antioxidant capacity, since it is widely used in both animal studies (242) and clinical studies (242–244).

According to the results given in Table 40, the fruit extracts did not cause statistically significant increase in the total antioxidant capacity of total blood. However, the experimental animals administered cornelian cherry leaf extract

exerted an increase of 20.51% ($p < 0.01$), besides the reference drug, silymarin also provided an increase of 25.38% ($p < 0.001$).

The liver total antioxidant capacities were observed different. Cornelian cherry, Japanese persimmon and cherry laurel fruit extracts showed increases of 12.87% ($p < 0.05$), 21.71% ($p < 0.01$) and 10.84% ($p < 0.05$), respectively. In addition to these results, the total antioxidant capacity of the livers in cornelian cherry leaf extract administered group had an increase of 29.19% ($p < 0.001$) and that of silymarin as 31.55% ($p < 0.001$).

The increased antioxidant capacity in total blood and liver tissues following the administration of fruit extracts or the leaf extract could indicate a direct absorption of antioxidant phytochemicals (244). It may also indicate an enhanced production of non–enzymatic antioxidants. The enhancement of enzymatic antioxidant production seems a low possibility, since no changes were observed in the levels of these enzymes.

Leite et al. investigated the TEAC in plasma of normal rats administered freeze-dried Jaboticaba fruit peel (*Myrciaria jaboticaba*), and reported an increase of 1.7 times (232). Gorinstein et al. found that TEAC of rat plasma treated with some Thai fruits exerted an increase up to 16.9% (241). Huang et al. studied the effect of sweet potato (*Ipomoea batatas*) in serum TEAC of healthy mice and reported an increase of 12.8%, compared to control group (245).

In addition to the *in vivo* studies, there are some clinical studies that used TEAC for measuring the total antioxidant capacity. Marnewick et al. studied the effects of *Aspalathus linearis* on oxidative stress in adults at risk for cardiovascular disease, but found no alteration (243). Sanchez et al. investigated the influence of whole and fresh-cut mango on plasma antioxidant capacity of healthy adults. They stated that both treatments increased plasma antioxidant capacity measured by TEAC method (244). In addition, Tulipani et al. studied the effect of strawberry consumption for 16 day on plasma

antioxidant status in humans, and reported a significant increase in fasting plasma total antioxidant capacity (242).

After accomplishing *in vitro* screening tests and *in vivo* antioxidant activity studies on healthy rats with normal metabolic conditions, the antioxidant potential was studied under elevated oxidative stress. The data obtained from all of the *in vitro* screening and *in vivo* antioxidant activity tests have clearly demonstrated that 80% methanolic leaf extract of *Cornus mas* L. (cornelian cherry) had by–far the highest antioxidant potential. Thus, we chose this extract for further *in vivo* studies under elevated oxidative stress.

A variety of different types of chemicals have been used in literature to induce increased oxidative stress. Some of the most commonly used of these chemicals are paracetamol (246–248), ethanol (199,249–251), lead (252), diquat (253) and methiocarb (234).

Carbon tetrachloride is a highly toxic chemical agent which is extensively used to study the antioxidant activity, and to evaluate the hepatoprotective effects of drugs and plant extracts. Its hepatotoxic effects are a result of its biotransformation process. CCl_4 is primarily accumulated in hepatic parenchyma cells and metabolized to trichloromethyl ($CCl_3^{\cdot}$) radical by cytochrome P450-dependent monooxygenases in liver. $CCl_3^{\cdot}$ radical reacts rapidly with oxygen to form the highly reactive trichloromethyl peroxyl radical ($CCl_3O_2^{\cdot}$). This radical binds to cellular macromolecules and causes peroxidative damage in lipid membranes of the adipose tissue. It also facilitates the removal of hydrogen atoms from unsaturated lipids. In addition, it causes auto–oxidation of the polyenic fatty acids found in the membrane phospholipids, leading to the lipid peroxidation. Consequently, by-products like MDA are generated in extremely high amount, resulting in the loss of cell membrane integrity and tissue damage (254–256).

For these reasons, we chose CCl_4 for inducing elevated oxidative stress. In order to evaluate the dose–response relationship between the antioxidant

activity and the extract, the leaf extract was administered in increasing doses of 100, 200 and 500 mg/kg body weight, per os.

Silymarin is one of the most commonly used reference drugs in *in vivo* antioxidant activity studies, along with vitamin E (200,234,257) and vitamin C (253). In the previous experiments, silymarin was administered in the various doses such as 25 (258), 50 (78,199,210,259,260), 75 (246), 100 (261) or 200 (262,263) mg/kg bw. Then, we decided to administer silymarin in the dose of 50 mg/kg, since it is the most frequently used dose.

After 5-days of treatment, the animals were decapitated; their fresh blood and liver samples were collected.

The tests indicated that CCl_4 administration caused a vast amount of increase in the MDA levels both in total blood ($p < 0.001$) and liver homogenates ($p < 0.001$), as expected. As mentioned above, elevated MDA level is a direct indicator of lipid peroxidation. Hsouna et al. (233), Öztürk et al. (200), Wang et al. (264) and Verma et al. (257) also reported increased levels of MDA levels after CCl_4 administration.

Although MDA levels tend to decrease in doses of 100 and 200 mg/kg, the differences were not statistically significant ($p > 0.05$). A decrease of 26.23% ($p < 0.05$) and 34.80% ($p < 0.01$) was seen in the dose of 500 mg/kg for leaf extract and 50 mg/kg of silymarin group, respectively. In terms of liver MDA levels, both 200 and 500 mg/kg administration caused statistically significant decrease ($p < 0.01$) (Table 54). The activity of 500 mg/kg administration in liver tissue seemed higher than that of total blood. We also found that silymarin administration caused a decrease of 41.92% ($p < 0.001$), which is very close to the decrease (45.45%) reported by Chen et al. at the same dose and period of treatment (199). Both Hsouna et al. (233). and Pradeep et al. (237) stated that restoration in the levels of lipid peroxidation could be related to the ability of samples to scavenge ROS, thus preventing further damage to membrane lipids.

As discussed in detail above, SOD, CAT and GSH-Px enzymes are the primary antioxidant enzymes, and referred as the first line of defense against oxidative stress.

As given in Tables 46, 47 and 48, the activities of all three enzymes were dramatically decreased both in total blood and liver tissues after CCl_4 administration. These data are in accordance with a vast number of studies that used CCl_4 as an oxidative stress inducer (200,257,258,259,261–263). However, there are some conflicting explanations in literature regarding the decrease in the activities of these enzymes. For example Öztürk et al. (200), Pradeep et al. (237) and Panda et al. (265) annotated the decreased enzyme activities with the elevated free radical generation during the metabolism of CCl_4; therefore the depletion of enzymes was a consequence of their excessive utilization in scavenging these radicals. In addition to these comments, Hsouna et al. (233) associated the reduced activity of these enzymes with the enhancement in the lipid peroxidation. Nevertheless, Verma et al. (257) suggested an opposite comment and stated that the declined activity of these enzymes causes lipid peroxidation. Another point of view is that the decrease in the antioxidant activity may be due to the inhibition of protein biosynthesis or oxidative modifications of these proteins. Free radicals generated might readily give reaction with amino acid residues of proteins leading to severe modifications in the structure of these proteins and consequently to inactivation of these enzymes. Deng et al. (262), Duh et al. (263), Luczaj et al. (236) and Augustyniak et al. (266) shared this point of view. A recent study by Li et al., confirming this perspective, explains the clear interaction of CCl_4 and the inhibition of protein synthesis in liver (267). Morever, Blum and Fridovich stated that the oxidation of the selenocysteine in the active center of GSH-Px, especially in metabolically active liver may cause irreversible inactivation of this enzyme (268).

Although there are some conflicting results about the mechanism of decrease in the enzyme activity, it is obviously related to elevated free radical production. The results given in Tables 46, 47 and 48 indicated that

administration of 80% methanolic leaf extract of cornelian cherry provided the restoration of these enzymes.

Administration in doses of 100 ($p < 0.05$), 200 ($p < 0.05$) and 500 ($p < 0.01$) mg/kg resulted in significant increases in the activity of SOD in total blood samples. In terms of liver SOD, all of the groups, except 100 mg/kg administered–group, again, showed statistically significant activities. It also should be remembered that cornelian cherry leaf extract also showed strong activity against superoxide radical according to our *in vitro* test results.

In 500 mg/kg–treated group of cornelian cherry leaf extract, GSH-Px activities of both total blood and liver tissues were significantly elevated ($p < 0.01$). The increase in the total blood GSH-Px levels (20.37%) was very close to that of liver tissues (19.51%). The increase in the activity of GSH-Px enzyme of this group was near to the activity in silymarin–treated. Although there were numerical increases of enzyme levels in 100 mg/kg and 200 mg/kg–administered groups, these differences were statistically not significant ($p > 0.05$).

The same results are valid for the CAT activities. There was a significant increase in the enzyme levels in both 500 mg/kg extract–administered group and the silymarin group. The differences were statistically significant.

We also investigated the TEAC of both total blood and liver tissues. As previously mentioned, it is important to measure the total antioxidant capacity of biological molecules, since the non–enzymatic antioxidant defenses also play a major role against oxidative stress. The TEAC results of total blood, given in Table 50**,** revealed that CCl_4–administration severely decreased the levels of TEAC in both total blood and liver tissues ($p < 0.001$). The reason for this decline was most probably due to the increased levels of free radical generation. Coudray et al. studied the lipid peroxidation and antioxidant status in experimental diabetes. They reported that streptozotocin–administration decreased the levels of plasma antioxidant capacities in rats significantly (239). According to these data, we can possibly assume that TEAC levels of biological

fluids severely decreases after the administration of oxidative stress inducing agents.

The results of TEAC experiments demonstrated that 200 mg/kg ($p < 0.01$), 500 mg/kg ($p < 0.01$) of the extract and 50 mg/kg of silymarin ($p < 0.001$) administration increased the TEAC of total blood. The increase in 500 mg/kg group (28.49%) was close to the increase in silymarin group (31.55%). The liver TEAC results also revealed similar results. 200 and 500 mg/kg of extract and silymarin administration increased the total antioxidant capacity significantly. Pradeep et al. found that silymarin treatment restored the depleted levels of non-enzymatic antioxidants caused by diethylnitrosamine (237). Fraschini (269) et al. reported that glutathione levels are elevated during silymarin treatment, and GSH homeostasis in the system was maintained. These data exerted that antioxidant molecules act not only in enzymatic defenses but also in non–enzymatic defense parameters. The reason for elevated TEAC values following the extract treatment was most probably due to the same reason. These results indicated that *in vivo* antioxidant activity of 500 mg/kg of cornelian cherry leaf extract was close to that of silymarin, a well-known antioxidant and antihepatotoxic component of *Silybum marianum*. Therefore, it may be postulated that the cornelian cherry leaf extract might be used as an alternative to silymarin. Although the dose of cornelian cherry leaf extract was 10 times higher (500 mg vs. 50 mg), it should be bear in mind that it was a crude extract when compared to silymarin, a mixture of flavonolignans. On the other hand, the leaves of cornelian cherry are used against diabetic complaints in Turkish folk medicine, indicating that they are probably safe for human use. However, further studies are required to obtain clearer results for toxicity profile.

Following the results of the *in vivo* activity tests, activity guided fractionation of 80% methanolic leaf extract of *C. mas* was conducted. *In vitro* assays were used for this purpose in order to avoid sacrificing more animals. DPPH radical scavenging activity and ferric reducing antioxidant activity were chosen because these tests cover the most important mechanisms of

antioxidant activity: free radical scavenging and metal ion reducing. The results indicated that ethyl acetate fraction had the highest activity on both tests as shown in Table 56**.** Further chromatographic studies led to the isolation of gallic acid, a well known antioxidant molecule. The activity studies demonstrated that gallic acid had higher activity than ethyl acetate fraction. Lin et al. also isolated the same molecule from the 70% acetone extract of fruits of *Cornus officinalis* (21).

After the isolation of the bioactive compound, we calculated the amount of gallic acid in 80% methanolic extract of *C. mas* by HPTLC, and found that the extract contains the molecule at a ratio of 0.585 ± 0.007 % (w/w).

6. CONCLUSION

This study was designed to investigate both *in vitro* and *in vivo* antioxidant potentials of widely consumed fruits of three plants (*Cornus mas* L., *Diospyros kaki* L., *Laurocerasus officinalis* Roem.). We also investigated the antioxidant potential of leaves of *C. mas* L. By this way, we aimed to compare the antioxidant capacity in two different organs. We chose the leaf of *C. mas* since it has been reported to be utilized traditionally used against diabetes in Turkey; and the relationship between diabetes and oxidative stress has been clearly demonstrated.

The results obtained from this study showed that all of the 80% methanolic extracts showed antioxidant activity potential. The *in vitro* tests displayed that samples had free radical scavenging activity and the capacity to reduce metal ions that participate in the free radical generation. Besides, the results of β-carotene bleaching assay stated that the samples have the capacity to inhibit *in vitro* lipid peroxidation.

In vivo activity studies also confirmed that all of the 80% methanolic extracts increased the total antioxidant capacity in healthy rats, whereas they did not induce any changes in the activities of antioxidant enzymes.

Studies on CCl_4–treated animals indicated that 80% methanolic extract of *C. mas* leaves restored the lowered activities of antioxidant enzymes, reduced the level of lipid peroxidation, and increased the deteriorated total antioxidant capacity of these animals.

In vitro activity guided fractionation studies of *C.mas* leaves, which demonstrated the highest activities in both *in vitro* and *in vivo* tests, revealed the presence of a well known antioxidant compound, gallic acid.

All of these results showed that the consumption of antioxidant–rich fruits might increase the total antioxidant capacity. On the other hand, *C. mas* leaves,

which is traditionally used against diabetes in Turkey, might have beneficial effects against elevated oxidative stress.

7. REFERENCES

1. Aydin A, Orhan H, Sayal A, Ozata M, Sahin G, Işimer A. Oxidative stress and nitric oxide related parameters in type II diabetes mellitus: Effects of glycemic control. Clin Biochem, 34: 65–70, 2001.

2. Sun L, Zhang J, Lu X, Zhang L, Zhang Y. Evaluation to the antioxidant activity of total flavonoids extract from persimmon (*Diospyros kaki* L.) leaves. Food and Chem Toxicol, 49: 2689–2696, 2011.

3. Willcox JK, Ash SL, Catignani GL. Antioxidants and prevention of chronic disease. Crit Rev Food Sci Nutr, 44: 275–295, 2004.

4. Chamberlain DF. In: Flora of Turkey and the Aegean Islands vol.4. Davis PH, editor. Edinburgh: Edinburgh University Press; 1972. pp. 539–540.

5. Yeşilada E, Sezik E, Honda G, Takaishi Y, Takeda Y, Tanaka T. Traditional medicine in Turkey IX: Folk medicine in north-west Anatolia. J Ethnopharmacol, 64: 195–210, 1999.

6. Pieroni A, Muenz H, Akbulut M, Başer KHC, Durmuşkahya C. Traditional phytotherapy and trans-cultural pharmacy among Turkish migrants living in Cologne, Germany. J Ethnopharmacol, 102: 69–88, 2005.

7. Baytop T. Türkçe Bitki Adları Sözlüğü. 3rd ed. Ankara: Türk Dil Kurumu Yayınları, 2007.

8. Pantelidis G, Vasilakakis M, Manganaris G, Diamantidis G. Antioxidant capacity, phenol, anthocyanin and ascorbic acid contents in raspberries, blackberries, red currants, gooseberries and Cornelian cherries. Food Chem, 102: 777–783, 2007.

9. Sultana N, Lee NH. Antielastase and free radical scavenging activities of compounds from the stems of *Cornus kousa*. Phytother Res, 21: 1171–1176, 2007.

10. Pieroni A, Giusti ME, Münz H, Lenzarini C, Turkovic GT, Turkovic A. Ethnobotanical knowledge of the Istro-Romanians of Zejane in Croatia. Fitoterapia, 74: 710–719, 2003.

11. Menković N, Savikin K, Tasić S, Zdunić G, Stesević D, Milosavljević S, Vincek D. Ethnobotanical study on traditional uses of wild medicinal plants in Prokletije Mountains (Montenegro). J Ethnopharmacol, 133: 97–107, 2011.

12. Pieroni A, Dibra B, Grishaj G, Grishaj I, Gjon Maçai S. Traditional phytotherapy of the Albanians of Lepushe, Northern Albanian Alps. Fitoterapia, 76: 379–399, 2005.

13. Baytop T. Türkiye'de Bitkilerle Tedavi. 2nd ed. Istanbul: Nobel Tıp Kitabevleri; 1999.

14. Kwon S-H, Park H-Y, Kim J-Y, Jeong I-Y, Lee M-K, Seo K-I. Apoptotic action of ursolic acid isolated from Corni fructus in RC-58T/h/SA#4 primary human prostate cancer cells. Bioorg Med Chem Lett, 20: 6435–6438, 2010.

15. Yamahara J, Mibu H, Sawada T, Fujimura H, Takino S, Yoshikawa M, Kitagawa I. Antidiabetic principles of Corni Fructus in experimental diabetes. Yakugaku Zasshi, 101: 86–90, 1981.

16. Graziose R, Rojas-silva P, Rathinasabapathy T, Dekock C, Grace MH, Poulev A, Ann M, Smith S, Raskin I. Antiparasitic compounds from *Cornus florida* L . with activities against *Plasmodium falciparum* and *Leishmania tarentolae*. J Ethnopharmacol, 142: 456–461, 2012.

17. Gao D, Li Q, Gao Z, Wang L. Antidiabetic effects of Corni Fructus extract in streptozotocin-induced diabetic rats. Yonsei Med J, 53: 691–700, 2012.

18. Yamabe N, Kang KS, Goto E, Tanaka T, Yokozawa T. Beneficial effect of Corni Fructus, a constituent of Hachimi-jio-gan, on advanced glycation end-product-mediated renal injury in streptozotocin-treated diabetic rats. Biol Pharm Bull, 30: 520–526, 2007.

19. Yamabe N, Noh JS, Park CH, Kang KS, Shibahara N, Tanaka T, Yokozawa T. Evaluation of loganin, iridoid glycoside from Corni Fructus, on hepatic and renal glucolipotoxicity and inflammation in type 2 diabetic db/db mice. Eur J Pharmacol, 648: 179–187, 2010.

20. Park CH, Tanaka T, Kim HY, Park JC, Yokozawa T. Protective effects of Corni Fructus against advanced glycation endproducts and radical scavenging. Evid-Based Compl Alt Med, 418953, 2012.

21. Lin M, Liu H, Huang W, Huang C, Wu T, Hsu F. Evaluation of the potential hypoglycemic and beta-cell protective constituents isolated from Corni Fructus to tackle insulin-dependent Diabetes Mellitus. J Agr Food Chem, 59: 7743–7751, 2011.

22. Chen C-C, Hsu C-Y, Chen C-Y, Liu H-K. Fructus Corni suppresses hepatic gluconeogenesis related gene transcription, enhances glucose responsiveness of pancreatic beta-cells, and prevents toxin induced beta-cell death. J Ethnopharmacol, 117: 483–90, 2008.

23. Jayaprakasam B, Olson LK, Schutzki RE, Tai M-H, Nair MG. Amelioration of obesity and glucose intolerance in high-fat-fed C57BL/6 mice by anthocyanins and ursolic acid in Cornelian cherry (*Cornus mas*). J Agr Food Chem, 54: 243–248, 2006.

24. Kim D, Park K-K, Lee SK, Lee S-E, Hwang J-K. *Cornus kousa* F.Buerger ex Miquel increases glucose uptake through activation of peroxisome proliferator-activated receptor γ and insulin sensitization. J Ethnopharmacol, 133: 803–809, 2011.

25. Gülçin İ, Beydemir Ş, Şat IG, Küfrevioğlu ÖI. Evaluation of antioxidant activity of cornelian cherry (*Cornus mas* L.). Acta Aliment, 34: 193–202, 2005.

26. Ersoy N, Bağcı Y, Gök V. Antioxidant properties of 12 cornelian cherry fruit types (*Cornus mas* L.) selected from Turkey. Sci Res Essay, 6: 98–102, 2011.

27. Hamid H, Yousef H, Jafar H, Mohammad A. Antioxidant capacity and phytochemical properties of cornelian cherry (*Cornus mas* L.) genotypes in Iran. Sci Horticult 129: 459–463, 2011.

28. Popović BM, Štajner D, Slavko K, Sandra B. Antioxidant capacity of cornelian cherry (*Cornus mas* L.)–Comparison between permanganate reducing antioxidant capacity and other antioxidant methods. Food Chem, 134: 734–741, 2012.

29. Tanaka T, Nishikawa K, Ishimaru K. Antioxidative capacity of extracts and constituents in *Cornus capitata* adventitious roots. J Agr Food Chem, ;51: 5906–5910, 2003.

30. Lee NH, Seo CS, Lee HY, Jung DY, Lee JK, Lee JA, Kim H, Ha H. Hepatoprotective and antioxidative activities of *Cornus officinalis* against acetaminophen-induced hepatotoxicity in mice. Evid-Based Compl Alt Med, 804924, 2012.

31. Turker AU, Yildirim AB, Karakas FP. Antibacterial and antitumor activities of some wild fruits grown in Turkey. Biotechnol Biotech Eq, 26: 2765–2772, 2012.

32. Dulger B, Gonuz A. Antimicrobial activity of some Turkish medicinal plants. Pak J Biol Sci, 7: 1559–1562, 2004.

33. Savikin K, Zdunic G, Jankovic T, Stanojkovic T, Juranic Z, Menkovic N. *In vitro* cytotoxic and antioxidative activity of *Cornus mas* and *Cotinus coggygria*. Nat Prod Res, 23: 1731–1739, 2009.

34. Vareed SK, Schutzki RE, Nair MG. Lipid peroxidation, cyclooxygenase enzyme and tumor cell proliferation inhibitory compounds in *Cornus kousa* fruits. Phytomedicine, 14: 706–709, 2007.

35. Jeong EJ, Kim TB, Yang H, Kang SY, Kim SY, Sung SH, Kim YC. Neuroprotective iridoid glycosides from *Cornus officinalis* fruits against glutamate-induced toxicity in HT22 hippocampal cells. Phytomedicine, 19: 317–321, 2012.

36. Lee D, Kang SJ, Lee SH, Ro J, Lee K, Kinghorn a D. Phenolic compounds from the leaves of *Cornus controversa*. Phytochemistry, 53: 405–407, 2000.

37. Xie X-Y, Wang R, Shi Y-P. Chemical constituents from the fruits of *Cornus officinalis*. Biochem Sys Eco, 45: 120–123, 2012.

38. Pawlowska AM, Camangi F, Braca A. Quali-quantitative analysis of flavonoids of *Cornus mas* L. (Cornaceae) fruits. Food Chem, 119: 1257–1261, 2010.

39. Lee D, Lee SH, Chung SR, Ro J, Lee K. Phenolic components from the leaves of *Cornus controversa* H. Korean J Pharmacog, 1995;26: 327–336, 1995.

40. Ma H, Li L, Seeram NP. Phenolics from *Cornus amomum* Mill. fruit. Biochem SysEco, 38: 1083–1084, 2010.

41. Vareed SK, Reddy MK, Schutzki RE, Nair MG. Anthocyanins in *Cornus alternifolia, Cornus controversa, Cornus kousa* and *Cornus florida* fruits with health benefits. Life Sci, 78: 777–784, 2006.

42. Seeram N, Schutzki RE, Handra AC, Nair MG. Characterization, quantification, and bioactivities of anthocyanins in *Cornus* species. Journal of Agricultural and Food Chem, 50: 2519–2523, 2002.

43. Bjorøy O, Fossen T, Andersen OM. Anthocyanin 3-galactosides from *Cornus alba* with glucosidation of the B-ring. Phytochemistry, 68: 640–645, 2007.

44. Slimestad R, Andersen OM. Cyanidin-3-(2-glucosylgalactoside) and other anthocyanins from fruits of *Cornus suecica*. Phytochemistry, 49: 2163–2166, 1998.

45. Stermitz FR, Krull RE. Iridoid glycosides of *Cornus canadensis*: A comparison with some other *Cornus* species. Biochem Sys Eco, 26: 845–849, 1998.

46. Browicz K. In: Flora of Turkey and the Aegean Islands vol.4. Davis PH, editor. Edinburgh: Edinburgh University Press; 1972. pp. 6–8.

47. Ayaz FA. Studies on water soluble sugar and sugar alcohol in cultivar and wild forms of *Laurocerasus officinalis* Roem. Pak J Bot, 29: 331–336, 1997.

48. Erdemoglu N, Kupeli E, Yesilada E. Anti-inflammatory and antinociceptive activity assessment of plants used as remedy in Turkish folk medicine. J Ethnopharmacol, 89: 123–129, 2003.

49. Akkol EK, Kırmızıbekmez H, Küçükboyacı N, Gören AC, Yeşilada E. Isolation of active constituents from cherry laurel (*Laurocerasus officinalis* Roem.) leaves through bioassay-guided procedures. J Ethnopharmacol, 139: 527–532, 2012.

50. Orhan IE, Akkol EK. Estimation of neuroprotective effects of *Laurocerasus officinalis* Roem. (cherry laurel) by *in vitro* methods. Food Res Int, 44: 818–822, 2011.

51. Kolaylı S, Küçük M, Duran C, Candan F, Dinçer B. Chemical and antioxidant properties of *Laurocerasus officinalis* Roem. (cherry laurel) fruit grown in the Black Sea region. J Agr Food Chem, 51: 7489–7494, 2003.

52. Orhan I, Aydın A, Çölkesen A, Şener B, Işımer AI. Free radical scavenging activities of some edible fruit seeds. Pharm Biol, 41: 163–165, 2003.

53. Tsiklauri GC, Shalashvili AG, Litvinenko VI. Kaempferol glycosides of the leaves of *Laurocerasus officinalis*. Chem Nat Comp 15: 84–85, 1979.

54. Weinges K, Shick H, Lautenschlager M, Schilling G. Isolation and NMR-spectroscopic investigations of (−)-1-O-benzyl-primveroside from green fruits of *Prunus laurocerasus*. Liebigs Ann Chem, 7: 703–705, 1991.

55. Ayaz FA, Kadıoğlu A, Reunanen M, Var M. Phenolic acid and fatty acidcomposition in the fruits of *Laurocerasus officinalis* Roem. and its cultivars. J Food Compos Anal, 10: 350–357, 1997.

56. Yaltırık F. In: Flora of Turkey and the Aegean Islands vol.6. Davis PH, editor. Edinburgh: Edinburgh University Press, 1972. pp. 142–143.

57. Kawase M, Motohashi N, Satoh K, Sakagami H. Biological activity of persimmon peel extracts. Phytother Res, 17: 495–500, 2003.

58. Chen G, Xu SX, Wang HZ, Zhang HQ. Kakispyrol, a new biphenyl derivative from the leaves of *Diospyros kaki*. J Asian Nat Prod Res, 7: 265–268, 2005.

59. Mallavadhani UV, Panda AK, Rao YR. Pharmacology and chemotaxonomy of *Diospyros*. Phytochemistry, 49: 901–951, 1998.

60. Matsushita Y, Jang IN, Imai T, Fukushima K, Lee SC. Antioxidant and cytotoxic activities of naphthalene derivatives from *Diospyros kaki*. J Wood Sci, 57: 161–165, 2011.

61 Jo KJ, Lee JM, Lee SC, Park HY. Anticancer activity of persimmon (*Diospyros kaki* L.) calyx extracts on human cancer cells. J Med Plants Res, 5: 2546–2550, 2011.

62. Sanchetti S, Sanchetti S, Bafna M, Lee SH, Seo SY. Persimmon leaf (*Diospyros kaki*), a potent α-glucosidase inhibitor and antioxidant: Alleviation of postprandial hyperglycemia in normal and diabetic rats. J Med Plants Res, 5: 1652–1658, 2011.

63. Chen G, Xue J, Xu SX, Zhang RQ. Chemical constituents of the leaves of *Diospyros kaki* and their cytotoxic effects. J Asian Nat Prod Res, 9: 347–353, 2007.

64. Ohguchi K, Nakajima C, Oyama M, inhibitory effects of flavonoid glycosides isolated from the peel of Japanese persimmon (*Diospyros kaki* 'Fuyu') on melanin biosynthesis. Biol Pharm Bull, 33: 122–124, 2010.

65. Thuong PT, Lee CH, Dao TT, Nguyen PT, Triterpenoids from the leaves of *Diospyros kaki* (persimmon) and their ınhibitory effects on protein tyrosine phosphatase 1B. J Nat Prod, 71: 1775–1778, 2008.

66. Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. Fourth Edi. New York: Oxford University Press Inc., 2007.

67. Aydın A, Sayal A, Işımer A. Serbest Radikaller ve Antioksidan Savunma Sistemi. Ankara: Gülhane Askeri Tıp Akademisi Basımevi; 2001.

68. Kohen R, Nyska A. Oxidation of biological systems: Oxidative stress phenomena, antioxidants, redox reactions, and methods. Toxicol Pathol, 30: 620–650, 2002.

69. Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell B, 39: 44–84, 2007.

70. Sorg O. Oxidative stress: A theoretical model or a biological reality?. C R Biologies, 327: 649–662, 2004.

71. Sies H. Oxidative stress: Oxidants and antioxidants. Exp Physiol, 82: 291–295, 1997.

72. Kehrer JP. Free radicals as mediators of tissue injury and disease. Crit Rev Toxicol, 23: 21–48, 1993.

73. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. P Natl Acad Sci USA, 90: 7915–7922, 1993.

74. Ames BN, Shigenaga MK, Hagen TM. Mitochondrial decay in aging. Biochim Biophys Acta, 1271: 165–170, 1995.

75. Cadenas E, Davies KJA. Mitochondrial free radical generation, oxidative stress, and aging. Free Radic Biol Med, 29: 222–230, 2000.

76. Ramallo IA, Zacchino SA, Furlan RLE. A rapid TLC autographic method for the detection of xanthine oxidase inhibitors and superoxide scavengers. Phytochem Analysis 17: 15–19, 2006.

77. McCord JM, Fridovich I. The biology and pathology of oxygen radicals. Ann Intern Med, 89: 122–127, 1978.

78. Rifkind JM, Ramasamy S, Manoharan PT, Nagababu E, Mohanty JG. Redox reactions of hemoglobin. Antioxid Redox Sign, 6: 657–666, 2004.

79. Sies H, De Groot H. Role of oxygen species in cell toxicity. Toxicol Lett, 64/65: 547–551, 1992.

80. Shadyro OI, Yurkova IL, Kisel MA. Radiation-induced peroxidation and fragmentation of lipids in a model membrane. Int J Radiat Biol. 78: 211–217, 2002.

81. Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: An overview. Method Enzymol, 186: 1–85, 1990.

82. Brent JA, Rumack BH. Role of free radicals in toxic hepatic injury I. Free radical biochemistry. Clin Toxicol, 31: 139–171, 1993.

83. Buechter D. Free radicals and oxygen toxicity. Pharm Res, 5: 253–260, 1988.

84. Gutteridge JMC. Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clin Chem, 41: 1819–1828, 1995.

85. Halliwell B, Chirico S. Lipid peroxidation: Its mechanism and significance. Am J Clin Nutr. 57: 715S–725S, 1993.

86. Halliwell B, Gutteridge JMC. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J, 219: 1–14, 1984.

87. Pastor N, Weinstein H, Jamison E, Brenowitz M. A detailed interpretation of OH radical footprints in a TBP-DNA complex reveals the role of dynamics in the mechanism of sequence-specific binding. J Mol Biol, 304: 55–68, 2000.

88. Mello Filho AC, Hoffmann ME, Meneghini R. Cell killing and DNA damage by hydrogen peroxide are mediated by intracellular iron. Biochem J, 218: 273–275, 1984.

89. Koppenol WH. The Haber-Weiss cycle – 70 years later. Redox Rep, 6: 229–234, 2001.

90. Halliwell B. Oxygen species in living systems: Source, biochemistry, and role in human disease. Am J Med, 91: 14S–22S, 1991.

91. Dukan S, Belkin S, Touati D. Reactive oxygen species are partially involved in the bacteriocidal action of hypochlorous acid. Arch Biochem Biophys, 367: 311–316, 1999.

92. Spencer JPE, Whiteman M, Jenner A, Halliwell B. Nitrite-induced deamination and hypochlorite-induced oxidation of DNA in intact respiratory tract epithelial cells. Free Radic Biol Med, 28: 1039–1050, 2000.

93. Cochrane CG. Cellular injury by oxidants. Am J Med, 91: 23S–30S, 1991.

94. Yin H, Xu L, Porter NA. Free radical lipid peroxidation: mechanisms and analysis. Chem Rev, 111: 5944–5972, 2011.

95. De Zwart LL, Meerman JHN, Commandeur JNM, Vermeulen NPE. Biomarkers of free radical damage applications in experimental animals and in humans. Free Radic Biol Med, 26: 202–226, 1999.

96. Moore K, Roberts II J. Measurement of lipid peroxidation. Free Radic Res, 28: 659–671, 1998.

97. Sugiyama M. Role of cellular antioxidants in metal-induced damage. Cell Biol Toxicol,10: 1–22, 1994.

98. Davies KJA. Protein damage and degradation by oxygen radicals. J Biol Chem, 262: 9895–9901, 1987.

99. Peng SL, Fatenejad S, Craft J. Scleroderma: A disease related to damaged proteins. Nat Med, 3: 276–278, 1997.

100. Cooke MS, Evans MD, Dizdaroglu M, Lunec J. Oxidative DNA damage: mechanisms, mutation, and disease. FASEB J, 17: 1195–1214, 2003.

101. Evans MD, Dizdaroglu M, Cooke MS. Oxidative DNA damage and disease: induction, repair and significance. Mutat Res, 567: 1–61, 2004.

102. Halliwell B. How to characterize a biological antioxidant. Free Radic Res Comm, 9: 1–32, 1990.

103. Niki E. Assessment of antioxidant capacity *in vitro* and *in vivo.* Free Radic Biol Med, 49: 503–515, 2010.

104. McCord J. M, Fridovich I. Superoxide dismutase: An enzymic function for erythrocuprein (hemocuprein). J Biol Chem, 244: 6049–6055, 1969.

105. Fridovich I. Superoxide radical and SODs. Annu Rev Biochem, 64: 97–112, 1995.

106. Öner G, Şentürk ÜK, İzgüt N. The effect of manganese on erythrocyte superoxide dismutase. Turk J Med Sci, 19: 283–286, 1993.

107. Yost FJ, Fridovich I. An iron-containing superoxide dismutase from *Escherichia coli.* J Biol Chem, 248: 4905–4908, 1973.

108. Duke MV, Salin ML. Purification and characterization of an iron-containing superoxide dismutase from a eukaryote, *Ginkgo biloba*. Arch Biochem Biophys, 243: 305–314, 1985.

109. Wuerges J, Lee J-W, Yim Y-I, Yim H-S, Kang S-O, Djinovic Carugo K. Crystal structure of nickel-containing superoxide dismutase reveals another type of active site. P Natl Acad Sci USA, 101: 8569–8574, 2004.

110. Sumner JB, Dounce AL. Crystalline Catalase. J Biol Chem, 121: 417–424, 1937

111. Reed TJ, Murthy MRN, Sicignanot A, Tanakat N, Musick WDL, Rossmann MG, Structure and heme environment of beef liver catalase at 2.5 A resolution. P Natl Acad Sci USA, 78: 4767–4771, 1981.

112. Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. Physiol Rev, 59: 527–605, 1979.

113. Mills GC. Hemoglobin catabolism: I. Glutathione peroxidase, an erythrocyte enzyme which protects hemoglobin from oxidative breakdown. J Biol Chem. 229: 189–197, 1957

114. Goodwin WJ, Lane HW, Bradford K, Marshall MV, Griffin C, Geopfert H. Selenium and glutathione peroxidase levels in patients with epidermoid carcinoma of the oral cavity and oropharynx. Cancer, 51: 110–115, 1983.

115. Lu SC. Regulation of hepatic glutathione synthesis: Current concepts and controversies. FASEB J, 13: 1169–1183, 1999.

116. Dickinson D a, Forman HJ. Cellular glutathione and thiols metabolism. Biochem Pharmacol, 64: 1019–1026, 2002.

117. Hanna PM, Mason RP. Direct evidence for inhibiton of free radical formation from Cu(I) and H_2O_2 by GSH and other potential ligands using the EPR spin technique. Arch Biochem Biophys, 295: 205–213, 1992.

118. Ames BN, Cathcart R, Schwiers E, Hochstein P. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. P Natl Acad Sci USA, 78: 6858–6862, 1981.

119. Squadrito GL, Cueto R, Splenser a E, Valavanidis A, Zhang H, Uppu RM. Reaction of uric acid with peroxynitrite and implications for the mechanism of neuroprotection by uric acid. Arch Biochem Biophys, 376: 333–337, 2000.

120. Okatani Y, Wakatsuki A, Shinohara K, Kaneda C, Fukaya T. Melatonin stimulates glutathione peroxidase activity in human chorion. J Pineal Res, 30: 199–205, 2001.

121. Albarrán MT, López-Burillo S, Pablos MI, Reiter RJ, Agapito MT. Endogenous rhythms of melatonin, total antioxidant status and superoxide dismutase activity in several tissues of chick and their inhibition by light. J Pineal Res, 30: 227–233, 2001.

122. Roche M, Rondeau P, Singh NR, Tarnus E, Bourdon E. The antioxidant properties of serum albumin. FEBS Lett, 582: 1783–1787, 2008.

123. Bourdon E, Blache D. The importance of proteins in defense against oxidation. Antioxid Redox Sign, 3: 293–311, 2001.

124. Halliwell B, Gutteridge JMC. The antioxidants of human extracellular fluids. Arch Biochem Biophys, 280: 1–8, 1990.

125. Prohaska JR, Gybina AA. Intracellular copper transport in mammals. J Nutr, 134: 1003–1006, 2004.

126. Biewenga GP, Haenen GR, Bast A. The pharmacology of the antioxidant lipoic acid. Gen Pharmacol, 29: 315–331, 1997.

127. Beckman JA, Goldfine AB, Gordon MB, Creager MA. Ascorbate restores endothelium-dependent vasodilation impaired by acute hyperglycemia in humans. Circulation, 103: 1618–1623, 2001.

128. Augustin a J, Böker T, Blumenröder SH, Lutz J, Spitznas M. Free radical scavenging and antioxidant activity of allopurinol and oxypurinol in experimental lens-induced uveitis. Invest Ophth Vis Sci 35: 3897–3904, 1994.

129. Escobar J, Pereda J, Arduini A, Sandoval J, Moreno ML, Pérez S. Oxidative and nitrosative stress in acute pancreatitis: Modulation by pentoxifylline and oxypurinol. Biochem Pharmacol, 83: 122–130, 2012.

130. Chauhan A, Chauhan V, Brown WT, Cohen I. Oxidative stress in autism: increased lipid peroxidation and reduced serum levels of ceruloplasmin and transferrin--the antioxidant proteins. Life Sci, 75: 2539–2549, 2004.

131. Ernster L, Dallner G. Biochemical, physiological and medical aspects of ubiquinone function. Biochim Biophys Acta, 1271: 195–204, 1995.

132. Gülçin İ. Antioxidant activity of food constituents: An overview. Arch Toxicol, 86: 345–391, 2012.

133. Sies H, Stahl W. Vitamins E and C , β-carotene , and other carotenoids as antioxidants. Am J Clin Nutr, 62 (Suppl): 1315–1321, 1995.

134. Diplock T, Charleux JL, Crozier-Willi G, Kok FJ, Rice-Evans C, Roberfroid M. Functional food science and defence against reactive oxidative species. Brit J Nutr, 80 (Suppl): S77–S112, 1998.

135. Traber MG, Atkinson J. Vitamin E, antioxidant and nothing more. Free Radic Biol Med, 43: 4–15, 2007.

136. Burton GW, Ingold KU. Autoxidation of biological molecules. 1. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants *in vitro.* J Am Chem Soc,103: 6472–6477, 1981.

137. Traber MG, Sies H. Vitamin E in humans: demand and delivery. Annu Rev Nutr, 16: 321–347, 1996.

138. Halliwell B. Antioxidants in human health and disease. Annu Rev Nutr, 16: 33–50, 1996.

139. Brewer MS. Natural antioxidants: Sources, compounds, mechanisms of action, and potential applications. Compr Rev in Food Sci Food Safety, 10:221–247, 2011.

140. Frei B, England L, Ames BN. Ascorbate is an outstanding antioxidant in human blood plasma. P Natl Acad Sci USA, 86: 6377–6381, 1989.

141. Padayatty SJ, Katz A, Wang Y, Eck P, Kwon O, Lee J-H. Vitamin C as an antioxidant: evaluation of its role in disease prevention. J Am Coll Nutr, 22: 18–35, 2003.

142. Prockop DJ, Kivirikko K. Collagens: Molecular biology, diseases, and potentials for therapy. Annu Rev Biochem, 64: 403–434, 1995.

143. Rebouche C. Ascorbic acid and carnitine biosynthesis. Am J Clin Nutr, 54: 1147S–1152S, 1991.

144. Kaufman S. Dopamine-beta-hydroxylase. J Psychiatr Res, 11: 303–306, 1974.

145. Halliwell B. Vitamin C: Antioxidant or prooxidant *in vivo*?. Free Radic Res, 25: 439–454, 1996.

146. Duarte TL, Lunec J. When is an antioxidant not an oxidant? A review of novel actions and reactions of vitamin C. Free Radic Res, 39: 671–686, 2005

147. Maiani G, Castón MJP, Catasta G, Toti E, Cambrodón IG, Bysted A. Carotenoids: actual knowledge on food sources, intakes, stability and bioavailability and their protective role in humans. Mol Nutr Food Res, 53 (Suppl 2): 194–218, 2009.

148. Stahl W, Sies H. Antioxidant activity of carotenoids. Mol Aspects Med, 24: 345–351, 2003.

149. Namitha KK, Negi PS. Chemistry and biotechnology of carotenoids. Crit Rev Food Sci Nutr, 50: 728–760, 2010.

150. Stahl W, Schwarz W, Sies H. Human serum concentrations of all *trans* β- and α-carotene but not 9-*cis* beta carotene increase upon ingestion of a natural isomer mixtureobtained from *Dunaliella salina* (Betatene). J Nutr, 123: 847–851, 1993.

151. Stahl W, Sies H. Physical quenching of singlet oxygen and *cis-trans* isomerization of carotenoids. Ann NY Acad Sci, 691: 10–19, 1993.

152. Liebler DC. Antioxidant reactions of carotenoids. Ann NY Acad Sci, 691: 20–31, 1993.

153. Burton GW, Ingold KU. β-carotene: An unusual type of lipid antioxidant. Science, 224: 569–573, 1984.

154. Leopoldini M, Russo N, Toscano M. The molecular basis of working mechanism of natural polyphenolic antioxidants. Food Chem, 125: 288–306, 2011.

155. Birt DF, Hendrich S, Wang W. Dietary agents in cancer prevention: Flavonoids and isoflavonoids. Pharmacol Therapeut, 90: 157–177, 2001.

156. Rechner AR, Spencer JPE, Kuhnle G, Hahn U, Rice-Evans C. Novel biomarkers of the metabolism of caffeic acid. Free Radic Biol Med, 30: 1213–1222, 2001.

157. Robbins RJ. Phenolic acids in foods: an overview of analytical methodology. J Agr Food Chem, 51: 2866–2887, 2003.

158. King A, Young G. Characteristics and occurence of phenolic phytochemicals. J Am Diet Assoc, 99: 213–218, 1999.

159. Winkel-Shirley B. Flavonoid biosynthesis: A colorful model for genetics, biochemistry, cell biology and technology. Plant Physiol, 126: 485–493, 2001.

160. Harborne JB. Phytochemical Methods. Third edit. London: Chapman & Hall; 1998.

161. Kandaswami C, Middleton E. Free radical scavenging and antioxidant activity of plant flavonoids. Adv Exp Med Biol, 366: 351–376, 1994.

162. Duarte J, Perez-Vizcainom F, Utrilla P, Jimenez J, Tanargo J, Zarzuello A. Vasodilatory effects of flavonoids in rat aortic smooth muscle, structure-activity relationships. Gen Pharmacol, 24: 857–862, 1993.

163. Rice-Evans C, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med, 20: 933–956, 1996.

164. Prior RL, Cao G. Antioxidant phytochemicals in fruits and vegetables: Diet and health implications. Hortic Sci, 35: 588–592, 2000.

165. Rice-Evans C, Miller NJ, Paganga G. Antioxidant properties of phenolic. Trends Plant Sci, 2: 152–159, 1997.

166. Jovanovic SV, Hara Y, Steenken S, Simic MG. Antioxidant potentials of theaflavins. A pulse radiolysis study. J Am Chem Soc, 119: 5337–5343, 1997.

167. Rice-Evans C. Flavonoid antioxidant. Curr Med Chem, 8: 797–807, 2001.

168. Pietta PG. Flavonoids as antioxidants. J Nat Prod, 63:1035–1042, 2000.

169. Prochazkova D, Bousova I, Wilhelmova N. Antioxidant and prooxidant properties of flavonoids. Fitoterapia, 82: 513–523, 2011.

170. Sroka Z, Cisowski W. Hydrogen scavenging, antioxidant and antiradical activity of some phenolic acids. Food Chem Toxicol, 41: 753–758, 2003.

171. Chimi H, Cillard J, Cillard P, Rahmani M. Peroxyl and hydroxyl radical scavenging activity of some natural phenolic antioxidants. J Am Chem Soc, 68: 307–312, 1991.

172. Heim KE, Tagliaferro AR, Bobilya DJ. Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships. J Nutr Biochem, 13: 572–584, 2002.

173. Chlebda E, Magdalan J, Merwid-Lad A, Trocha M, Kopacz M. Influence of water-soluble flavonoids, quercetin-5'-sulfonic acid sodium slat and morin-5'-sulfonic acid sodium salt, on antioxidant parameters in the subacute cadmium intoxication mouse model. Exp Toxicol Pathol, 62: 105–108, 2010.

174. Plumb GW, Price KR, Williamson G. Antioxidant properties of flavonol glycosides from tea. Redox Rep, 4: 13–16, 1999.

175. Mora A, Paya M, Rios JL, Alcaraz MJ. Structure-activity relationships of polymethoxyflavones and other flavonoids as inhibitors of non-enzymic lipid peroxidation. Biochem Pharmacol, 40: 793–797, 1990.

176. Limasset B, Le Doucen C, Dore JC, Ojasoo T, Damon M, De Paulet AC. Effects of flavonoids on the release of reactive oxygen species by stimulated human neutrophils. Biochemical Pharmacol, 46: 1257–1271, 1993.

177. Halliwell B. Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies?. Arch Biochem Biophysics 476: 107–112, 2008.

178. Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agr Food Chem, 53: 4290–4302, 2005.

179. Ou B, Hampsch-Woodill M, Prior RL. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. J Agr Food Chem, 49: 4619–4626, 2001.

180. Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J Agr Food Chem, 53: 1841–1856, 2005.

181. Ou B, Hampsch-Woodill M, Flanagan J, Deemer EK, Prior RL, Huang D. Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. J Agr Food Chem, 50: 2772–2777, 2002.

182. Winston GW, Regoli F, Dugas A J, Fong JH, Blanchard K A. A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids. Free Radic Biol Med, 24: 480–493, 1998.

183. Miller HM. A simplified method for the evaluation of antioxidants. J Am Oil Chem Soc, 45: 91, 1971.

184. Hossain MA, Rahman SMM. Total phenolics, flavonoids and antioxidant activity of tropical fruit pineapple. Food Res Int, 44: 672–676, 2011.

185. Benzie IFF, Strain JJ. The ferric reducingability of plasma (FRAP) as a measure of “antioxidant power”: The FRAP assay. Anal Biochem, 239: 70–76, 1996.

186. Pulido R, Bravo L, Saura-Calixto F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. J Agr Food Chem, 48: 3396–3402, 2000.

187. Apak R, Güçlü K, Özyürek M, Karademir SE. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. J Agr Food Chem, 52: 7970–7981, 2004.

188. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature, 26:1199–1200, 1958.

189. Miller NJ, Diplock AT, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin Sci, 84: 407–412, 1993.

190. Re R, Pelligrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med, 26: 1231–1237, 1999.

191. Miller NJ, Sampson J, Candeias LP, Bramley PM, Rice-Evans C. Antioxidant activities of carotenes and xanthophylls. FEBS Lett, 384: 240–242, 1996.

192. Cano A, Alcaraz O, Acosta M, Arnao MB. On-line antioxidant activity determination: Comparison of hyrophilic and lipophilic antioxidant activity using the $ABTS^{\cdot+}$ assay. Redox Rep, 7: 103–109, 2002.

193. Awika JM, Rooney LW, Wu X, Prior RL. Screening methods to measure antioxidant capacity of sorghum (*Sorghum bicolor*) and sorghum products. Food Chem, 51: 6657–6662, 2003.

194. Halliwell B, Zhao K, Whiteman M. The gastrointestinal tract: A major site of antioxidant action. Free Radic Res, 33: 819–830, 2000.

195. Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A. Biomarkers of oxidative damage in human damage. Clin Chem, 52: 601–623, 2006.

196. Weber LWD, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: Carbon tetrachloride as a toxicological model. Criti Rev Toxicol, 33: 105–136, 2003.

197. Wikipedia. http://en.wikipedia.org/wiki/Carbon_tetrachloride. Wikipedia.

198. Manibusan MK, Odin M, Eastmond DA. Postulated carbon tetrachloride mode of action: A review. J Environ Sci Health C, 25: 185–209, 2007.

199. Chen Y, Huang B, He J, Han L, Zhan Y, Wang Y. *In vitro* and *in vivo* antioxidant effects of the ethanolic extract of *Swertia chirayita*. J Ethnopharmacol, 136: 309–315, 2011.

200. Ozturk F, Gul M, Ates B, Ozturk IC, Cetin A, Vardi N, Otlu A, Yılmaz I. Protective effect of apricot (*Prunus armeniaca* L.) on hepatic steatosis and damage induced by carbon tetrachloride in Wistar rats. Brit J Nutr, 102: 1767–1775, 2009.

201. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Viticult, 16: 144–158, 1965.

202. Woisky R, Salatino A. Analysis of propolis: Some parameters and procedures for chemical quality. J Apic Res, 37: 99–105, 1998.

203. Kiranmai M, Kumar CBM, Ibrahim M. Comparison of total flavanoid content of Azadirachta indica root bark extracts prepared by different methods of extraction. Res J Pharm Biol Chem Sci, 2: 254–261, 2011.

204. Ariffin F, Chew SH, Bhupinder K, Karim AA. Antioxidant capacity and phenolic composition of fermented *Centella asiatica* herbal teas. J Sci Food Agr 91: 2731–2739, 2011.

205. Nakamura Y, Tsuji S, Tonogai Y. Analysis of proanthocyanidins in grape seed extracts. J Health Sci, 49: 45–54, 2003.

206. Akter S, Ahmed M, Eun JB. Solvent effects on antioxidant properties of persimmon (*Diospyros kaki* L. cv. Daebong) seeds. Int J Food Sci Tech, 45: 2258–2264, 2010.

207. Guo T, Wei L, Sun J, Hou CL, Fan L. Antioxidant activities of extract and fractions from *Tuber indicum* Cooke & Massee. Food Chem, 127: 1634–1640, 2011.

208. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific

application to the determination of vitamin E. Anal Biochem, 269: 337–341, 1999.

209. Zovko Končić M, Kremer D, Karlović K, Kosalec I. Evaluation of antioxidant activities and phenolic content of *Berberis vulgaris* L. and *Berberis croatica* Horvat. Food Chem Toxicol, 48: 2176–2180, 2010.

210. Huang B, Ban X, He J, Zeng H, Zhang P, Wang Y. Hepatoprotective and antioxidant effects of the methanolic extract from *Halenia elliptica*. J Ethnopharmacol, 131: 276–281, 2010.

211. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin Phenol Reagent. J Biol Chem, 193: 265–275, 1951.

212. Pleban PA, Munyani A, Beachum J. Determination of selenium concentration and glutathione peroxidase activity in plasma and erythrocytes. Clin Chem, 28: 311–316, 1982.

213. Aebi H. Catalase *in vitro*. Method Enzymol, 105: 121–126, 1984.

214. Jamall IS, Smith JC. Effects of cadmium on glutathione peroxidase, superoxide dismutase, and lipid peroxidation in the rat heart: A possible mechanism of cadmium cardiotoxicity. Toxicol Appl Pharmacol, 80: 33–42, 1985.

215. Sonawane SD, Nirmal S a., Patil a. N, Pattan SR. Development and validation of HPTLC method to detect curcumin and gallic acid in polyherbal formulation. J Liq Chromatogr Rel Tech, 34: 2664–2673, 2011.

216. Chanwitheesuk A, Teerawutgulrag A, Kilburn JD, Rakariyatham N. Antimicrobial gallic acid from *Caeselpinia mimosoides* Lamk. Food Chem, 100: 1044–1048, 2007.

217. Gaziano JM, Mansoın JE, Buring JE, Hennekens CH. Dietary antioxidants and cardiovascular disease. Ann NY Acad Sci, 669: 249–259, 1992.

218. Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Willet WC. Vitamin E consumption and the risk of coronary heart disease in men. New Engl J Med, 328: 1450–1460, 1993.

219. Block G, Patterson BH, Subar AF. Fruit, vegetables and cancer prevention: A review of the epdemiological evidence. Nutr Cancer,18: 1–29, 1992.

220. Lean ME, Noroozi M, Kelly I, Burns J, Talwar D, Sattar N. Dietary flavonols protect diabetic human lymphocytes against oxidative damage to DNA. Diabetes, 48: 176–181, 1999.

221. Proteggente AR, Pannala AS, Paganga G, Buren LV, Wagner E, Wiseman S. The antioxidant activity of regularly consumed fruit and vegetables reflects their phenolic and vitamin C composition. Free Radic Res. 36: 217–233, 2002.

222. Ndhlala AR, Moyo M, Van Staden J. Natural antioxidants: Fascinating or mythical biomolecules?. Molecules, 15: 6905–6930, 2010.

223. Ercisli S, Akbulut M, Ozdemir O, Sengul M, Orhan E. Phenolic and antioxidant diversity among persimmon (*Diospyrus kaki* L.) genotypes in Turkey. Int J Food Sci Nutr, 59: 477–482, 2008.

224. Naczk M, Shahidi F. Extraction and analysis of phenolics in food. J Chromatogr A, 54: 95–111, 2004.

225. Liyana-Pathirana CM, Shahidi F, Alasalvar C. Antioxidant activity of cherry laurel fruit (*Laurocerasus officinalis* Roem.) and its concentrated juice. Food Chem, 99: 121–128, 2006.

226. Han J, Kang S, Choue R, Kim H, Leem K, Chung S. Free radical scavening effect of *Diospyros kaki*, *Laminaria japonica* and *Undaria pinnatifida*. Fitoterapia, 73: 710–712, 2002.

227. Sakanaka S, Tachibana Y, Okada Y. Preparation and antioxidant properties of extracts of *Japanese persimmon* leaf tea (kakinoha-cha). Food Chem, 89: 569–575, 2005.

228. Yilmaz K, Ercisli S, Zengin Y, Sengul M, Kafkas E. Preliminary characterisation of cornelian cherry (*Cornus mas* L.) genotypes for their physico-chemical properties. Food Chem, 114:408–412, 2009.

229. Aktumsek A, Zengin G, Guler GO, Cakmak YS, Duran A. Screening for *in vitro* antioxidant properties and fatty acid profiles of five *Centaurea* L. species from Turkey flora. Food Chem Toxicol, 49: 2914–2920, 2011.

230. Lotito SB, Frei B. Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: Cause, consequence, or epiphenomenon?. Free Radic Biol Med, 41: 1727–1746, 2006.

231. Kong AN, Owuor E, Yu R, Hebbar V, Chen C, Hu R, Mandlekar S. Induction of xenobiotic enzymes by the MAP kinase pathway and the antioxidant or electrophile response element (ARE/EpRE). Drug Metabol Rev, 33: 255–271, 2001.

232. Leite AV, Malta LG, Riccio MF, Eberlin MN, Pastore GM, Maróstica Júnior MR. Antioxidant potential of rat plasma by administration of freeze-dried jaboticaba peel (*Myrciaria jaboticaba* Vell Berg). J Agr Food Chem, 59: 2277–2283, 2011.

232. Hsouna AB, Saoudi M, Trigui M, Jamoussi K, Boudawara T, Jaoua S, Feki AE. Characterization of bioactive compounds and ameliorative effects of *Ceratonia siliqua* leaf extract against CCl_4 induced hepatic oxidative damage and renal failure in rats. Food Chem Toxicol, 49: 3183–3191, 2011.

234. Ozden S, Catalgol B, Gezginci-Oktayoglu S, Arda-Pirincci P, Bolkent S, Alpertunga B. Methiocarb-induced oxidative damage following subacute exposure and the protective effects of vitamin E and taurine in rats. Food Chem Toxicol, 47: 1676–1684, 2009.

235. Pandanaboina SC, Kondeti SR, Rajbanshi SL, Kunala PN, Pandanaboina S, Pandanaboina MM. Alterations in antioxidant enzyme activities and oxidative damage in alcoholic rat tissues: Protective role of *Thespesia populnea*. Food Chem, 132: 150–159, 2012.

236. Luczaj W, Stankiewicz-Kranc A, Milewska E, Roszkowska-Jakimiec W, Skrzydlewska E. Effect of sweet grass extract against oxidative stress in rat liver and serum. Food Chem Toxicol, 50: 135–140, 2012.

237. Pradeep K, Mohan CVR, Gobianand K, Karthikeyan S. Silymarin modulates the oxidant-antioxidant imbalance during diethylnitrosamine induced oxidative stress in rats. Eur J Pharmacol, 560: 110–116, 2007.

238. Rattmann YD, Mendéz-Sánchez SC, Furian AF, Paludo KS, de Souza LM, Dartora N, Costa MD. Standardized extract of *Dicksonia sellowiana* Presl. Hook (Dicksoniaceae) decreases oxidative damage in cultured endothelial cells and in rats. J Ethnopharmacol, 133: 999–1007, 2011.

239. Feillet-Coudray C, Rock E, Coudray C, Grzelkowska K, Azais-Braesco V, Dardevet D. Lipid peroxidation and antioxidant status in experimental diabetes. Clin Chim Acta, 284: 31–43, 1999.

240. Rice-Evans C, Miller NJ. Total antioxidant status in plasma and body fluids. Methods Enzymol, 234: 279–293, 1994.

241. Gorinstein S, Poovarodom S, Leontowicz H, Leontowicz M, Namiesnik J, Vearasilp S. Antioxidant properties and bioactive constituents of some rare exotic Thai fruits and comparison with conventional fruits. Food Res Int, 44: 2222–2232, 2011.

242. Tulipani S, Alvarez-Suarez JM, Busco F, Bompadre S, Quiles JL, Mezzetti B. Strawberry consumption improves plasma antioxidant status and erythrocyte resistance to oxidative haemolysis in humans. Food Chem. 128: 180–186, 2011.

243. Marnewick JL, Rautenbach F, Venter I, Neethling H, Blackhurst DM, Wolmarans P, et al. Effects of rooibos (*Aspalathus linearis*) on oxidative stress and biochemical parameters in adults at risk for cardiovascular disease. J Ethnopharmacol 133: 46–52, 2011.

244. Robles-Sánchez M, Astiazarán-García H, Martín-Belloso O, Gorinstein S, Alvarez-Parrilla E, de la Rosa L A. Influence of whole and fresh-cut mango intake on plasma lipids and antioxidant capacity of healthy adults. Food Res Int, 44: 1386–1391, 2011.

245. Huang G, Chang H, Chen H, Lu T, Chang Y, Sheu M. Effects of trypsin inhibitor on plasma antioxidant activity and lipid levels in mice from sweet potato roots. J Sci Food Agr, 88: 2556–2562, 2008.

246. Nagalekshmi R, Menon A, Chandrasekharan DK, Nair CKK. Hepatoprotective activity of *Andrographis paniculata* and *Swertia chirayita*. Food Chem Toxicol, 49: 3367–3373, 2011.

247. Olaleye MT, Akinmoladun AC, Ogunboye A, Akindahunsi AA . Antioxidant activity and hepatoprotective property of leaf extracts of *Boerhaavia diffusa* Linn against acetaminophen-induced liver damage in rats. Food Chem Toxicol, 48: 2200–2205, 2010.

248. Janbaz KH, Gilani AH. Potentiation of paracetamol and carbon tetrachloride-induced hepatotoxicity in rodents by the food additive vanillin. Food Chem Toxicol, 37: 603–607, 1999.

249. Faremi TY, Suru SM, Fafunso MA, Obioha UE. Hepatoprotective potentials of *Phyllanthus amarus* against ethanol-induced oxidative stress in rats. Food Chem Toxicol, 46: 2658–2664, 2008.

250. Khanal T, Choi JH, Hwang YP, Chung YC, Jeong HG. Saponins isolated from the root of *Platycodon grandiflorum* protect against acute ethanol-induced hepatotoxicity in mice. Food Chem Toxicol, 47: 530–535, 2009.

251. Pari L, Suresh A. Effect of grape (*Vitis vinifera* L.) leaf extract on alcohol induced oxidative stress in rats. Food Chem Toxicol, 46: 1627–1634, 2008.

252. Liu C-M, Ma J-Q, Sun Y-Z. Protective role of puerarin on lead-induced alterations of the hepatic glutathione antioxidant system and hyperlipidemia in rats. Food Chem Toxicol, 49: 3119–3127, 2011.

253. Lu T, Piao XL, Zhang Q, Wang D, Piao XS, Kim SW. Protective effects of *Forsythia suspensa* extract against oxidative stress induced by diquat in rats. Food Chem Toxicol, 48: 764–770, 2010.

254. Recknagel RO. A new direction in the study of carbon tetrachoride hepatotoxicity. Life Sci, 33: 401–408, 1983.

255. Recknagel RO, Glende EA, Dolak JA, Waller RL. Mechanism of carbon tetrachloride toxicity. Pharmacol Therapeut, 43: 139–154, 1989.

256. Thabrew MI, Joice PD, Rajatissa WA. Comparative study of the efficay of *Paetta indica* and *Osbeckia octandra* in the treatment of liver dysfunction. Planta Med, 53: 239–241, 1987.

257. Verma AR, Vijayakumar M, Mathela CS, Rao CV. *In vitro* and *in vivo* antioxidant properties of different fractions of *Moringa oleifera* leaves. Food Chem Toxicol, 47: 2196–2201, 2009.

258. Desai SN, Patel DK, Devkar RV, Patel PV, Ramachandran V. Hepatoprotective potential of polyphenol rich extract of *Murraya koenigii* L.: An *in vivo* study. Food Chem Toxicol, 50: 310–314, 2012.

259. Khan MR, Siddique F. Antioxidant effects of *Citharexylum spinosum* in CCl_4 induced nephrotoxicity in rat. Exp Toxicol Pathol, 64: 349–355, 2012.

260. Maheshwari P, Baburao B, Reddy RN. Hepatoprotective activity of methanolic extract of *Hiptage bengalensis* leaves against CCl_4-induced hepatotoxicity in rats. Toxicol Mech Method, 22: 483–487, 2012.

261. Srinivasan R, Chandrasekar MJN, Nanjan MJ, Suresh B. Antioxidant activity of *Caesalpinia digyna* root. J Ethnopharmacol, 113: 284–291, 2007.

262. Deng JS, Chang YC, Wen CL, Liao JC, Hou WC, Amagaya S. Hepatoprotective effect of the ethanol extract of *Vitis thunbergii* on carbon tetrachloride-induced acute hepatotoxicity in rats through anti-oxidative activities. J Ethnopharmacol, 142: 795–803, 2012.

263. Duh PD, Lin SL, Wu SC. Hepatoprotection of *Graptopetalum paraguayense* E. Walther on CCl_4-induced liver damage and inflammation. J Ethnopharmacol, 134: 379–385, 2011.

264. Wang CY, Ma FL, Liu JT, Tian JW, Fu FH. Protective effect of salvianic acid a on acute liver injury induced by carbon tetrachloride in rats. Biol Pharm Bull, 30: 44–47, 2007.

265. Panda VS, Ashar HD. Antioxidant and hepatoprotective effects of *Garcinia indica* Choisy fruits in carbon tetrachloride-induced liver injury in rats. J Food Biochem, 36: 240–247, 2012.

266. Augustyniak A, Waszkiewicz E, Skrzydlewska E. Preventive action of green tea from changes in the liver antioxidant abilities of different aged rats intoxicated with ethanol. Nutrition, 21: 925–932, 2005

267. Li X-W, Zhu R, Li B, Zhour M, Sheng OJ, Yang YP. Mechanism underlying carbon tetrachloride-inhibited protein synthesis in liver. World J Gastroentero. 16: 3950–3956, 2010.

268. Blum J, Fridovich I. Inactivation of glutathione peroxidase by superoxide radical. Arch Biochem Biophys 240: 500, 1985.

269. Fraschini F, Demartini G, Esposti D. Pharmacology of silymarin. Clin. Drug. Invest, 22: 51–65, 2002.

8. APPENDIXES

YEDİTEPE ÜNİVERSİTESİ

T.C. YEDİTEPE ÜNİVERSİTESİ, DENEY HAYVANLARI ETİK KURULU (YÜDHEK)

ETİK KURUL KARARI

Toplantı Tarihi	Karar No	İlgi	Proje Yürütücüsü
24.02.2012	246	07.02.2012 tarihli yazı	Prof.Dr.Erdem YEŞİLADA

'Gıda olarak kullanılan bazı meyvelerin sağlıklı hayvanlarda vücut antioksidan kapasitesi üzerine etkilerinin araştırılması' başlıklı bilimsel araştırma Etik Kurulumuzda görüşülmüş olup, çalışmanın etik kurallara uygun olduğuna oybirliği ile karar verilmiştir.

Etik Onay Geçerlilik Süresi: 1Yıl

GÖREVİ	ADI SOYADI	İMZA
Başkan	Prof. Dr. M. Ece GENÇ	
Başkan Yardımcısı	Prof. Dr. Erdem YEŞİLADA	KATILMADI
Raportör	Prof. Dr. Işıl Aksan KURNAZ	
Üye	Prof. Dr. Bayram YILMAZ	
Üye	Prof. Dr. Ertuğrul KILIÇ	
Üye	Doç. Dr. C. Narter YEŞİLDAĞLAR	
Üye	Yrd. Doç. Dr. Ediz DENİZ	KATILMADI
Üye	Hatice ÖZTÜRK	
Üye	Semra TECÜMEN	KATILMADI

YEDİTEPE ÜNİVERSİTESİ

T.C. YEDİTEPE ÜNİVERSİTESİ, DENEY HAYVANLARI ETİK KURULU (YÜDHEK)

ETİK KURUL KARARI

Toplantı Tarihi	Karar No	İlgi	Proje Yürütücüsü
24.02.2012	245	07.02.2012 tarihli yazı	Prof.Dr. Erdem YEŞİLADA

'Kızılcık bitkisinin (*Cornus mas* L.) yapraklarının karbon tetraklorür ile indüklenmiş oksidatif hasara karşı etkilerinin araştırılması' başlıklı bilimsel araştırma Etik Kurulumuzda görüşülmüş olup, çalışmanın etik kurallara uygun olduğuna oybirliği ile karar verilmiştir.

Etik Onay Geçerlilik Süresi: 1Yıl

GÖREVİ	ADI SOYADI	İMZA
Başkan	Prof. Dr. M. Ece GENÇ	
Başkan Yardımcısı	Prof. Dr. Erdem YEŞİLADA	KATILMADI
Raportör	Prof. Dr. Işıl Aksan KURNAZ	
Üye	Prof. Dr. Bayram YILMAZ	
Üye	Prof. Dr. Ertuğrul KILIÇ	
Üye	Doç. Dr. C. Narter YEŞİLDAĞLAR	
Üye	Yrd. Doç. Dr. Ediz DENİZ	KATILMADI
Üye	Hatice ÖZTÜRK	
Üye	Semra TECÜMEN	KATILMADI

9. CURRICULUM VITAE

Engin Celep was born in November, 1985 in Istanbul, Turkey. He graduated from Hüseyin Avni Sözen Anadolu Lisesi in 2003. He entered Yeditepe University Faculty of Pharmacy in the same year and graduated in July, 2007 and attended Yeditepe University Institute of Health Sciences Pharmacognosy PhD. Programme in August, 2007. He started to work as a research associate in Yeditepe University Faculty of Pharmacy Department of Pharmacognosy in 2007. He is currently employed in the same job.