FIRAT UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
TÜRKİYE

THE DETERMINATION OF SOME BIOLOGICAL ACTIVITIES OF *CUCURBITA PEPO* LEAVES

Lara SAEED

Master's Thesis

DEPARTMENT OF CHEMİSTRY

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THESIS APPROVAL

This thesis, which was prepared according to the thesis writing rules of the Graduate School of Natural and Applied Sciences, Fırat University, was evaluated by the committee members who have signed the following signatures and was unanimously approved after the defense exam made open to the academic audience.

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This thesis was approved by the Administrative Board of the Graduate School on

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Director of the Graduate School

DECLARATION

I hereby declare that I wrote this Master's Thesis titled " The Determination of some Biological Activities of Cucurbita Pepo Leaves " in consistent with the thesis writing guide of the Graduate School of Natural and Applied Sciences, Firat University. I also declare that all information in it is correct, that I acted according to scientific ethics in producing and presenting the findings, cited all the references I used, express all institutions or organizations or persons who supported the thesis financially. I have never used the data and information I provide here in order to get a degree in any way.

18 January 2022

Lara SAEED

PREFACE

All praises for Allah.

This thesis was about demonstrating that *Cucurbita pepo* leaves have antioxidant and antimicrobial activities that have an important role in preventing and curing various diseases. There was no difficulties or limits to the thesis. There was also contributions, explanations and assists during the thesis period.

I would like to thank my supervisor Doç. Dr. Mehmet Mürşit TEMÜZ from Firat University, Faculty of Science, Department of Chemistry, Elazığ/TURKEY.

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For their guidance, assistance, and support.

Special thanks to my mother to whom i dedicate my thesis. Special thanks to my family and freinds who were always there for me.

Lara SAEED

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ABSTRACT

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This thesis aims to conclude the antioxidant activity of Cucurbita pepo (a kind of pumpkin fruit) leaves in vitro to grade Cucurbita pepo leaves as a potential source of antioxidant phytochemicals vegetables because of containing many vitamins and minerals that can be utilized for decreasing or treatment of the threat of various diseases such as cardiac diseases, nervous disorders, cancer, diabetes mellitus and gouty arthritis, etc.

Material and methods: this study is accomplished for determining antioxidant activity for ethanol, as well as acetone extracts of Cucurbita pepo leaves, the antioxidant, the antioxidants vitamin contents, the anticancer, as well as the antimicrobial activities of the leaves were determined.

Results: The results of our study revealed that the Cucurbita pepo leaves acetone and ethanolic extracts had high radical scavenging activities against ABTS+•, OH•, and DPPH• radicals, Furthermore the results of our study showed that the leaves extracts had a high total flavonoid, total phenolic content.

The results of our study also revealed that the leaves extracts content of vitamins had only trace amounts of vitamin A and E, but had no vitamin C content. And the vitamins showed no antioxidant effect from vitamins (C, A, and E) of the extracts samples treated with saccharomyces cerevisiae yeast and against MDA.

The results of our study also revealed that the acetone and ethanolic leaves extracts samples had no effect on hepatocellular carcinoma, while the acetone leaves extracts decreasd breast cancer effects, and the ethanolic extract showed a higher effect decreasing human breast cancer than Doxorubicin which is a breast cancer therapy.

And finally the antimicrobial activity was as the following: the acetone extract of the leaves showed activity against all the bacteria and fungus strains, the antimicrobial activity of the acetone extract against the E.coli strain was: 47.36%, the K. pneumonia: 75%, the B. megaterium: 64%, the S. aureus: 57.8%, and the C. albicans: 58.3% . While the antimicrobial activity of the ethanolic extract of the leaves showed activity only against the B. megaterium: 60%, the S. aureus: 47.36%, and the C. albicans: 58.3%, but showed no effects against the E.coli and the K. pneumonia strains.

Conclusion: Our experiments, reviled that Cucurbita pepo leaves contain significant phytochemicals, that have numerous significant health benefits, as well as having the ability to reduce or inhibit several diseases. And finally Our experimental results showed that Cucurbita pepo leaves have antibacterial and fungicidal properties, due to the saponins, tannins, flavonoids, and alkaloids contents of Cucurbita pepo leaves.

Keywords: Antioxidant, Cucurbita pepo leaves, Phytochemicals, Vitamins, Minerals.

ÖZET

Cucurbita Pepo Yapraklarının bazı Biyolojik Aktivitelerinin Belirlenmesi

Lara SAEED

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Kimya Anabilim Dalı

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Bu tez, Cucurbita pepo (bir tür balkabağı meyvesi) yapraklarının in vitro olarak antioksidan aktivitelerini sonuçlandırmayı amaçlamaktadır kalp hastalıkları, sinir bozuklukları, kanser, şeker hastalığı ve gut artriti gibi çeşitli hastalıkların tehdidi.

Gereç ve yöntemler: Bu çalışma, etanolün antioksidan aktivitesinin yanı sıra Cucurbita pepo yapraklarının aseton ekstraktlarının antioksidan, antioksidan vitamin içerikleri, antikanser ve ayrıca yaprakların antimikrobiyal aktivitelerinin belirlenmesi amacıyla yapılmıştır.

Bulgular: Çalışmamızın sonuçları, Cucurbita pepo yapraklarının aseton ve etanolik ekstraktlarının $ABTS^{+\bullet}$, OH• ve DPPH• radikallerine karşı yüksek radikal süpürücü aktiviteye sahip olduğunu ortaya koydu. , toplam fenolik içerik.

Çalışmamızın sonuçları ayrıca, vitaminlerin yaprak özü içeriğinin sadece eser miktarda A ve E vitamini içerdiğini, ancak C vitamini içeriğine sahip olmadığını ortaya koydu. Ayrıca saccharomyces cerevisiae mayası ile muamele edilen ekstrakt örneklerinin vitaminlerinden (C, A ve E) ve MDA'ya karşı vitaminler hiçbir antioksidan etki göstermedi.

Çalışmamızın sonuçları ayrıca aseton ve etanolik yaprak ekstresi örneklerinin hepatosellüler karsinom üzerinde hiçbir etkisinin olmadığını, aseton yaprağı ekstrelerinin meme kanseri etkilerini azalttığını ve etanolik özütün insan meme kanserini azaltan bir meme olan Doksorubisine göre daha yüksek bir etki gösterdiğini ortaya koydu kanser tedavisi.

Ve son olarak antimikrobiyal aktivite şu şekildeydi: yaprakların aseton özütü tüm bakteri ve mantar türlerine karşı etkinlik gösterdi, aseton özütünün E.coli suşuna karşı antimikrobiyal etkinliği: %47.36, K. pnömoni: 75 %, B. megaterium: %64, S. aureus: %57.8 ve C. albicans: %58.3. Yaprakların etanolik ekstraktının antimikrobiyal aktivitesi sadece B. megaterium: %60, S. aureus: %47.36 ve C. albicans: %58.3'e karşı aktivite gösterirken, E.coli ve K. pnömoni suşları.

Sonuç: Deneylerimiz, Cucurbita pepo yapraklarının önemli fitokimyasallar içerdiğini, çok sayıda önemli sağlık yararına sahip olduğunu ve ayrıca çeşitli hastalıkları azaltma veya engellemek yeteneğine sahip olduğunu ortaya koydu.

Ve son olarak deneysel sonuçlarımız Cucurbita pepo yapraklarının saponinler, tanenler, flavonoidler ve alkaloidler içeriğinden dolayı antibakteriyel ve mantar öldürücü özelliklere sahip olduğunu göstermiştir.

Anahtar Kelimeler: Antioksidan, Cucurbita pepo yaprağı, Fitokimyasallar, Vitaminler, Mineraller.

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SYMBOLS AND ABBREVIATIONS

Symbols

α	: Alpha
β	: Beta
°C	: Degree Celsius
ē	: Electron
µL	: Micro
γ	: Gamma

Abbreviations

α-TO	: Alpha-Tocopherol radical
α-TOH	: Alpha-Tocopherol
ABTS	: 2,2′-Azinobis-(3-Ethylbenzothiazolin-6-Sulfonic Acid)
ABTS•+	: 2,2′-Azinobis-(3-Ethylbenzothiazolin-6-Sulfonic Acid) radical
ATP	: Adenosine Tri-Phosphate
Al	: Aluminum
B. megaterium	: *Bacillus megaterium*
BHT	: Butylated Hydroxy Tooluene
Ca	: Calcium
C. albicans	: *Candida albicans*
CA	: Caffeic acid
CAT	: Catalase
C-C	: Carbon-Carbon Bonds
CE	: Catechin Equivalent
CFM	: Colony-forming Unit
cm	: Centimeters
C-O-C	: Carbon-Oxygen-Carbon Bonds
Co enzyme Q	: Ubiquinone (the Q refers to the constitutive Quinone group)
copper (II)	: Copper +2 ion
copper (III)	: Copper +3 ion
CPL	: *Cucurbita Pepo Linnaeus*
Cu	: Copper
Cu^{+2}	: Cupric ion
Cu^{+3}	: Copper +3 cation
D-glucos	: Dextro-Glucose
Difco™	: Detroit Infusion and Fermentation Company
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribo Nucleic Acid
D.W.	: Distilled Water
DPPH	: 1,1-Di-Phenyl-2-Picryl-Hydrazyl or 2,2-Di-Phenyl-2-Picryl-Hydrazyl
E. coli	: *Escherichia coli*
ELISA	: Enzyme Linked Immunosorbent Assays
EDTA	: Ethylene Diamine Tetra Acetic acid
Fe^{+2}	: Ferrous ion
Fe^{+3}	: Ferric ion
$FeCl_3$	: Ferric Chloride
g	: Grams

GAE : Gallic Acid Equivalent
Gla : Gama-carboxyglutamate
Glu : Glutamte
GGCX : Gama-Glutamyl Carboxylase
GPx : Glutathione Peroxidase
GR : Glutathione Reductase
GSH : Glutathione
GSSG : Glutathione disulfide (oxidized glutathione)
H-atom : Hydrogen atom
H^+ : Hydrogen cation (Proton)
$HClO_4$: Perchloric acid
HEPC 2 : Hepatocellular carcinoma
H_2O : Dihydrogen Oxide (water molecule)
H_2O_2 : Hydrogen Peroxide radical
HO• : Hydroxyl radical
HO_2• : Perhydroxyl radical
HPLC : High Performance Liquid Chromatography
H_2SO_4 : Sulfuric acid
iron (II) : Iron +2 ion
iron (III) : Iron +3 ion
IP6 : Inositol HexaPhosphate
K. pneumonia : *Klebsiella pneumonia*
KH_2PO_4 : Potassium dihydrogen phosphate
KOH : Potassium hydroxide
$K_2S_2O_8$: Potassium persulphate
$L^{\cdot}$: Alkyl radical
L-arginine : Levo-arginine
L-ascorbic acid : Levo-ascorbic acid
L-ascorbate : Levo-ascorbate
LDL : Low Density Lipoprotein
LH : Lipid substrate (Linoleic acid)
L : Liter
mAU : Milli Absorbance Unit
MDA : Malon Di Aldehyde
MCF 7 : Human breast cancer
mL : Milliliter
mm : Millimeter
mM : Millimolar
min : Minute
nm : Nanometer
MT : Metallo Thionein
Myo : Muscle
Myo-inositol hexaphosphate :so-called because it present in muscle tissues
Na : Natrium (Sodium)
Na_2CO_3 : Sodium carbonate
NADH : Nicotinamide Adenine Di-Nucleotide
NADPH : Nicoteinamide Adenine Di-Nucleotide Phosphate
$NADP^+$: Nicoteinamide Adenine Di-Nucleotide Phosphate (oxidized form of NADPH)
NAD(P)H : Quinone oxidoreductase
NF-$_K$B : Nuclear Factor-Kappa- light-chain enhancemPent of activated B lymphocytes
NO : Nitric Oxide
NO• : Nitric Oxide radical

O_2 : Di Oxygen (Oxygen molecule)
O_2^- : Superoxide radical
$O_2^{\bullet-}$: Superoxide radical
OH^- : Hydroxide ion
$ONOO^-$: Peroxynitrite
O-quinone : Ortho-Quinone
P : Phosphorus
P-coumaric acid: Para-Coumaric acid
PA : Phytatic acid
1,2,3,4,6- Penta-O-galloyl- β-D-glucose: 1,2,3,4,6- Penta-Oxygen-Galloyl- Beta-Dextro-Glucose
pH : Potential Hydrogen (indicates the activity of hydrogen ion)
PhOH : Phenolic compound
$PhO^{\bullet}$: Phenoxide compound
PRDX : Peroxyredoxine
Prx : Peroxiredoxin
PTSD : Post Traumatic Stress Disorder
RA : Retinoic Acid
Rdh : Retinol DeHydrogenase
RNA : Nuclic Acid
RNS : Reactive Nitrogen Species
ROS : Reactive Oxygen Species
ROO• : Peroxyl radical
ROOH : Hydroperoxide
RPE : Retinal Pigment Epithelium
RPM : Revolutions Per Minute
SO4 2- : Sulfate
$SO_4^{\bullet-}$: Sulfate radical
SOD : Super Oxide Dismutase
SOD1 : cytoplasmic enzyme,
SOD2 : mitochondrial enzyme , and
SOD3 : extracellular enzyme
SP. : Species (when the species name is not specified)
S. aureus : *Staphylococcus aureus*
T : Thionein
TBA : Tert Butyl Alcohol
TCA : Tri Chloro Acetic acid
Trx : Thioredoxin
VKD : Vitamin K Dependant
VKR : Vitamin K E Reductase
VKOR : Vitamin K Epoxide Reductase
UV : Ultra violet radiation
UVA : Long wavelength Ultra violet radiation (320-400 nm)
UVB : Medium wavelength Ultra violet radiation (280- 320 nm)
Zn : Zinc

1. INTRODUCTION

A medicinal plant is a plant with definite activities that are used in herbalism. They are regarded as sources of phytochemicals [1]. Phytochemicals are secondary bioactive metabolites that supply plants with flavor, colour, and natural toxicity against pests. Some examples of phytochemicals are phenolic compounds, carotenoids, and glucosinolates, etc. [2]. Antioxidant phytochemicals prime role is to protect from oxidative stress that results from pathogens, UV radiation, parasites, etc. [3]. The consumption of these substances decreases the risks of heart defects, stroke, hypertension, cancer, and many other diseases. Phytochemicals are recently drawing the attention of nutrition science that studies deficiency diseases, their sources, and treatments, It also studies the health benefits of vitamins, minerals, antioxidants, etc., and their effect on preventing or treating diseases [4]. *Cucurbita pepo* (a kind of pumpkin fruit) leaves are antioxidant-rich phytochemicals [5]. *Cucurbita pepo* leaves have a dark green color and taste like a mixture of spinach, broccoli, asparagus, and green *beans* [6]. They possess several health benefits as they contain many vitamins and minerals besides antioxidant and anti-inflammatory properties: *Cucurbita pepo* leaves have anti-diabetic properties because of the existence of polyphenols [7]. They enhance and encourage the immune system by including vitamin A and β-carotene. They reduce blood cholesterol, the threat of heart attack, and stroke, due to the phenolic compounds [8]. They are vital for regrowth and attenuation of skin for containing antioxidant vitamins [9]. They prohibit anemia because of being rich in Folic acid, besides the excellent iron content. They prevent and heal migraines because of the presence of vitamin B2 [10]. They have antimicrobic, antihepatotoxic, and anticancer properties, because of the phytochemicals content of the leaves [11, 12].

Cucurbita pepo leaves contain water, protein, fat, carbohydrate, fiber, Calcium, Iron (Ferrum), Potassium, Sodium, Magnesium, Phosphorous, Zinc, L-ascorbic acid (vitamin C), Thiamine (vitamin B1), Riboflavin (vitamin B2), Niacin (vitamin B3), Pyridoxine (vitamin B6), Folate (vitamin B9), β-carotene, vitamin A, saturated fatty acids, as well as polyunsaturated fatty acids [13].

Seven phenolic compounds were determined by water hydrolysis of pumpkin leaves [14].

Guangpu Zeng et al. reported in their experimental results that pumpkin leaves contain plenty of vitamins and minerals, besides alkaloids, saponins, phytates, and polyphenols that were responsible for the pumpkin leaf extract antioxidant capacity [15].

Srianthie A Deraniyagala et al. reported that the Sri Lankan *Cucurbita maxima* leaves extract gave the highest rate in the reducing power assay for ferric ion. The leaves extract also had an antimicrobial effect against *Bacillus subtilis* bacterium [16].

Parsa Dar et al. reported that *Cucurbita pepo L.* (CPL) leaves ethyl acetate extract had flavonoid concentration in the highest level, while the highest level of phenolics was found in the n-Butanolic extract; both acted as a strong antioxidant active solution [17].

Oboh et al. reported that the fluted pumpkin leaf extract phytochemical screening reveild the presence of flavonoids, tannins, alkaloids, saponins and glycosides. They also reported that the ethanolic extract of the leaves showed antimicrobial activity against *E. coli*, *S. typhi*, and *Strept. Faecalis* [18].

Mondal et al. reported that *Cucurbita pepo* Linn can be regarded as a significant source of phytochemicals that possess antioxidant activities [19].

1.1. The aims of the study

The study aims to declare the benefits of *Cucurbita pepo* leaves, especially being a source of antioxidants, for providing novel nutrients antioxidants whether consumed as food or for pharmaceutical purposes as antioxidants do an essential function in neutralizing free radicals and thus preventing nervous disorder, cancer, diabetes, gouty arthritis and heart diseases. In addition to possessing antimicrobial activities. This thesis aims to determine some biological activities of *Cucurbita pepo* leaves through:

1. Determination of the radicals scavenging activities of the ethanolic and acetone extracts as well as determining the antioxidant vitamins C, E, and A.
2. Determining anticancer activity.
3. Determining the antimicrobial activity of ethanolic and acetone extracts of the leaves.

1.2. Free radicals

A free radical or an oxidant is an ion or a molecule with an unpaired electron (a paramagnetic ordered ion or molecule). Free radicals may afford these unpaired electrons or receive electrons from other molecules, therefore, they have continuous activity and are unsteady. That is why Free radicals are rare and are produced only under restricted conditions. Free radicals can cause damaging as well as advantageous biological outcomes. For example, free radicals that produce during cellular metabolism and functions play an essential part in many vital processes like cell signaling, metabolism pathways, immune response, gene expression, apoptosis, and many pathological disorders [20]. But excessive production of free radicals attacks the nucleic acid bases, proteins amino acid chains, and unsaturated fatty acids' double bonds leading to oxidative stress [21].

1.2.1. Free radicals sources

1.2.1.1. Endogenous free radicals

These are radicals that are produced within the body:

Natural metabolism depends on oxygen, and oxygen is the primary source of free radicals inside living systems because the major source of energy (ATP) in mammals comes from cellular respiration in mitochondria reducing oxygen to produce H_2O. During these reactions, other radicals are produced from oxygen and are called reactive oxygen species (ROS), and they are superoxide radical (O_2•−), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO•) and others. As can be seen in figure 1.1. Below:

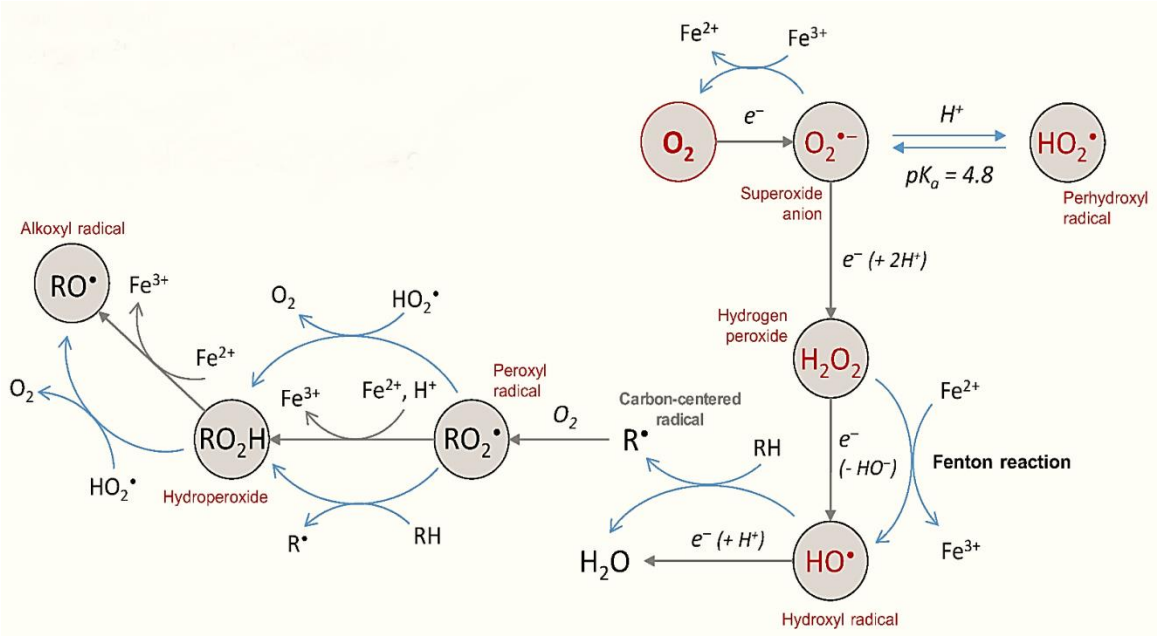

Figure 1.1. Reactive oxygen species (ROS) that produce during mitochondrial respiration [22].

As can be seen in figure 1.1. the superoxide radical (O_2•−) is produced from molecular oxygen (O_2) by gaining an electron from the electron transport chain during mitochondrial respiration, and when the enzyme superoxide dismutase (SOD) converts the superoxide radical to hydrogen peroxide H_2O_2 and a water molecule, the hydrogen peroxide has the possibility to change to hydroxyl radical (HO•) (reaction 1) and cause damages if not neutralized to water molecules by glutathione, ascorbic acid and other antioxidants.

Under the condition of excess ferrous ions (otherwise cupric ion) [23], or during oxidative stress (when the amounts of hydrogen peroxide exceed the ability of the antioxidant enzymes to change it to water molecule), a reaction between the hydrogen peroxide and ferrous ion takes place. It is called the Fenton reaction (according to the British chemist Henry J.H. Fenton, who reported the reaction in 1894), and the reaction is:

$H_2O_2 + Fe^{+2} / Cu^{+2} \longrightarrow OH^- + OH\bullet + Fe^{+3} / Cu^{+3}$ …reaction 1 (Fenton reaction) [24].

The super oxide radical under other conditions, becomes protonated, and the resulting product is called perhydroxy radical [25].

But the protonated form of the superoxide radical only exists at 0.6% at pH 7 (standard conditions), and the reaction route is not favorable [26], as shown in figure 1.2. Furthermore, the superoxide radical can react with hydrogen peroxide in the presence of iron ions (catalyze the reaction) to form hydroxyl radical and other products as can be seen in reaction 2, and the reaction is called the Haber-Weiss reaction (according to the German chemist Fritz Haber and his student Joseph Joshua Weiss in 1932):

$H_2O_2 + O_2\bullet^- \longrightarrow OH^- + OH\bullet + O_2$ …reaction 2 (Haber-Weiss reaction)

And finally, superoxide can react with nitric oxide radical that produced by nitric oxide synthases enzymes by the conversion reaction of L-arginine to L-citrulline, and NO• has many significant functions, for example: regulating apoptosis (programmed cell death to remove dead cells that cannot be repaired, also prevent cancer cells), neurotransmitter, antimicrobial agent, and many other significant functions [27]. The product of the reaction of super oxide with nitric oxide radical is the highly reactive peroxynitrite (ONOO-) that causes oxidative tissue damages. The production of peroxynitrite increases as the production of nitric oxide radical increase, or when mitochondrial superoxide radical production increase [28]. As can be seen in figure 1.2.

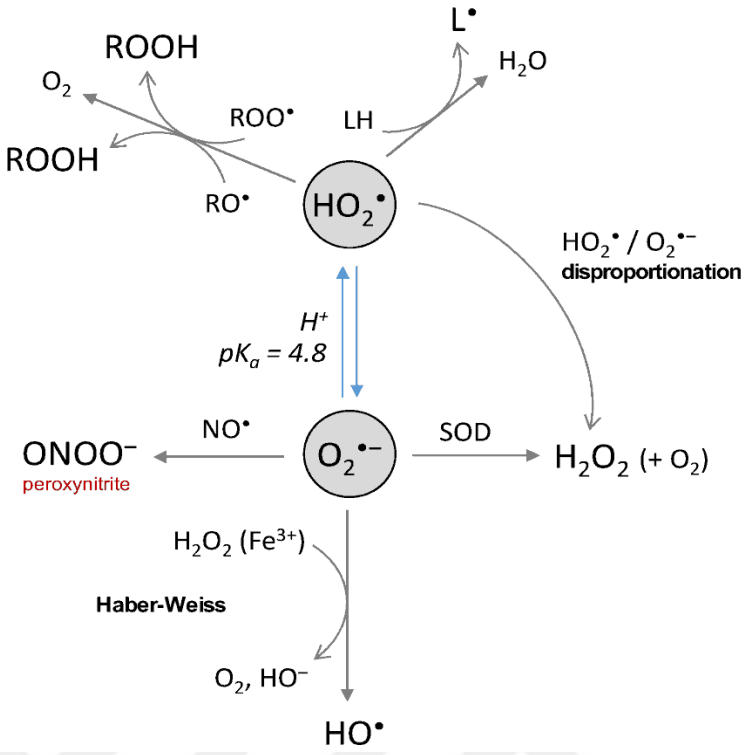

Figure 1.2. All the possible reactive oxygen species that produce from superoxide ions [22].

Free radicals can also produce after cerebral ischemia and reperfusion in the form of ROS and reactive nitrogen species (RNS). And through stress conditions, acute physical effort, psychological trauma (PTSD), because of elevated respiratory oxygen consumption that is required to meet energy requirements [29].

Under uncontrolled volumes, (oxidative stress conditions that will be declared in oxidative stress section), ROS and RNS cause damages to proteins, DNA, and lipids. Lipid peroxidation is an example of radical damage [30]. As shown in figure 1.3.

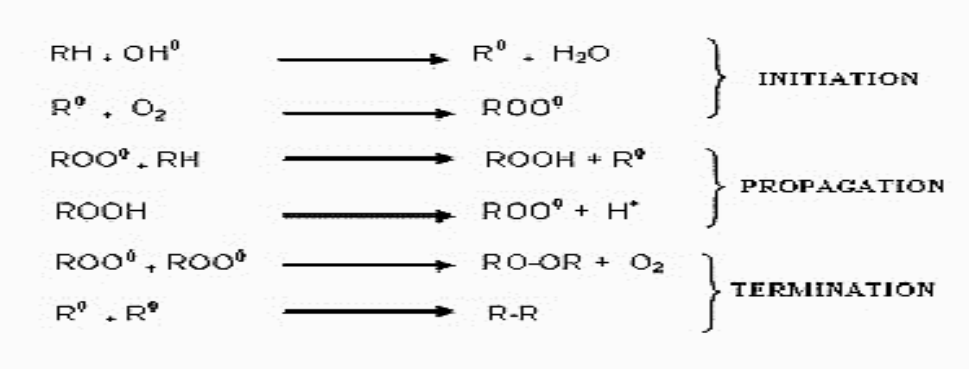

Figure 1.3. The reaction of hydroxyl radical with unsaturated producing lipid peroxide (lipid peroxidation) [31].

The reaction of radicals with proteins leads to oxidation of amino acids, protein cross-linking and denaturation, as well as DNA oxidation. DNA oxidation causes breaking DNA strands and the release of oxidized DNA bases. Other examples are oxidative damage to the mitochondrial membrane, which causes the release of cytochrome and damages mitochondria.

1.2.1.2. Exogenous free radicals

These come from external causes as cigarettes smoke, air contamination, ultraviolet radiation, x-ray radiation, electron emission, ozone exposure, and heavy metals, etc. [32].

1.3. Oxidative stress

Consumption of molecular oxygen by aerobic organisms ends with the formation oxidants (free radicals) RNS and ROS that can cause cellular damages. In addition, that is why these organisms have developed an antioxidant system to defense their biomolecules against the cellular damages caused by the free radicals.

All living systems have antioxidant defense mechanisms within their cells: the antioxidant defense system is composed of antioxidants (free radical scavengers) that work to prevent oxidative damage through deactivating oxidants [33]. This mechanism is preserved by oxidative metabolism together with the activity of antioxidant enzymes like superoxide dismutase (SOD), thioredoxin hydroperoxidases, or other substances such as glutathione, Vitamin E, C, and others (as will be declared in antioxidant section).

Free radicals and antioxidants are generally in balance under physiological conditions. Still, with the extended formation of free radicals during physical activities or weakened antioxidant defenses, (free radicals exceed the cell antioxidant ability), the balance becomes distorted, leading to oxidative stress. Oxidative stress means that the antioxidants amount in cells, as well as tissues, is increased due to exposure to oxidants. Oxidative stress causes oxidative injuries such as heart disease, cancer, and atherosclerosis [34]. Oxidative stress also causes diabetes, hypertension, preeclampsia, acute renal failure [33].

1.4. Antioxidant system

Diverse processes as growth, immune response, and others in aerobic organisms call for low concentrations of oxidants to avoid the generation of oxidative stress that causes cellular defect and damages. Therefore an antioxidant system is crucial for neutralizing oxidants and maintain appropriate cellular functions [35]. Furthermore, the cellular submission to free radicals tempts the activation of an internal defense system to eliminate the free radicals and their derivatives, this is

called the mechanism of antioxidant protection or defense, and the mechanism includes these processes:

- Prevention of free radicals and their derivatives activity with the aid of biological constituents in the body.
- Repair and disruption of oxidation reactions.
- Inactivation of free radical reactions yields through repairing or eliminating structural damages.

Antioxidants are compounds that inhibit, decrease or mend the damages caused via ROS and RNS.

The antioxidant defense system is a network of all the antioxidants that function interchangeably, as shown in figure 1.4.

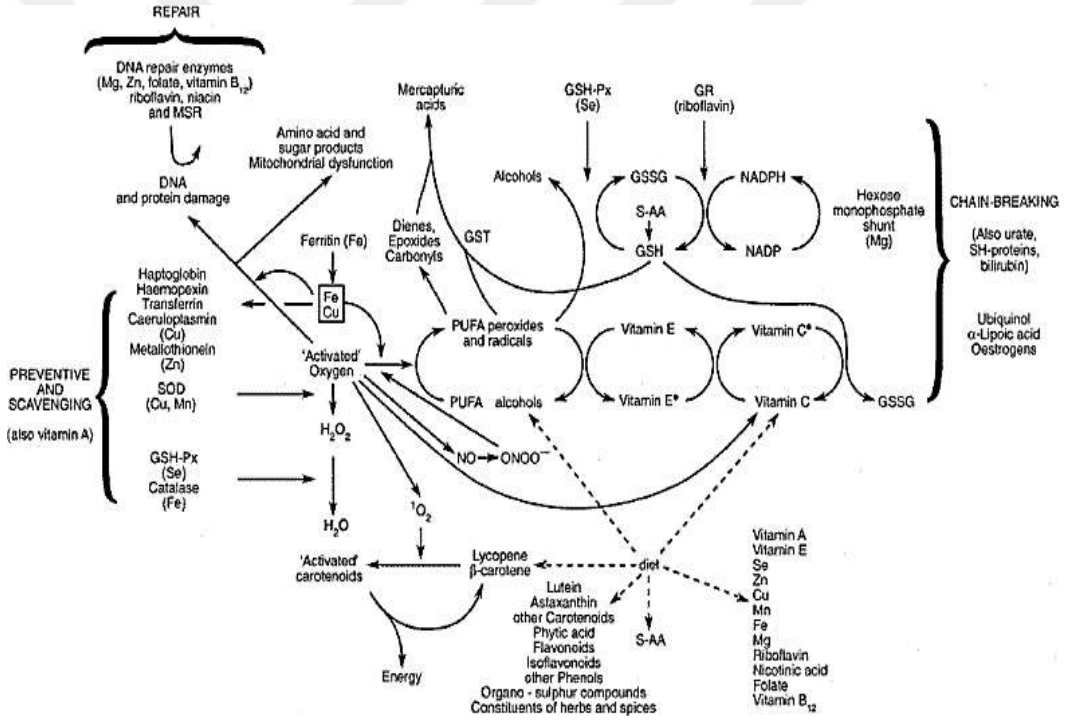

Figure 1.4. The antioxidant defense network [36].

The superoxide radical (O_2•−) that is produced from molecular oxygen (O_2), as mentioned before in the free radicals section, is neutralized by the enzymatic antioxidant superoxide dismutase (SOD) that converts the superoxide radical to hydrogen peroxide H_2O_2. Furthermore, the hydrogen peroxide has the possibility to change to hydroxyl radical HO•. Still, the enzymatic antioxidant glutathione peroxidase (GPx) changes hydrogen peroxide to water molecule through the glutathione cycle. Other antioxidants that can reverse hydrogen peroxide to water molecules are

the enzymatic antioxidants catalase (CAT) and peroxiredoxins (PRDX), as shown in figure 1.5. [37].

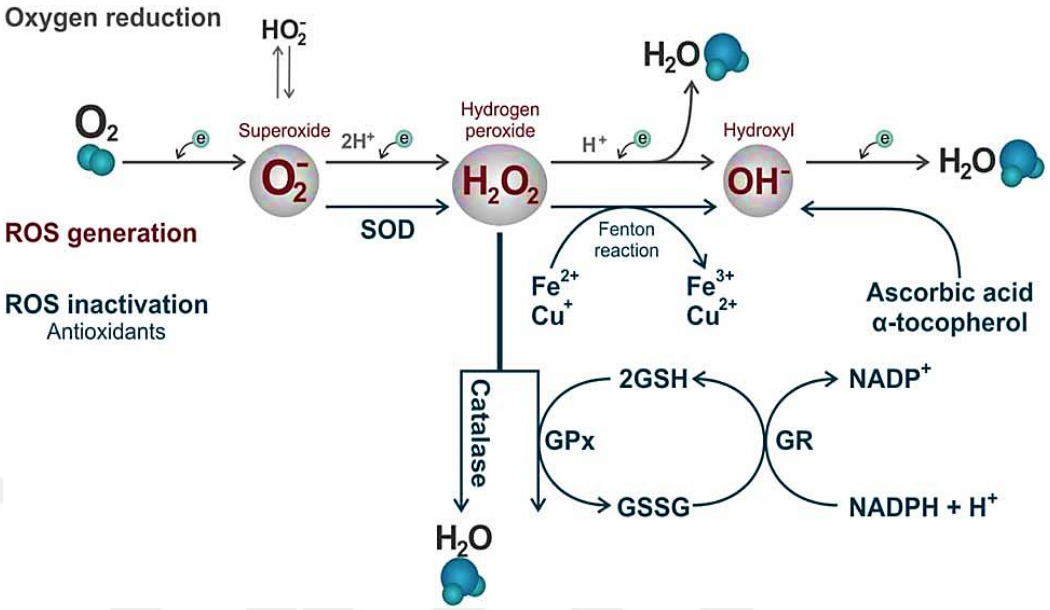

Figure 1.5. Mitochondrial antioxidant defenses against ROS [37].

In addition, non-enzymatic antioxidants like ascorbic acid and α-tocopherol, etc., can scavenge the hydroxyl radical, as shown in figure 1.5.

Furthermore, the hydroxyl radical that forms from the hydrogen peroxide can be scavenged by the enzymatic antioxidants: glutathione peroxidase (GPx), peroxiredoxin (Prx), and thioredoxin (Trx), as can be seen in figure 1.6.

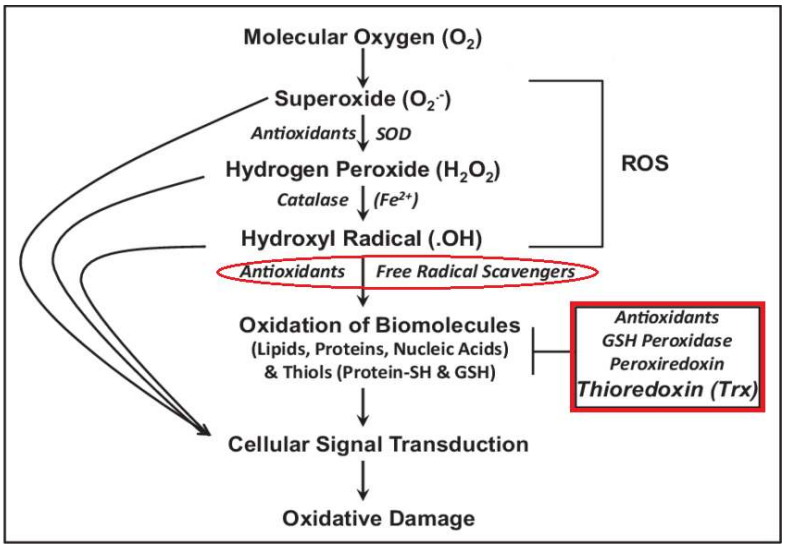

Figure 1.6. The antioxidants that scavenge the hydroxyl radical [38].

Another example of the antioxidant defense system appears with the defense against the peroxynitrite can be seen in figure 1.7.

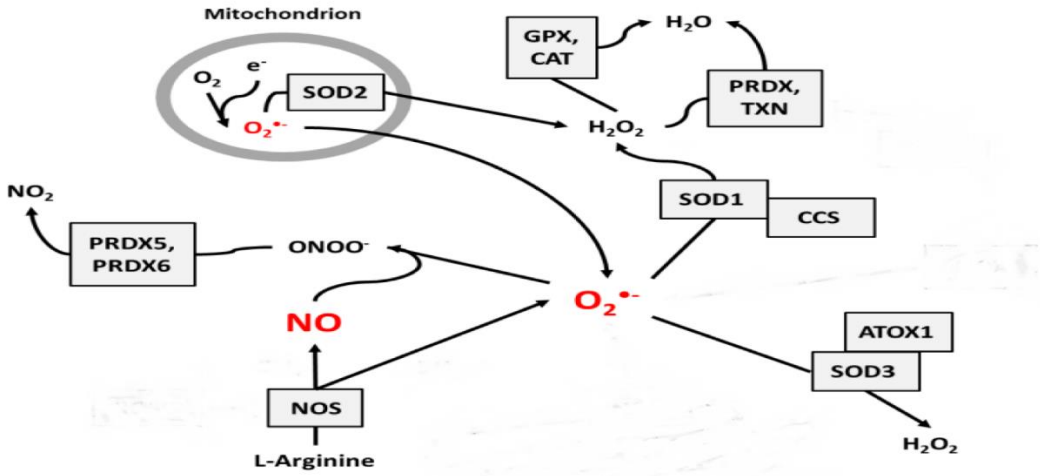

Figure 1.7. The antioxidants pathways against super oxide radicals [39].

PRDX are thiol-specific enzymes that scavenge H_2O_2 and ONOO-, in addition PRDX also decomposes ONOO- and oxidases nitric oxide to nitrite [40].

1.4.1. Types of antioxidants

1.4.1.1. Natural antioxidants

These are further divided into two groups (endogenous and exogenous): Endogenous antioxidants: are substances that present inside the human body.

A/ Endogenous antioxidants additionally can be classified to protein antioxidants and non-protein antioxidants) [41]:

• Protein antioxidants: that include enzymatic as well as non-enzymatic protein antioxidants: The enzymatic protein antioxidant that is doing a significant function within the defense mechanism is superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR). As can be seen in figure 1.7.

Other enzymatic protein antioxidants are glutathione-synthesizing enzyme, glutathione s-transferase, glutaredoxin, thioredoxin, thioredoxin reductase, peroxiredoxin, sulfiredoxin, methionine sulfoxide reductase, heme oxygenase, NAD(P)H: quinone oxidoreductase, paraoxonase, and glucose-6-phosphate dehydrogenase [3, 42].

And non-enzymatic protein antioxidants that restrict the yielding of reactive species through forming bonds with metal ions are ceruloplasmin, transferrin, ferritin, albumin, lactoferrin, and metallothionein [43].

- Non-protein antioxidants: this includes glutathione, bilirubin, coenzyme Q, estrogens, α-lipoic acid, melatonin, pyruvate, uric acid

B/ Exogenous antioxidants: These substances are not made by cells but are able to be taken out from plant or animal tissues. Examples are:

- Vitamins: C, E, A, and B vitamins
- Trace elements: zinc, selenium
- Carotenoids: like β-carotene
- Phenolic compounds: like caffeic acid [44].

1.4.1.2. Synthetic antioxidants:

These are synthesized in laboratories. Examples are antioxidant enzyme mimetics, cerium oxide nanoparticles, and spin traps [45].

1.5. Prooxidants

Antioxidants perform as prooxidants (any ingredient that stimulates oxidative stress whether through generating ROS or through inhibiting antioxidants function) under certain conditions that are declared as in activation of prooxidants below [46].

1.5.1. Activation of prooxidants

- Vitamin C can become a prooxidant in case of the existence of extra quantities (high dose) of iron and Copper: vitamin C reduces ferric ion to ferrous ion, and copper (III) to copper (II) leading to reduction of hydrogen peroxide and yielding hydroxyl radicals as shown in Fenton reaction (reaction 1) above [47].
- α-tocopherol at high concentrations: once reaction with free radicals occur α-tocopherol itself turns into a radical, and when not enough ascorbic acid is available for the revival of α-tocopherol it becomes a prooxidant [48].
- A high dose of vitamin A was indicated to have prooxidant activity and enhanced lipid peroxidation, and release oxygen radicals by liver cells in rats [49].
- Zinc excess prompts oxidative stress by reducing cellular energy and increasing mitochondrial ROS production [50].

• Another compound that is able to behave as prooxidants is dietary phenolic compounds, when at high concentrations in a system containing iron and copper, ROS are produced as well as phenoxyl radicals that cause destruction to DNA, lipids, etc. [51].

1.6. Antioxidants in *Cucurbita pepo leaves*

1.6.1. Vitamin C

Also known as L-ascorbic acid, have the chemical formula $C_6H_8O_6$. Vitamin C can act as a reducing agent. Ascorbic acid was known as hexuronic acid at the beginning of its discovery, but later after discovering the compounds preventing scurvy, it was renamed ascorbic acid; that's why vitamin C is a term that should be used for compounds that have ascorbic acid activity.

The human body cannot produce vitamin C. That is why it should come directly from the diet [52].

Ascorbate is a conjugated base of L-ascorbic acid. Ascorbate is a water-soluble compound, a vital microorganism that stands essential for the metabolism in nearly all organisms [53]. As can be seen in figure 1.8.

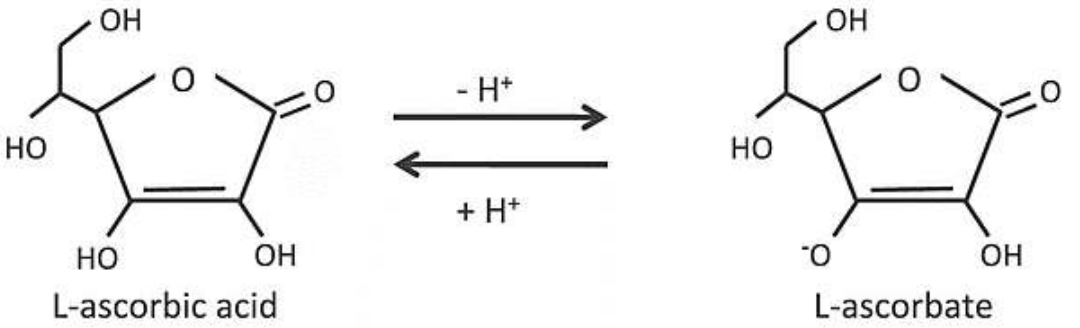

Figure 1.8. L-ascorbic acid and L-ascorbate [53].

1.6.1.1. Vitamin C as an antioxidant

• Vitamin C and ascorbate counteract free radicals and preserve DNA, lipids, and proteins from oxidative damage [54]. As can be seen in figure 1.9.

• Vitamin C defends the eyes against oxidative damage that results from radiations [55].

• Vitamin C prohibits nitrosamines (a cancer-causing compound) that is synthesized in guts or occupied from food [56].

• Vitamin C reduces tetrahydrobiopterin which catalysis the synthesis of the free radical nitric oxide [57].

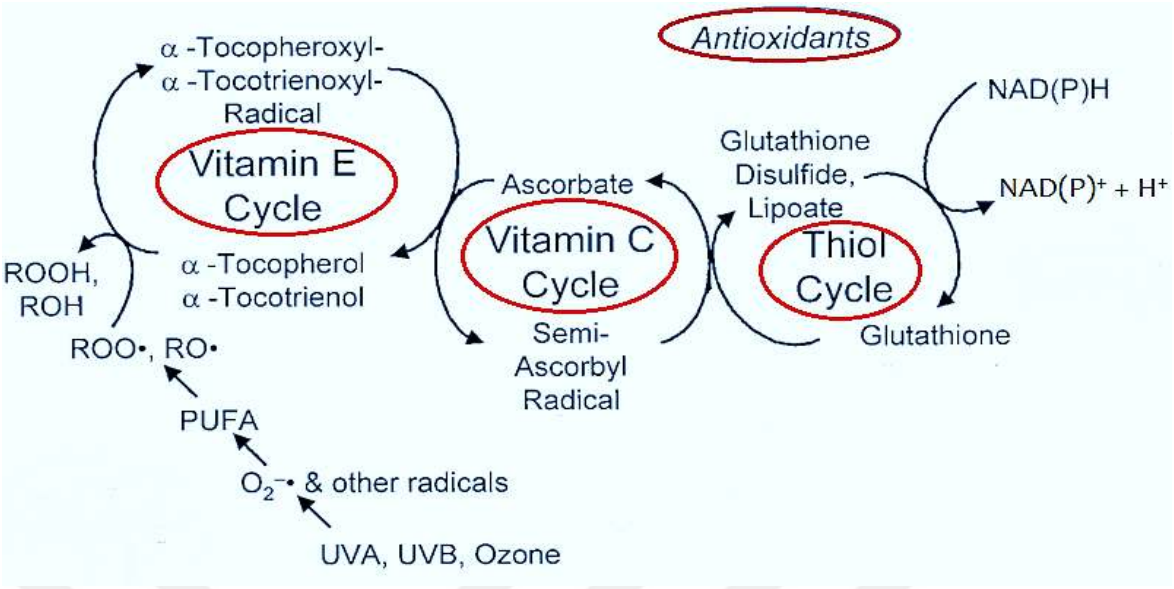

Figure 1.9. The antioxidant network including vitamins C, E with the thiol redox cycles [58].

1.6.2. Vitamin A

Also, named retinol has the chemical formula: $C_{20}H_{30}O$. Vitamin A is consumed from diety sources either in the form of preformed vitamin A from animal products as retinol or retinyl ester, or in the form of pro-vitamin A carotenoids from fruits and vegetables.

Retinol is converted in the retinal pigment epithelium (RPE) by a reversible reaction by retinol dehydrogenase (Rdh) to retinaldehyde (retinal) that is utilized as a portion of the pigment rhodopsin, and rhodopsin is involved in the ability to see under dim conditions after it is taken up by the retina cells. Moreover, retinal further converted into retinoic acid by retinal oxidase by irreversible reaction, because both retinal and retinoic acid are needed for biotic processes. As can be seen in figure 1.10. [59].

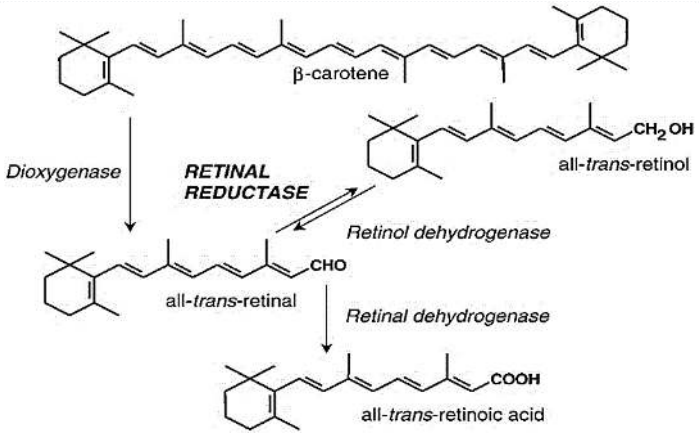

Figure 1.10. Biosynthesis of retinal, retinoic acid [60].

1.6.2.1. Vitamin A and carotenoids as antioxidants

- Retinol exhibits an effective peroxyl radical forager through restriction of peroxidation, which is more effective than tocopherol. Vitamin A can be oxidized straight with radicals, stabilizing the liposome lipid bilayer radical [61].
- Carotenoids have numerous actions as an antioxidant through foraging singlet oxygen and peroxyl radicals, and thus protecting α-tocopherol and inhibiting lipid peroxidation in human plasma [62].
- Vitamin A protect human low-density lipoprotein (LDL) from free-radical injury. Oral supplementation with vitamin A improves resistance of LDL toward copper-stimulated oxidation. [63].
- β-carotene reduces ischemic heart disease, the necessity of thrombolysis, as well as heart failure [64].

1.6.3. Vitamin E

Also called tocopherol, has the chemical formula: $C_{29}H_{50}O_2$. Vitamin E is a fat-soluble vitamin. Like vitamin A, vitamin E is not synthesizable inside the body and must be consumed from the diet [65]. Vitamin E naturally exist in the following forms: the α, β, γ, as well as the σ-tocopherols along with their related corresponding tocotrienols (has a similar structure to α -tocopherols apart from that tocotrienol own three unsaturated double bonds), as can be seen in figure 1.11. [66].

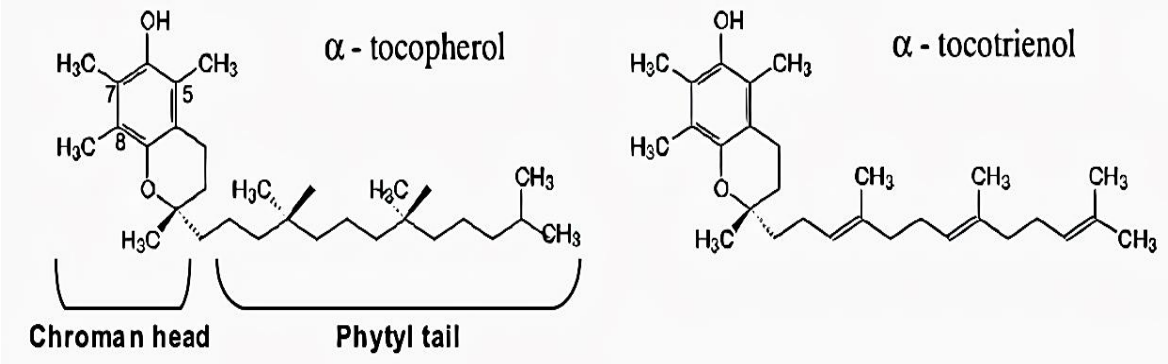

Figure 1.11. The structure of α-tocopherol and α-tocotrienol [67].

The most abundant tocopherol in the diet is γ-tocopherol. Still, the only form that adjusts human requirements is the α-tocopherol form, and most researchers study the α-tocopherol because it is retained within human tissues and sera [68].

1.6.3.1. Vitamin E as an antioxidant

- The vitamin E part accountable to the antioxidant activity is the chroman head group, while the phytyl group (figure 1.6.) has no role in the antioxidant activity [69].
- The chroman group is able to donate H-atom, as shown in reaction 3, α-tocopherol (α-TOH) has anti-inflammatory activity and is an active peroxyl radical scavenger that restricts the distribution of radical damages within living membranes [70]. As shown in reactions 3 and 4:

$$ROO\bullet + \alpha\text{-}TOH \longrightarrow ROOH + \alpha\text{-}TO\bullet \quad \ldots \text{ reaction 3}$$

$$ROO\bullet + \alpha\text{-}TO\bullet \longrightarrow \text{inactive products} \quad \ldots \text{ reaction 4}$$

- The free radical foraging activity of the α, β, γ, σ tocopherols have been measured [71].
- The tocotrienols have neuroprotective, cholesterol-lowering, and anti-cancer properties [72].
- Vitamin E acts as antioxidant intercepting damage to cell walls as well as proteins and adjusts their action by foraging ROS as well as RNS, or by regulating specific enzymes (e.g. pancreatic lipase) [73].

1.6.4. Vitamin K

Vitamin K is a fat-soluble naphthoquinone compound. There are two types of vitamin K: a natural type which includes:

Vitamin K1 (phylloquinone) is manufactured by plants and has the chemical formula: $C_{31}H_{46}O_2$.

Vitamin K2 (menaquinone) is made by bacteria and has the chemical formula: $C_{31}H_{40}O_2$.

It mainly presents in fermented food such as cheese, but human gut bacteria microbiota is capable of synthesizing vitamin K2.

The other type is synthetic K3 (menadione) with the chemical formula: $C_{11}H_8O_2$ [74]. As can be seen in figure 1.12.

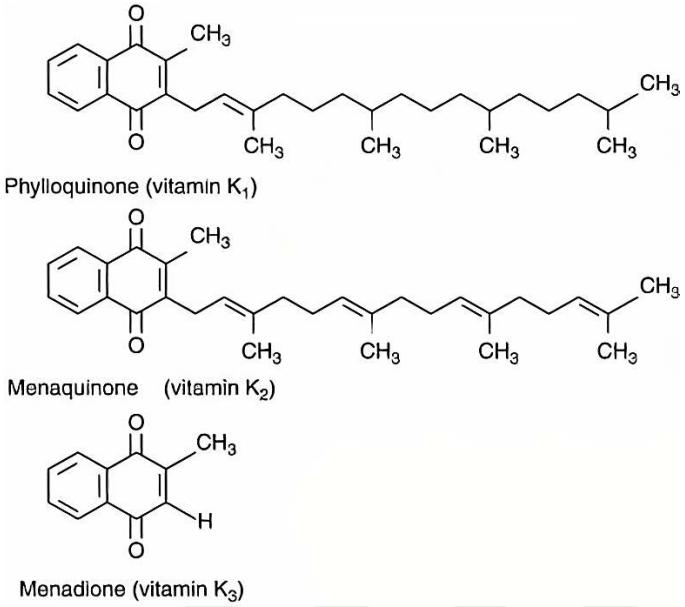

Figure 1.12. The chemical structure of vitamin K1, K2, K3 [75].

The type of vitamin that exists in *Cucurbita pepo* leaves is K1. The body does not form vitamin K1; therefore, it must be consumed from plants, as it is essential for the posttranslational adjustment of definite proteins helping blood clotting [76].

1.6.4.1. Vitamin K1 as an antioxidant

- Vitamin K1 inhibits cell death that results from oxidative stress in oligodendrocytes and neurons [77].
- All vitamin K types display levels of anti-inflammatory activity [78].
- The vitamin K series that generates vitamin K hydroquinone have strong antioxidant potentials [79]. As can be seen in figure 1.13.

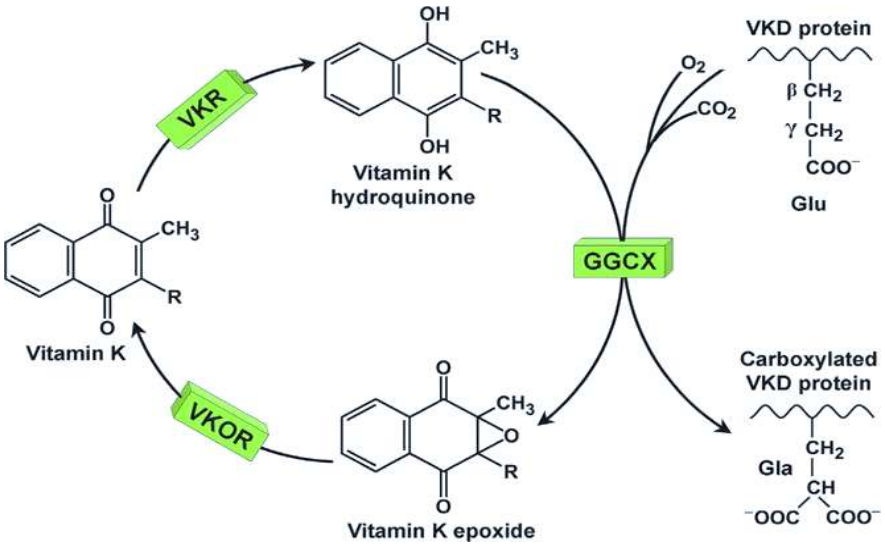

Figure 1.13. The vitamin K series [80].

- Vitamin K1 and K2 inhibit lipid peroxidation of lecithin liposomes [81].
- Vitamin K1 is capable of reducing type 1 diabetes by adjusting free radical stress [82].

1.6.5. Zinc

Zinc is an essential nutritional trace mineral, zinc is not cytotoxic, and it's also not carcinogenic or mutagenic [83]. Because zinc exists in organs, tissues, and the body's fluids, its deficiency is vigorous for human health. Zinc deficiency causes growth retardation, immune malfunction, a rise in oxidative stress, besides increased production of provocative cytokines [84].

1.6.5.1. Zinc as an antioxidant

- Zinc is a basic constituent of the enzyme SOD that stimulates the transformation of two molecules of superoxide into one molecule of hydrogen peroxide together with an oxygen molecule, and thus reducing ROS and reducing oxidative stress [85]. As shown in reaction 5:
- $2O_2^- + 2H + SOD \longrightarrow H_2O_2 + O_2$ hydrogen peroxide … reaction 5
- The SOD enzyme is an anti-carcinogenic enzyme, and since zinc is an essential constituent of the enzyme, it protects against genetic mutations
- Zinc inhibits the phagocytes enzyme NADPH oxidases, which catalyze producing of superoxide radical from NADH:

$$NADPH + 2O_2 \xrightarrow[\text{oxidase}]{\text{NADPH}} 2O_2^{\cdot-} + NADP^+ + H^+ \quad \text{… reaction 6}$$

- Zinc deficiency is able to cause growth retardation, weakened wound healing, hair loss, etc. And zinc provisions is used for the treatment of a number of diseases as dwarfism, skin and intestine inflammation, sexual immaturity, anorexia nervosa as well as bulimia nervosa [86].
- Supplying humans with zinc with normal healthy issues lower the products of oxidative stress, besides increasing the activity of NF-$_K$B: a mitochondrial protein complex known as nuclear factor kappa-light-chain-enhancer of activated B cells (activated white blood cells that are portion of the immune system). NF-$_K$B regulates many inflammatory and immune function genes like type one T helper cells that motivate the damaging of pathogens [87].
- Supplying with zinc together with vitamin C reduces osmotic fragility as well as reducing the amount of the yields of the lipid peroxidation in plasma [88].
- Zinc deficiency is found in diabetes type 2 patients [89]. And zinc deficiency is also found in chronic kidney disease [90].
- Zinc binds to Metallothionein (which is a superb OH• scavenger) and increase its structure and function, and thus zinc takes part in antioxidant mechanism either straight by glutathione or secondarily by being a glutathione peroxidase cofactor [91]. As is shown in figure 1.14.

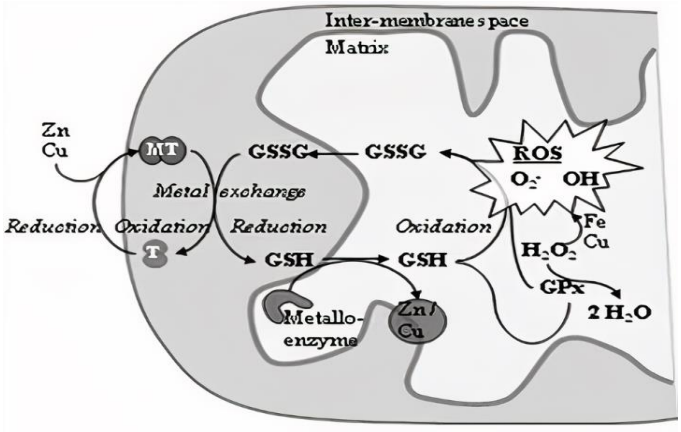

Figure 1.14. The cycle of the metallothionein-glutathione redox and metal exchange [91].

1.6.6. Phenolic compounds

The conception that phenolic compounds have the action of scavenging free radicals is constructed on the capacity to contribute a hydrogen atom to free radicals and form a less harmful radical structure that can be neutralized [92], as can be seen in figure 1.15.

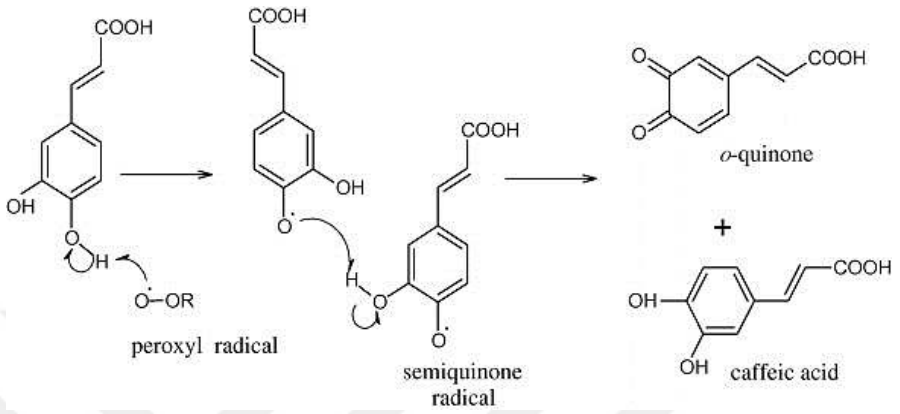

Figure 1.15. The free radical foraging action of caffeic acid [93].

Phenolic compounds are classified to phenolic acids and poly phenols; the poly phenols are: lignans, stilbenes, tannins, and flavonoids.

The phenolic compounds existing in *Cucurbita pepo* leaves are:

1.6.6.1. Phenolic acids

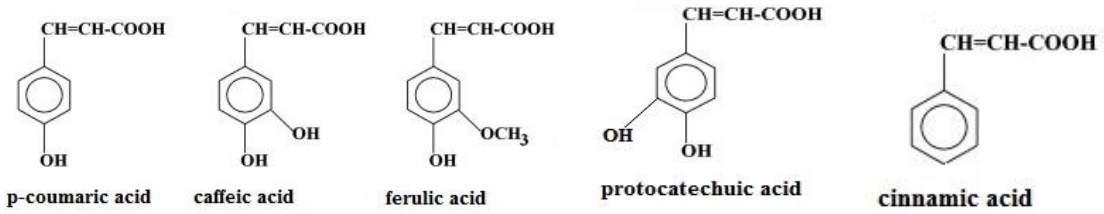

Figure 1.16. Phenolic acids that found in *Cucurbita pepo* leaves [94].

Plants manufacture phenolic acids as byproducts from the breakdown product of membrane polymers of the vascular plants [95].

Phenolic acids have a carboxyl group and a hydroxyl group joined to the aromatic ring that defines phenolic acids and differentiates them from other phenolic compounds. As shown in figure 1.16.

Phenolic acids exhibit activities in diabetes, memory loss, skin whitening, wound healing, anti-aging, as well as anti-wrinkle, etc. Besides being used in food, cosmetics, pharmaceutical applications, and chemical industries [96].

1.6.6.1.1. Phenolic acids as antioxidants

- Phenolic acids act as reducing agents donating hydrogen, which makes them potent antioxidants [97].
- Phenolic acids prevent or disturb free radical chain reactions [98].
- Intaking foods rich in caffeic acid inhibit nitro compounds formation and thus protecting normal cells from becoming cancer cells [99].
- Gallic acid and its derivatives have antitumoral properties [100].
- Gentisic acid forages radicals that cause lipid peroxidation [101].

1.6.6.2. Tannins

Tannins can be defined as water-soluble polyphenols that are made by plants [102].

In addition to having antioxidant activities, tannins differ from other plant polyphenols with binding to proteins, pigments, metal ions, and others [103].

1.6.6.2.1. Classification of tannins

Tannins can be categorized into four groups as can be seen in figure 1.17.

A/ Hydrolysable tannins: include gallotannins together with ellagitannins:

Gallotannins: a simple hydrolysable tannin that contain a polyphenolic and a polyol residue that is derivative of D-glucose.

Ellagitannins: these are made by a combination of two galloyl units of the gallotannins.

B/ Condensed tannins (proanthocyanidins): these exist as oligomers or polymers that are regularly formed from the linkage of the catechin C-4 to a C-8 of the next one.

C/ Phlorotannins: they are composed of the basic unit 1,3,5-trihydroxybenzene (phloroglucinol), and the phloroglucinol units can polymerize to form oligomers or polymers.

Phlorotannins can be further classified to Fucols, phloroethols, and fucophloroethols, as can be seen in figure 1.18.

D/ Complex tannins: these are developed by a combination of a gallotannin or an ellagitannin unit with a catechin part. [104].

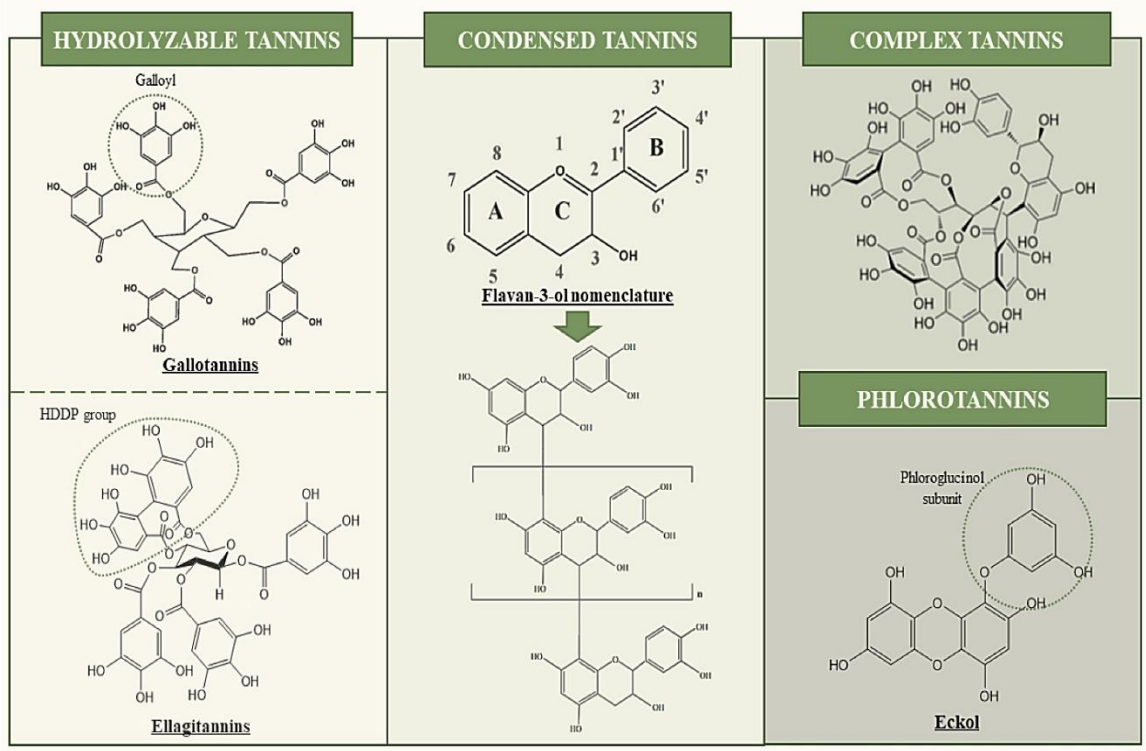

Figure 1.17. Classification of tannins [105].

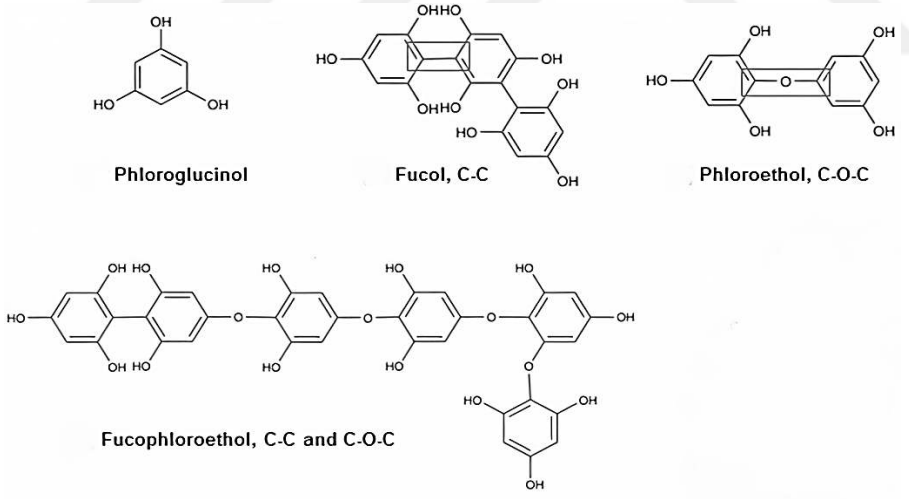

Figure 1.18. The types of phlorotannins [106].

1.6.6.2.2. Tannins as antioxidants

- Tannins have the ability to reduce peroxyl radicals [107].
- The gallotannin 1,2,3,4,6-penta-O-galloyl-β-D-glucose own appreciated role as antibacterial and antioxidant agent [108]. As can be seen in figure 1.19.

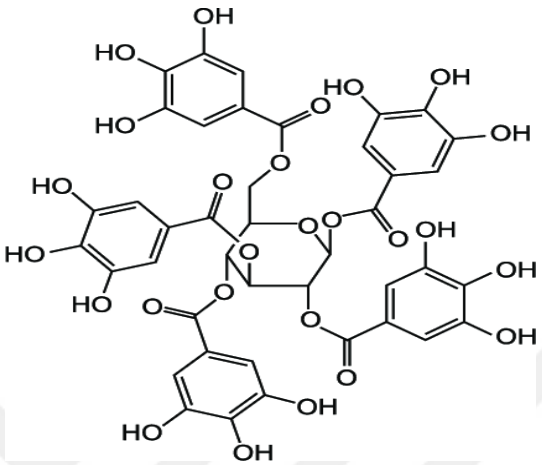

Figure 1.19. 1,2,3,4,6-penta-O-galloyl-β-D-glucose [109].

- Tannins can scavenge DPPH radicals, besides foraging the superoxide radical and prevent lipid peroxidation [110].
- Tannins demonstrate several health benefits like cardioprotective, antitumor, anti-inflammatory as well as antimicrobial activities [111].

1.6.6.3. Flavonoids

The name flavonoid is derived from the Latin word “flavus” meaning yellow Flavonoids are a class of polyphenols, they are found in fruits and vegetables. Flavonoids are liable for the colour and aroma of flowers and fruits, they protect plants from UV rays performing as filters, they role as signal molecules, they have detoxing and antimicrobial activities, as well as resisting dryness [112].

1.6.6.3.1. Classification of flavonoids

Flavonoids are classified to flavones, isoflavones, flavonols, flavanones, chalcones, as well as anthocyanidins. As can be seen in figure 1.20.

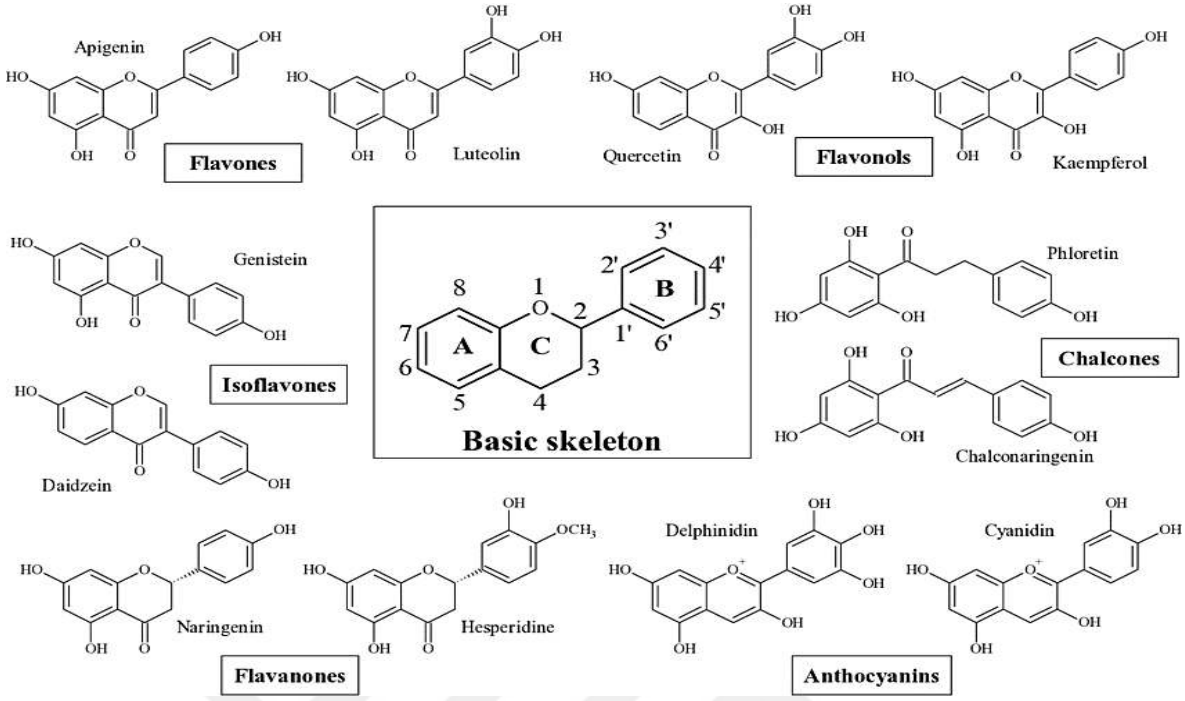

Figure 1.20. Flavonoids sub groups [112].

1.6.6.3.2. Flavonoids as antioxidants

- Flavonoids have antioxidant aptitude that is more potent than antioxidant activity of vitamin E and C.
- Flavonoids avert damages caused by free radicals through scavenging ROS and RNS, triggering antioxidant enzymes, chelating with excessive metals that implicate in oxidative stress.
- Flavonoids prompt detoxifying enzymes, and prevent cancer and mutation [113].
- Flavonoids prevent lipid peroxidation, and reduce and inhibit free radicals.
- Flavonoids have hepatoprotective, antibacterial and antiviral (specially catechins) potentials.
- Flavonoids have anit-inflammatory properties [114].

1.6.7. Alkaloids

Plant alkaloids are natural organic compounds produced by plants as secondary metabolites and have a nitrogen atom in their construction that has a cyclic system. Alkaloids are colorless crystalline solids that are nonvolatile and have a bitter taste. The name alkaloid comes from alkali-like because they react with acids to form salts [115].

1.6.7.1. Classification of alkaloids

Alkaloids include:

A/ Heterocyclic (true-alkaloids): derived from an amino acid.

Heterocyclic alkaloids are subdivided into 14 groups as can be seen in figure 1.21.

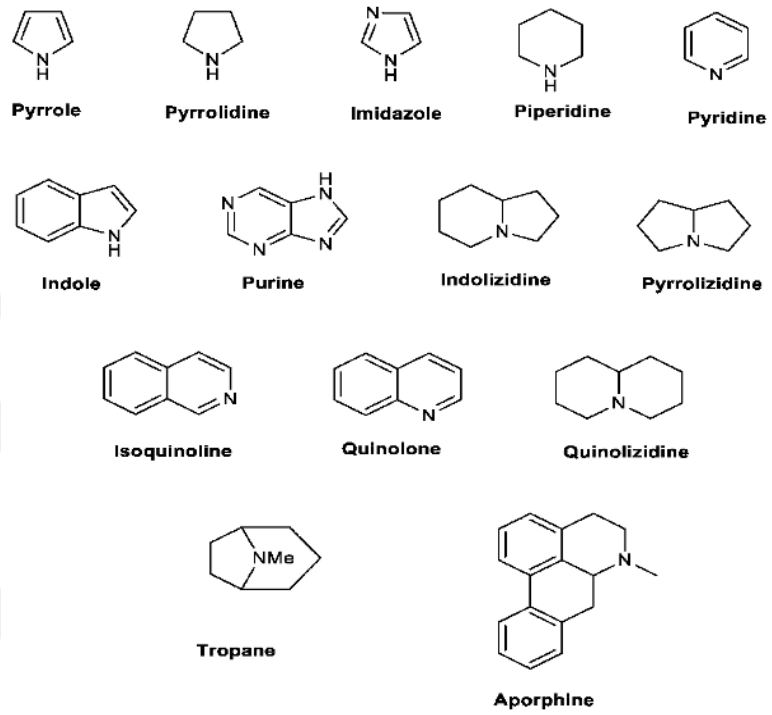

Figure 1.21. Alkaloids sub-groups [116].

B/ Non-heterocyclic (proto-alkaloids): derived from an amino acid. Some examples are shown in figure 1.22.

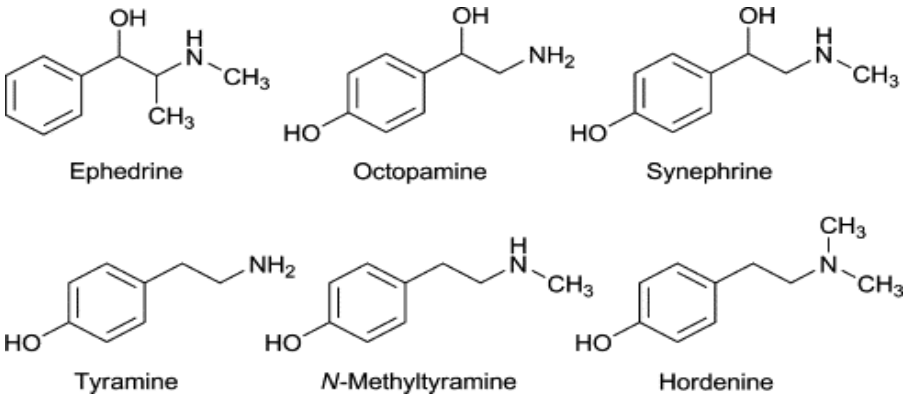

Figure 1.22. Some proto-alkaloids [117].

C/ Pseudo- alkaloids: not derived from an amino acid as given in figure 1.23.

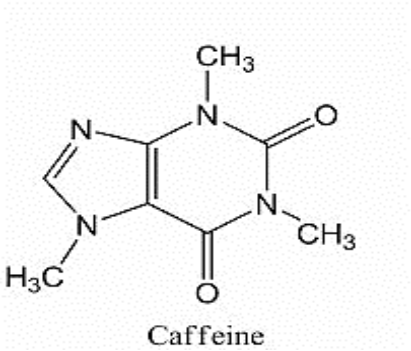

Figure 1.23. The structure of caffein a pseudo-alkaloids [118].

1.6.7.2. Alkaloids as antioxidants

The isoquinoline alkaloids have antimicrobial, anti-human immunodeficiency, antimalarial activities, and possible antitumor activity associated with their radical foraging action in contradiction of DPPH radical [119].

Antioxidant activities such as scavenging DPPH radical and inhibiting lipid peroxidation of three phenolic alkaloids isolated from the *Portulaca oleracea* plant have been determined [120].

Alkaloids have important antioxidant activities, primarily through scavenging radicals to hinder or break chain propagation and secondarily through the strong binding effects of metal ions [121].

1.6.8. Saponins

The name saponin originates from the Latin word "Sapo" because the saponins produce a soapy state forms when plants holding saponins are stirred in water. Saponins have the molecular formula: $C_{58}H_{94}O_{27}$ [122].

1.6.8.1. Classification of saponins

Saponins are:

A/ Glycosides of triterpenoids (compounds with carbon skeleton that have a complex cyclic structure formed from the condensation of six isoprene units), as can be seen in figure 1.24.

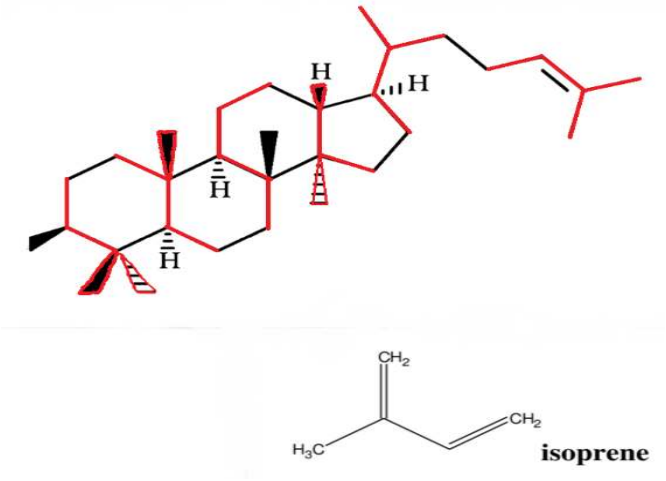

Figure 1.24. A triterpenoid saponin is made of six isoprene units [123].

B/ glycosides of steroids (isoprene derivative compounds including seventeen carbon atoms that configure four fused rings: three cyclohexane rings and one cyclopentane ring and act as a cell membrane component or signaling molecules) show various biological activities [124]. As shown in figure 1.25.

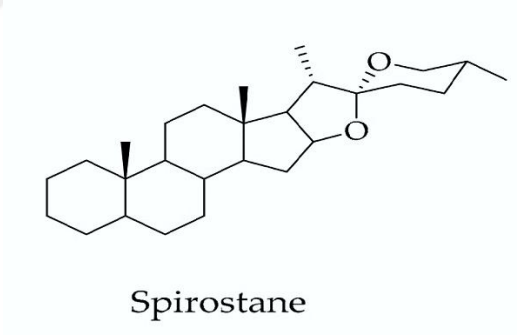

Figure 1.25. An example of a steroid molecule: spirostane [124].

An example of saponin that exist in *Cucurbita pepo* leaves is cucurbitane, can be seen in figure 1.26.

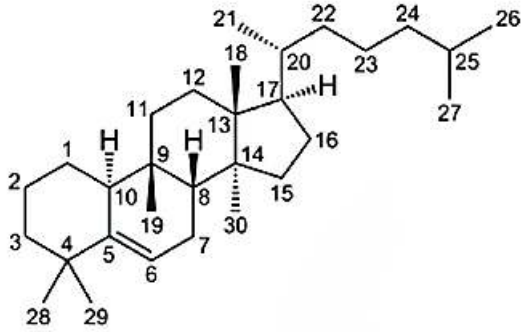

Figure 1.26. The structure of a cucurbitane [125].

1.6.8.2. Saponins as antioxidants

- Saponins have direct antioxidant activity decreasing the dangerous causes of atherosclerosis [126].
- Saponins have the capacity to scavenge superoxide anion, besides chelating ferrous (Fe+2) ions and scavenging hydrogen peroxide radical [127].
- Saponins have antitumor activities [128].
- Saponins have antibiotic, fungicidal and insecticidal properties [129].

1.6.9. Phytates

Phytate has the chemical formula: $C_6H_{18}O_{24}P_6$ and the chemical name: Myo-inositol hexaphosphate (IP6). As can be seen in figure 1.27.

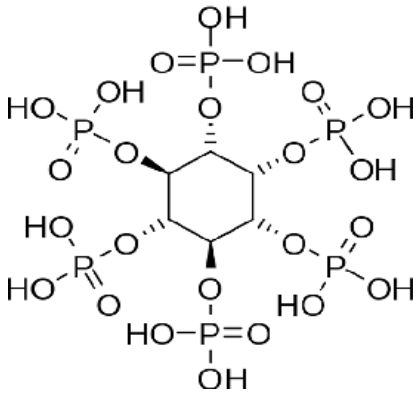

Figure 1.27. Myo-inositol hexaphosphate structure [130].

Phytate is the main phosphorus and inositol storing formula in a plant; phosphate is bounded to inositol, mainly forming phytate. The phosphate group's location on the inositol ring makes phytate extremely charged as well as being a superb chelator [131].

1.6.9.1. Phytates as antioxidants

- Phytates have antitumor and anti-cancer properties [132].
- Phytatic acid (PA) inhibit iron-catalyzed hydroxyl radical development from Fentons reaction (as mentioned earlier in the free radicals' section) because it has a good iron-chelating probability, and it reacts with all the available iron, as can be seen in reaction7, and in figure 1.28.

PA + Fe+2 / Fe+3 ⟶ PA . Fe+2 / Fe+3 … reaction 7

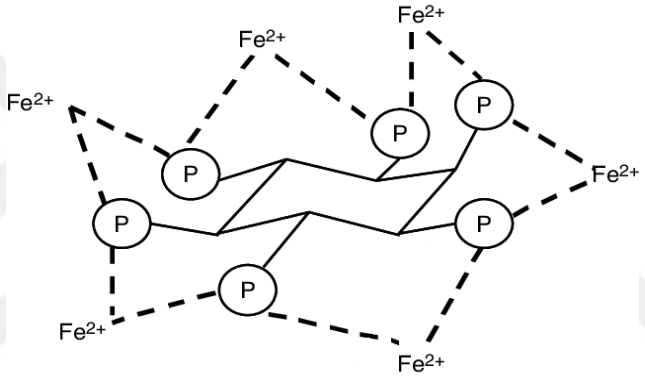

Figure 1.28. Phytic acid-binding six iron ions [133].

Phytic acid, by this role, also inhibits lipid peroxidation by hydroxyl radicals [134].

- Phytic acid (myo-inositol hexaphosphoric acid) can protect the heart against ischemic and reperfusion harms [135].
- Phytates act as a vital signaling molecule through: governing cell propagation and diversity, RNA transfer, DNA reparation, energy transformation, as well as ATP renewal besides lowering the serum cholesterol amount, regulate pathological platelet action, as well as inhibiting kidney stone formation [136].

2. MATERIALS AND METHODS

2.1. Plant material collection

Green leaves of *Cucurbita pepo* were obtained from Aşağıdemirtaş countryside that is established on the old Malatya Road, 4 km from Hankendi town and 15 km away from the center of Elazığ. It is in the west of Elazığ province center, as can be seen in figure 2.1.

Figure 2.1. The fresh leaves and the fruits of *Cucurbita pepo*.

The leaves were identified by Prof. Dr. Şemsettin CİVELEK at the biology department of Firat University, Elazığ, Turkey.

The leaves were dried using air drying at shade and room temperature (25°C) method, for two weeks, as can be seen in figure 2.2.

Figure 2.2. Air drying of the leaves.

• Later the dried leaves were grounded to make a fine powder using an electric hand blender, as shown in figure 2.3.

Figure 2.3. The powder product of the leaves.

2.2. Plant extracts preparation

The sample extraction was made following Pekdemir et al. procedures [137].

- Four sample beakers were prepared as the following: 20 g of the leaves powder sample were weighed using sensitive balance and added to each beaker, after that, 200 mL of ethanol was added to each beaker.
- The four beakers were sonicated for 10 minutes. As can be seen in figure 2.4.

Figure 2.4. Sonication of the leaves sample.

- After that, the samples were stirred using a magnetic stirrer for 2 hours, with the temperature set according to room temperature. As can be seen in figure 2.5.

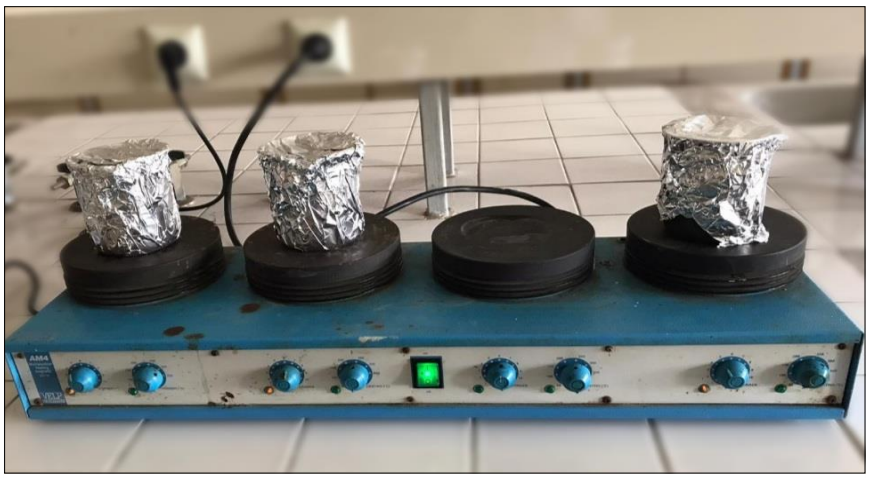

Figure 2.5. Stirring the samples with a magnetic stirrer.

- Then the samples were shaken in a laboshake shaker machine for 18 hours: prog: 60 (shaking speed), heat: 38.5°C. As can be seen in figure 2.6.

Figure 2.6. Laboshake shaker machine.

- Then the samples were stirred again for another 2 hours.
- After that, the samples were centrifuged with a laboratory centrifuge machine for 10 min. As can be seen in figure 2.7.

Figure 2.7. Centrifuge machine.

- Then the samples were filtered using filter paper, and the supernatant liquid was evaporated with a rotary evaporator machine at 40°C. As can be seen in figure 2.8.

Figure 2.8. Evaporation of the supernatant liquid.

- Finally, the samples were left to dry at room temperature and getting all ethanol evaporated.
- The same procedures were done for acetone extraction.
- After evaporation, the extracts were kept in the freezer at -18°C.

• Later the samples became a gel-like product, and the weight of the samples was calculated with a sensitive balance, the products were two beakers for each extract, one beaker of each extract has been used, the other beakers have been reserved for backup.

1. The Ethanol extract:

The weight of the empty beaker1 = 49.4595 g

The weight of the sample with the beaker1 = 51.8350 g

The weight of the sample= 51.8350 g – 49.4595 g = 2.3755 g

2. The acetone extract:

The weight of the empty beaker1 = 49.0705 g

The weight of the sample with the beaker1 = 50.3930g

The weight of the sample= 50.3930 g – 49.0705 g = 1.3225 g

Later the extract samples were stored at 4°C in the refrigerator until the determination of antioxidant and antimicrobial activities time.

2.3. Preparing the samples for antioxidant and antimicrobial activities

When the time of determining the extract samples activities came, they were taken out from the refrigerator, the sample extracts that were gel like were dissolved in 50 mL of dimethyl sulfoxide (DMSO) as following: acetone extract sample: 1.3225 g sample extract/50 mL DMSO (26450 ppm), ethanolic sample extract: 2.3755 g sample extract/50 mL DMSO (47510 ppm). And thus they were ready to be used.

2.4. Determination of antioxidants activity

2.4.1. ABTS radical scavenging activity

• The ABTS radical scavenging activity was determined by spectrophotometric analysis following Re et. al. procedures [138].

• The cation radical ABTS•+ was initiated by the reaction of 2 mM ABTS in water with 2.45 mM Potassium persulphate $K_2S_2O_8$ with keeping in the dark for 2 hours at room temperature.

• Later the ABTS•+ solution was diluted with 0.1 M sodium phosphate buffer (pH= 7.4) to attain an absorbance of 1.520 ± 0.025 at 734 nm.

• Then 1mL of ABTS•+ solution was added to 3mL (500 µg/mLfrom the prepared acetone and ethanol sample extracts), and the absorbance was measured with laboratory UV–visible spectrophotometer at 734 nm after a half of an hour. As shown in figure 2.9.-2.10. and figure 2.11.

Figure 2.9. UV–visible spectrophotometer.

Figure 2.10. The the colourless ABTS sample, and the formation of the bluish green ABTS•+ radical.

Figure 2.11. The samples before, and after neutralizing ABTS•+ radical (the colour change on the right).

2.4.2. Hydroxyl radical (OH•) scavenging activity

The capacity of the extracts to hinder non-site-specific hydroxyl radical-mediated peroxidation was measured following Halliwell et. al. procedures [139].

- 3.6 mM of 2-deoxy-D-ribose in 20 mM (pH=7.4) KH_2PO_4–KOH buffer, along with 200 µL of 100 µM $FeCl_3$, and 200 µL of 104 mM EDTA, as well as 100 µL of 1.0 mM H_2O_2 and finally 100 µL of 1.0 mM aqueous ascorbic acid was added to 500 µL of our acetone and ethanolic extracts.
- Then the mixture was mixed using laboratory Clifton™ Cyclone Vortex Mixer, as shown in figure 2.12. and incubated at 37°C in laboratory incubator for one hour. As can be seen in figure 2.13.

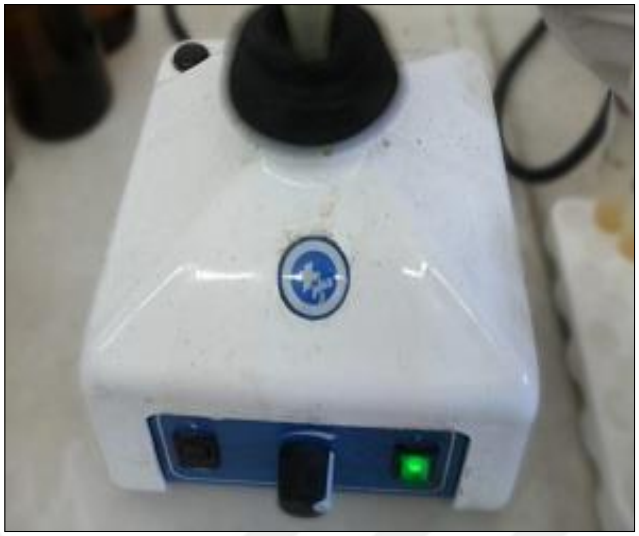

Figure 2.12. Clifton™ Cyclone Vortex Mixer.

Figure 2.13. Laboratory incubator.

- Later 1 mL of 2.8% TCA and 1 mL of 1.0% TBA was added to the samples and mixed again with laboratory Clifton™ Cyclone Vortex Mixer, and placed in water bath at 50°C for 30 minutes.
- The range of 2-deoxyribose oxidation was valued by the solution absorbance at 532 nm. As can be seen in figure 2.14.

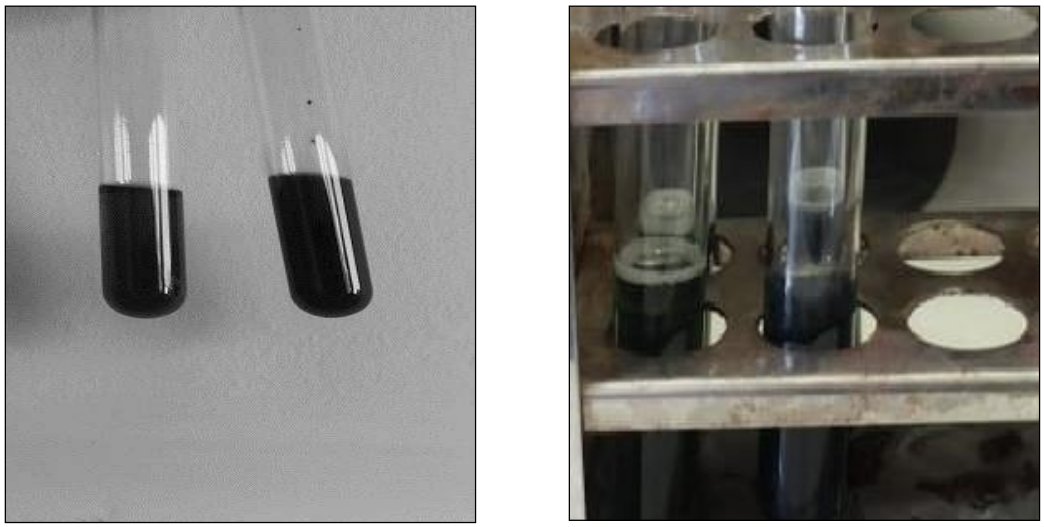

Figure 2.14. The samples before, and after scavenging hydroxyl radical (on the right).

2.4.3. DPPH radical scavenging activity

The DPPH radical scavenging ability of the extracts was measured following Brand-Williams et al. procedures [140]. And using 2,2-diphenyl-1-picryl-hydrazil as following:

- 4 mL of a previously prepared solution of 25 mg/L DPPH was mixed with 500 µg/mL of our acetone and ethanolic extracts. And the mixture was stored at room temperature for 30 minutes in the dark.
- Later the absorbance of the mixtures was measured at 517 nm with laboratory UV–visible spectrophotometer. The samples before and after adding DPPH can be seen in figure 2.15.

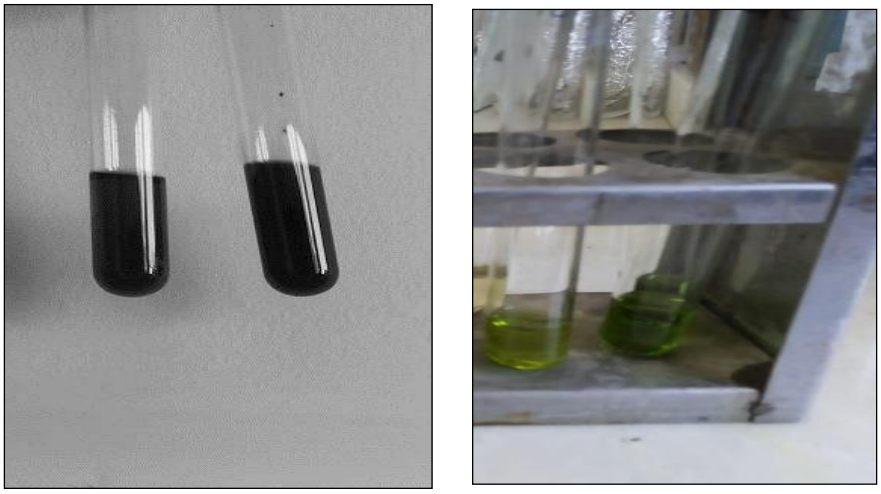

Figure 2.15. The samples befor, and samples after scavenging DPPH radical (on the right).

2.4.4. Determination of total phenolic content

The total phenolic content was determined following Slinkard and Singleton procedures [141]. And using Folin-Ciocalteu reagent. Gallic acid was used as a standard phenolic compound.

- 1mL from our (500 µg/mL) acetone and ethanoic extracts was diluted with 46 mL distilled water, and 1 mL of Folin-Ciocalteu reagent was added and the mixture was mixed thoroughly.
- Later after three minutes 3 mL of 2% Na_2CO_3 was added and the mixture was randomly shacked for two hours. The absorbance was measured at 760 nm.

2.4.5. Determination of total flavonoid content

- The determination of the total flavonoid content was accomplished following Kim et al. Procedures[142].
- To our 0.5 mL (500 µg/mL) extracts 4 mL of distilled water was added, then 0.3 mL of 5% sodium nitrite solution was added, as well as 0.3 mL of 10% aluminum chloride solution, the test tubes were incubated in for 5 minutes at room temperature.
- Later the mixture was mixed using laboratory Clifton™ Cyclone Vortex Mixer and the absorbance was measured at 510 nm.

2.4.6. Determination of proanthocyanidin content

The determination of proanthocyanidin content was carried out following Amaeze et al. procedures [143].

- 0.5 mL of our (500 µg/mL) extracts was mixed with 1.5 mL of 4% vanillin-methanol solution along with 0.75 mL of concentrated hydrochloric acid, then the mixture was let to stand for 15 min, later the absorbance was measured at 500 nm.

2.4.7. Vitamin content determination

2.4.7.1. Applied cells

Saccharomyces cerevisiae which belongs to the Fungi kingdom is a single celled yeast that has a high level of vitamins and is used to determine antioxidant activity and as a model for molecular response to oxidative stress [144].

- The dry sample of *saccharomyces cerevisiae* was kept at 4°C throughout the experiment. Cultivation of the yeast was done at the microbiology laboratory: the yeast was imbued in Difco™ Malt extract broth for one day at 25°C.

- Later the yeast culture was immunized into YEDP (1g yeast in 100mL extract, 2g Bacto™ Peptone, 2g glucose, 2g agar) and adjusted to a standard density at a rate of 1% (10000 CFU per mL for the yeast) using DEN-1 McFarland densitometer (that is Shawn in figure 2.14.) then incubated at 25°C for 2 days. As can be seen in figure 2.16.

Figure 2.16. DEN-1 McFarland densitometer.

2.4.7.2. Extracts samples treatment with *saccharomyces cerevisiae* for antioxidant activity

2 mL from *saccharomyces cerevisiae* (at density of 10000 CFU per mL) samples was added to 50 µL and 100 µL from the prepared samples extract and incubated for one day.

2.4.7.3. Determination of MDA and vitamin C

Malondialdehyde and vitamin C level were determined using the procedures of Karatepe procedures [145].

- First of all, the liquid treated samples were agitated using a syringe to keep the medium in a mixed form.
- Then 1 mL of 0.5 M $HClO_4$ was added to our samples extracts to precipitate proteins and release MDA bound to amino groups and other amino compounds, along with 0.5 mL D.W and mixed using Clifton™ Cyclone Vortex Mixer for few minutes.
- Then the samples were centrifuged using centrifuge machine for 5 min.
- the supernatant was obtained and 20 µL from it was injected into HPLC instrument.

The mobile phase was 17.5% methanol, 85.5% (30M potassium phosphate buffer pH=3.6) (v/v).

2.4.7.4. HPLC quantification for vitamins A, B

The liquid soluble vitamins of the extracts samples were analyzed following Catignani procedures [146].

- To 100 µL of the treated extracts samples, 200 µL of ethanol along with 99:1 H_2SO_4 and 100 µL D.W were added in a test tube for proteins precipitation, then the samples were mixed with laboratory Clifton™ Cyclone Vortex Mixer, and later centrifuged at 4500 RPM for 5min.
- After centrifugation100 µL of n-hexane along with 0.05% BHT were added to the mixture, so that to extract the lipid soluble vitamins in the samples.
- The mixture was mixed and centrifuged again.
- Then the hexane layer was obtained and transferred to another test tube.
- Another 100 µL of n-hexane was added to the sample residue, mixed, centrifuged and added to the previous hexane layer.
- Then the hexane layer was evaporated by nitrogen gas, and to test tube residues 50 µL methanol, 25 µL acetonitrile, 25 µL chloroform, was added.
- Finally, 20 µL from the test tube mixture was injected into the HPLC instrument. The mobile phase was (47:42:11) (v/v) methanol/ acetonitrile/chloroform.

2.4.7.5. Apparatus

The high performance liquid chromatography (HPLC) system consisted of CTO-10AS VP column oven, LC-20AD pumps, DGU-20A5 degassers, SIL 20A auto sampler, SPD-M20A DAD system. Communication model: Model CBM-20A which connects the other apparatus together, LC Solution Workstation (Shimadzu, Kyoto, Japan) controller. And Shimadzu Shim-pack vp-ODS column (150L× 4.6). As shown in figure 2.17.

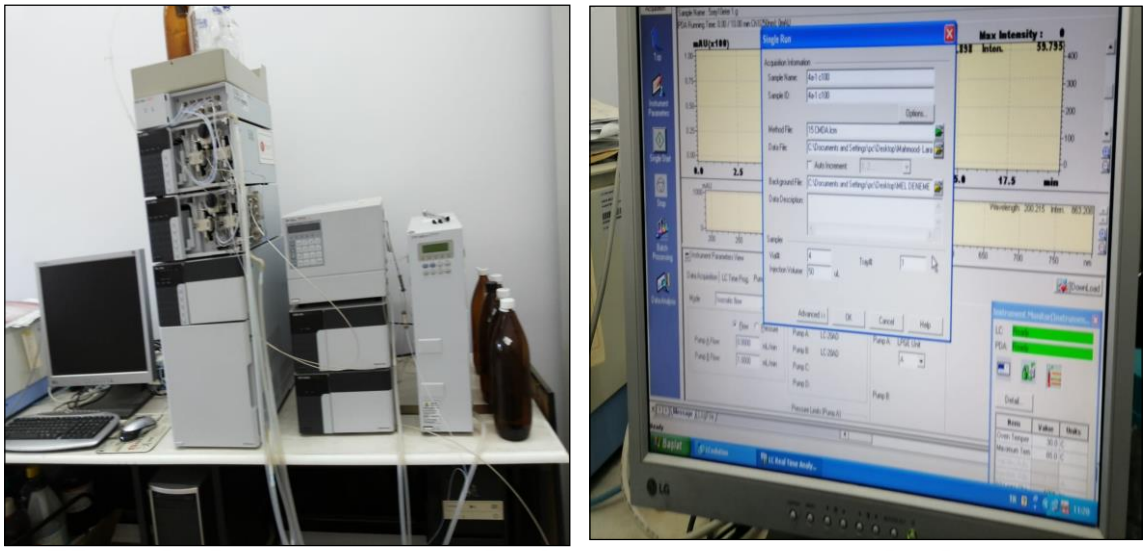

Figure 2.17. The HPLC device.

2.4.7.6. Statistical analysis

Statistical analysis was made using SPSS 22.0 for windows software. The statistical significance was at $p< 0.05$, and the results were expressed as mean ±S.D. Independent sample t-test were used for evaluation of mean values.

2.5. Anticancer activity determination

The anticancer activity determination was done at the faculty of medicine- Firat university.

2.5.1. Supply of cell lines

The cell lines: human breast cancer (MCF 7) and hepatocellular carcinoma (HEPC 2) were achieved from Ankara University Biotechnology Institute.

2.5.2. Cell culture and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test

2.5.2.1. Cell culture

The anticancer activity of *Cucurbita pepo* leaves extracts was tested through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide method.

- Human breast cancer and hepatocellular carcinoma cells were developed in 25 cm² flasks in Dulbecco's modification of Eagle medium (1% L-glutamine, 10% fetal bovin serum, and 1% Penicillin-Streptomycin) medium, at 37°C beneath 5% CO_2 atmosphere conditions

- After the development of the cells in the flasks and covering the flask surface the medium was removed from the cells and the cells were washed with sterile phosphate-buffered saline solution, then 1 mL of trypsin-EDTA was added to the flasks and incubated for 2 min at 37°C in an oven with 5% CO_2
- Later the cells were separated from the surface, 5mL of the medium was added to inactivate Trypsin-EDTA and the cells were occupied from the flask and centrifuged at 1200 RPM for 5min, the supernatant was discarded and the pellet was dissolved in 10 mL of the medium
- After that 100µL from the cells in the medium was embedded in 96-well plates, while the first row was embedded with the medium alone as a blank. The plates were incubated for one day at 37°C in an oven with 5% CO_2
- 2.5 ng/mL doxorubicin was used as positive control while the medium is used as negative control. After incubation 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide test was applied [147, 148].

2.5.2.2. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide test is used to measure cell feasibility, viability, and cytotoxicity. The test is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, a salt that can pass through the cell membrane of lively cells mitochondria and receiving electrons inside the cell and changing into water soluble formazan crystals [149, 150].

- 20 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution was added to stock prepared in sterile phosphate buffer (pH: 7.2) cells in well plates, and incubated for 4 hours at 37°C with 5% CO_2 in the dark
- Later the medium was removed and the salt crystals were dissolved with 100 µL DMSO
- 100 µl of the salt solution was added to 96-well plates and kept in the dark for 10 min
- Later the absorbance of the salt solution was determined at 450 nm with a plate reader mashine
- The average of the control wells absorbance was obtained and accepted as 100% viable cells
- The gained absorbance from the *Cucurbita pepo* leaves extracts wells was proportional to the control absorbance and the percent of viability was calculated [147].
- The measured absorbance values were compared with the control and the data were transported to diagrams

2.5.3. Analysis

The absorbance values were quantified with Enzyme Linked Immunosorbent Assays (ELISA) plate reader at 450 nm and the average of the absorbance values was quantified and after comparing with the control the control the percentage was calculated.

2.6. Determination of antimicrobial activity

The antimicrobial activity was determined following SÖNMEZ et al. procedures [151]:

Using disk diffusion method, the antimicrobial activity of the extracts was evaluated against:

A/ Bacteria strains: *Escherichia coli* (ATCC 25322), a gram-negative bacterium, *Klebsiella pneumoniae* (ATCC 700603), another gram-negative bacterium, *Staphylococcus aureus* (ATCC 25923), a gram-positive, *Bacillus megaterium* (DSM 32) also a gram-positive, were cultivated into Difco™ nutrient broth medium, for one day at 35°C. (Nutrient broths are biological media used for providing nutrition requirements of bacteria).

B/ Yeast strain: *Candida albicans* (FMC 17), a gram-positive fungus, was imbued in Difco™ Malt extract broth for two days at 25°C.

- The cultivated cells of bacteria and yeast were imbued into Mueller-Hinton agar together with Sabouraud dextrose agar and well mixed then adjusted to standard density at a rate of 1% (1000000 CFU per mL for bacteria, and 10000 CFU per mL for yeast) using DEN-1 McFarland densitometer.
- Then 25mL of the cells were poured into sterilized petri dishes (diameter = 9 cm) with homogenous spreading until the agar was hardened.
- Oxoid™ antimicrobial discs (diameter = 6mm) were soaked with 100 µL of (4.751 g) ethanol extract and 100 µL of (2.645 g) acetone extract respectively and were placed on the hardened agar and stored in the laboratory incubator for two hours at 4°C to permit the extracts dispersion into the agar media.
- Later the petri dishes with the bacterial strains were incubated in a laboratory incubator at 37°C for one day. In contrast, the petri dishes with the yeast strain were incubated at 25°C for two days.
- Streptomycin sulfate 10 µg/ disc was used as a control disk for bacteria, while Nystatin 10 µg/ disc was used as a control disk for yeast, and dimethyl sulfoxide (DMSO) was used as a negative control.
- On the next day, the zone inhibitions were measured in millimeters.

3. RESULTS AND DISCUSSION

3.1. The antioxidant activities:

Our experiment results coincide with other previous studies: (Dissanayake et al., 2018) [152], (Mondal et al., 2017) [153], (Oboh et al., 2010) [154], (Dar et al., 2017) [155], (Nwanna et al., 2007) [156], in that Cucurbita pepo leaves can be considered as a potent source of antioxidants due to the phytochemical contents of the leaves: phenolic compounds, vitamins, etc. as been mentioned earlier in antioxidants in Cucurbita pepo leaves section. The activity and concentration of the antioxidant phytochemicals in the leaves differ in accord with environmental conditions, genetic aspects, the aptitude of the plant to produce the active phytochemicals besides to decrease in antioxidant activity either due to demolition of antioxidant compounds or formation of prooxidants with the antioxidants. And it is dose dependent [152, 157]. The poly phenols dissolve in polar and semi-polar solvents like our semi-polar acetone and ethanol solvents [158].

The ABTS+• radical, hydroxyl radical, and DPPH• radical scavenging activity is shown in table 1, both ethanolic and acetone extracts had a high radical scavenging activity, but the ethanolic extract had a higher radical scavenging activity, and these results point out that phytochemical compounds that are responsible for the radical scavenging activity have higher content and thus more activity in the ethanolic medium rather than the acetone medium.

The ABTS+• radical scavenging by the extract antioxidants is based on electron transfer [159]. The $ABTS^{+\bullet}$ radical is produced by oxidizing ABTS molecule with potassium persulfate:

$$ABTS + S_2O_8^{2-} \longrightarrow ABTS^{+\bullet} + SO_4^{\bullet -} + SO_4^{2-}$$ ……reaction 8 [160].

The ABTS sample is colourless while the ABTS+• radical has a bluish green colour as shown in figure 2.10. And ABTS+• radical is decolourized when its reduced by the sample that contains antioxidant phytochemicals (mainly phenolic compounds) as can be seen in reaction 9, and in figure 3.1.

$$ABTS^{+\bullet} + PhOH \longrightarrow ABTS + PhO^{\bullet} + H^{+}$$ …… reaction 9 [160].

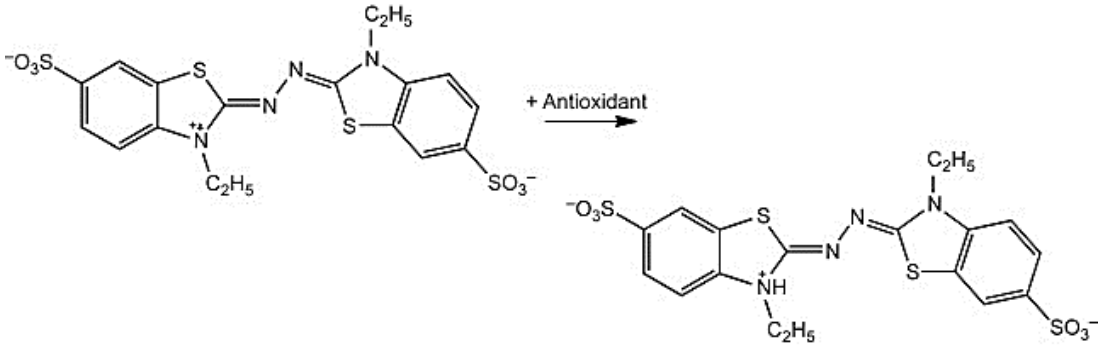

Figure 3.1. Neutralizing $ABTS^{+\bullet}$ radical by an antioxidant compound [161].

The percentage of The ABTS+• radical is indicated by the equation:

% Scavenging Activity = [(A° - A1)/A°] x 100

Where A° is the absorbance of control, and A1 is the absorbance of the extracts samples.

The hydroxyl radical is produced by Fenton reaction between ferrous ion and hydrogen peroxide as mentiond in reaction 1, in the free radicals sources section. And the 2-deoxy-D-ribose is used to signalize the damage caused by hydroxyl radical, and the degree of this reduction in proportional with the antioxidants concentration, the antioxidants in the extracts sample protect the 2-deoxy-D-ribose from hydroxyl radical damage, the mechanism of the reaction of the antioxidants in the extracts with the hydroxyl radical may be one of three mechanisms [162], as is shawn in the reactions bellow, Caffeic acid (CA) is used as an example of a phenolic compound antioxidant:

$CA + OH\bullet \longrightarrow CA^{+\bullet} + OH^{-}$ …reaction 10, mechanism1 (based on electron transfer).

$CA + OH\bullet \longrightarrow CA\bullet + H_2O$ …reaction 10, mechanism 2 (based on hydrogen atom transfer).

$CA + OH\bullet \longrightarrow CA(OH)\bullet$ …reaction 10, mechanism 3 (based on radical adduct formation).

The percentage of the hydroxyl radical scavenging activity is determined using the equation:

% Scavenging Activity= [(A° - A1)/A°] x 100

Where A° is the absorbance of control, and A1 is the absorbance of the extracts samples.

The DPPH (2,2-diphenyl-1-picryl-hydrazil) radical is a free radical with an unpaired electron and has a violet colour, the DPPH assay is based on hydrogen atom transfer from the antioxidant compound to the DPPH molecule as can be seen in figure 3.2.

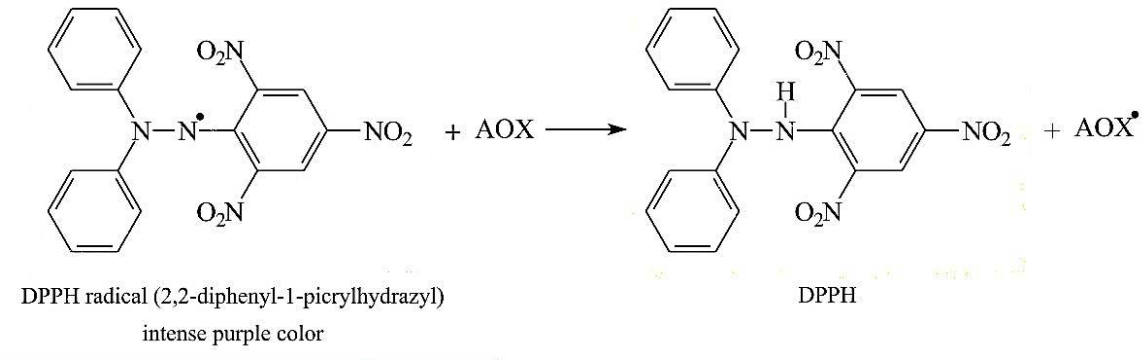

Figure 3.2. Neutralizing DPPH radical by an oxidant molecule [163].

And neutralizing DPPH radical will turn its colour to light yellow, as can be seen in the colour change of the extracts samples in figure 2.16.

The scavenging activity of DPPH by the phytochemicals in the sample extracts is determined using the equation:

% Scavenging Activity= [(A° - A1)/A°] x 100

Where A° is the absorbance of control, and A1 is the absorbance of the extracts samples. As can be seen in table 3.1.

Table 3.1. The absorbance of the control, standard, and the samples:

Control, standard, and samples	ABTS•+ test absorbance	DPPH test Absorbance	OH˙ Radical test absorbance	Flavonoids absorbance	Phenolic compounds absorbance	Proanthocyanidins absorbance
Control	1.519	3.135	1.555			
BHT	0.032	0.145	0.054			
Acetone extract	0.134	1.232	0.483	3.01	1.612	1.135
Ethanolic extract	0.115	1.005	0.325	2.767	1.436	1.338

Using the absorbance given in table 3.1 as well as using the equation:

% Scavenging Activity= [(A° - A1)/A°] x 100, for determining the radical scavinging activity, the following results were optained in table 3.2.

Table 3.2. Radicals scavenging activities, and contents of the leaves extracts:

Extracts	ABTS+• Scavenging (%)	OH• Scavenging (%)	DPPH• Scavenging (%)	Total Flavonoid (µg CE/g)	Total Proanthocyanidin (µg CE/g)	Total Phenolic (mg GAE/g)
Acetone extract	91.18	68.94	60.70	6013.40	1289.67	62.51
Ethanolic extract	92.43	79.10	67.94	5527.40	1515.22	55.14
BHT	97.89	96.53	95.37	-	-	-

The antiradical activity results were calculated for 500 µg/mL extract concentrations. Total flavonoid and total proanthocyanidin contents were expressed as µg catechin equivalent/g extract, and total phenolic content were expressed as mg gallic acid equivalent/g extract. From the table it is clear that the ethanolic and acetone extracts exhibited lower scavenging activity than the BHT standard.

For the total flavonoids content was determined using catechin as a standard and calibration was done using different concentrations of catechin (10, 30, 50, 70) (mg/mL) and the standard equation: y= 0.0005x + 0.0033 with the correlation coefficient (R^2=1) was used to find the total flavonoids, whereas y= the absorbance of the samples at 510 nm, and x= total flavonoids content

in the samples. The total flavonoids in the ethanolic extract of *Cucurbita pepo* leaves was 5527.40 µg CE/g extract. While the total flavonoids in the acetone extract was 6031.40 µg CE/g extract. As can be seen in table 3.3. And in figure 3.3.

Table 3.3. Standard catechin acid absorbance.

Catechin standard	Concentration (mg/mL)	Absorbance At 510 nm
1	10	0.0083
2	30	0.0183
3	50	0.0283
4	70	0.0383

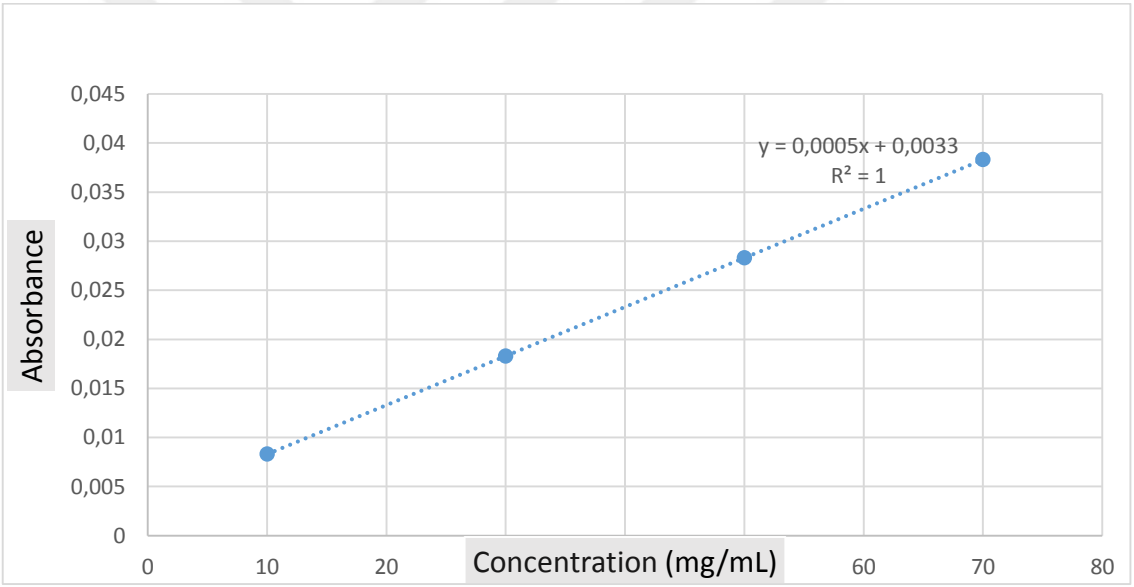

Figure 3.3. Catechin standard calibration curve.

For the total phenolic amount gallic acid was used as a standard, calibration was done using different concentrations of gallic acid (5, 10, 15, 20) (µg/mL) and the standard equation from the calibration curve was used: y= 0.0239x + 0.1181 with the correlation coefficient (R^2=1) was used to find the total phenolics, whereas y= the absorbance of the samples at 760 nm, and x= total phenolic content in the samples. As can be seen in table 3.4. And in figure 3.4.

Table 3.4. Standard gallic acid absorbance.

Gallic acid standard	Concentration (µg/mL)	Absorbance At 760 nm
1	5	0.2376
2	10	0.3571
3	15	0.4766
4	20	0.5961

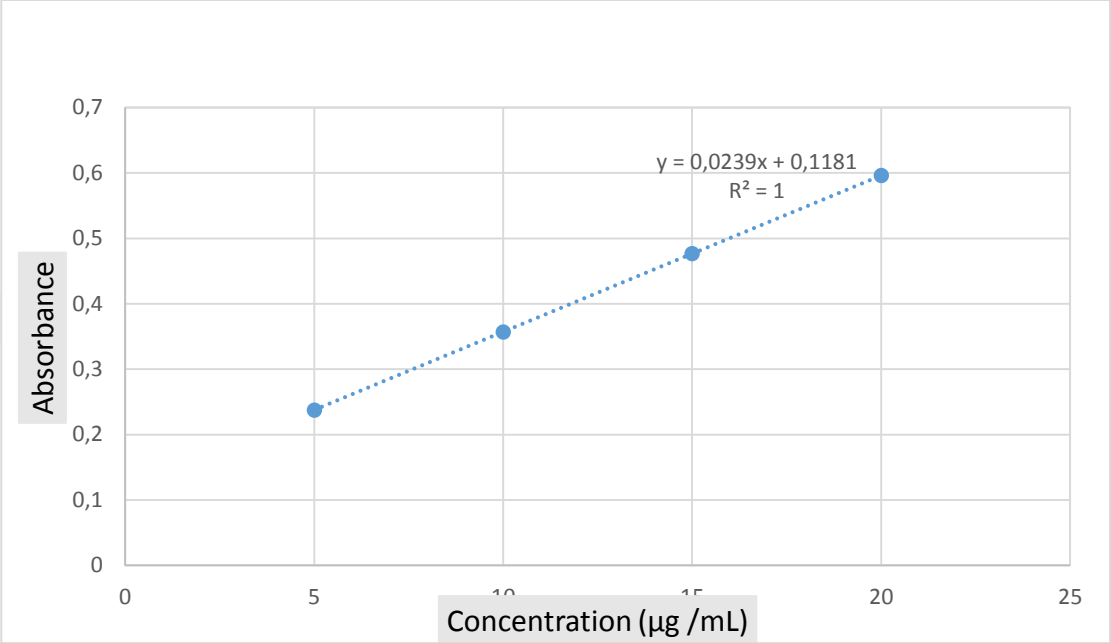

Figure 3.4. Gallic acid standard calibration curve.

The total phenolic content in the ethanolic extract of *Cucurbita pepo* leaves was 55.14 mg GAE/g extract. While the total phenolic content in the acetone extract was 62.51 GAE/g extract.

For the total proanthocyanidin content was determined using catechin as a standard and calibration was done using different concentrations of catechin (10, 20, 30, 40) (µg/mL) and the standard catechin equation: y= 0.0009x - 0.0257 with the correlation coefficient (R^2=1) was used to find the total proanthocyanidin, whereas y= the absorbance of the samples at 510 nm, and x= total proanthocyanidin content in the samples. The total proanthocyanidin in the ethanolic extract of *Cucurbita pepo* leaves was 1515.22 µg CE/g extract. While the total proanthocyanidin in the acetone extract was 1289.67 µg CE/g extract. As can be seen in table 3.5. And in figure 3.5.

Table 3.5. Standard catechin acid absorbance.

Catechin standard	Concentration (µg/ml)	Absorbance At 510 nm
1	10	-0.0167
2	20	-0.0077
3	30	0.0013
4	40	0.0103

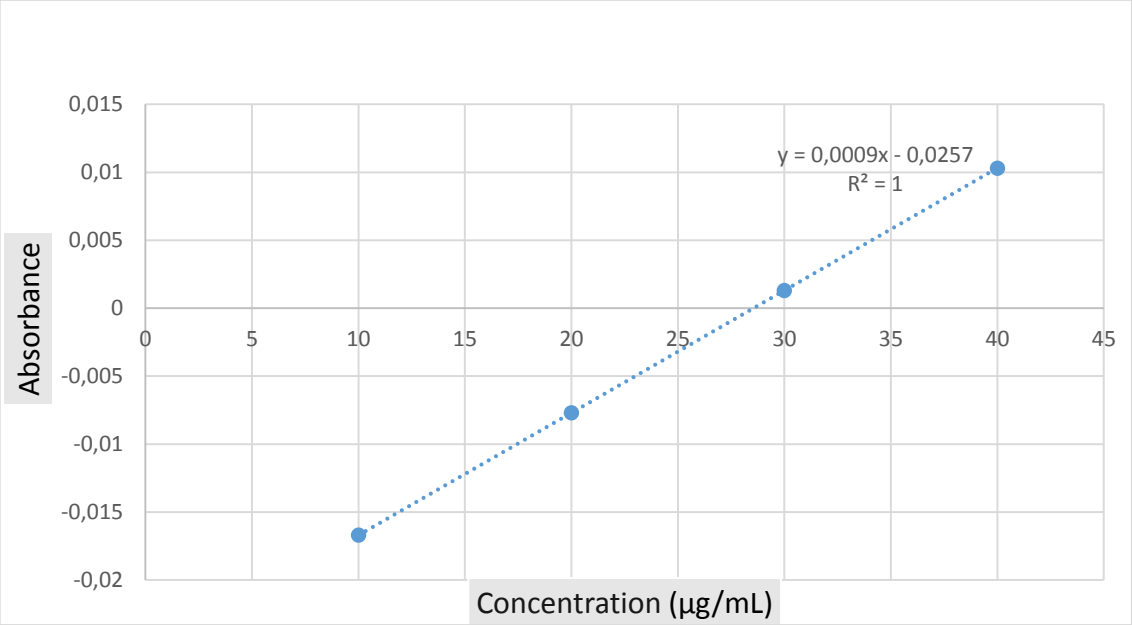

Figure 3.5. Catechin standard calibration curve.

3.2. Vitamin content determination:

The HPLC chromatograms for MDA with 7.179 retention time, vitamins C with 4.099 retention time, vitamin E with 3.922 retention time, vitamin A with 2.342 retention time, were like the followings in the figures bellow, in figure (3.6.), (3.7.), (3.8.), and figure (3.9.):

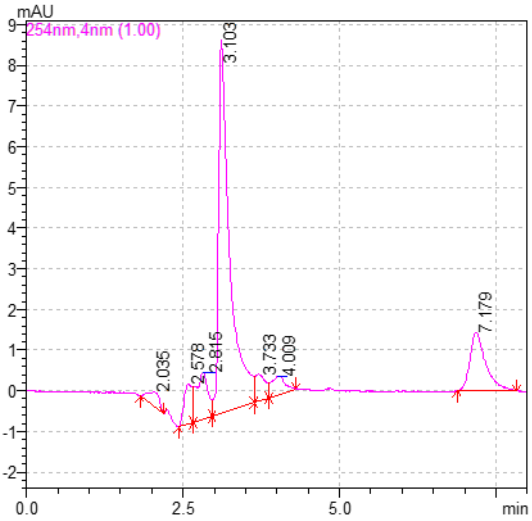

Figure 3.6. HPLC chromatogram for MDA

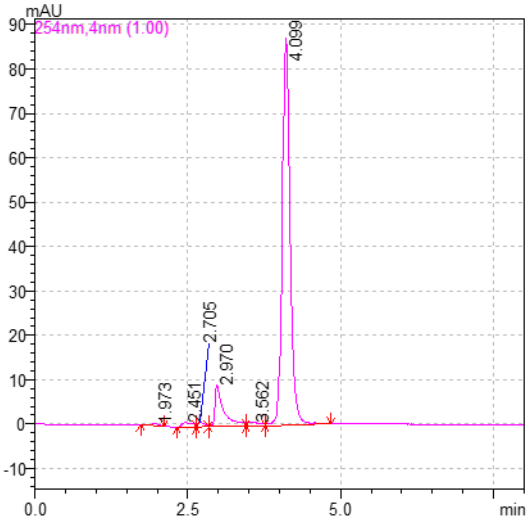

Figure 3.7. HPLC chromatogram for vitamin C

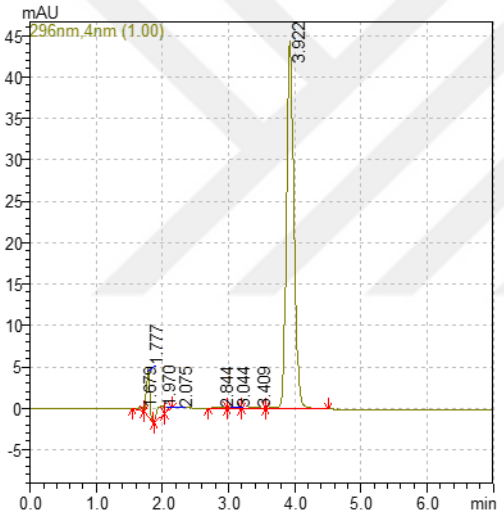

Figure 3.8. HPLC chromatogram for vitamin E

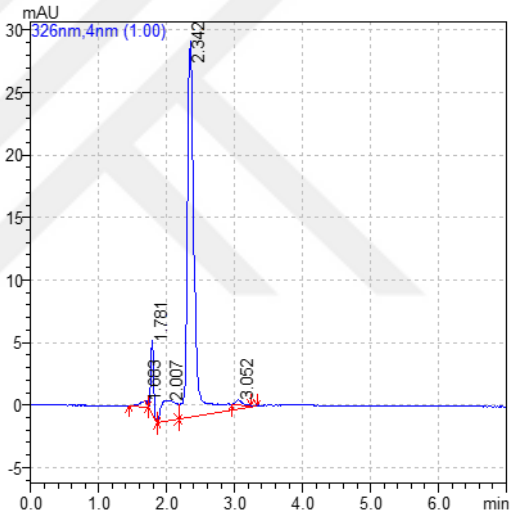

Figure 3.9. HPLC chromatogram for vitamin A

While the mean values of the extracts samples treated with *Saccharomyces cerevisiae* were as the following in table 3.6.

Table 3.6. The mean values of MDA, vitamins C, A, E for the extracts at $p < 0.05$.

Control and Extracts	MDA (ppm)	Vitamin C (ppm)	Vitamin A (ppm)	Vitamin E (ppm)
Control	23±0.26	0.82±0.02	0.08±0.009	0.79±0.06
Acetone extract (50 µl)	22.67±1.12	0.79±0.05	0.06±0.11	0.75±0.05
Acetone extract (100 µl)	21.34±0.88	0.82±0.04	0.08±0.001	0.84±0.06
Ethanolic extract (50 µl)	21.83±1.26	0.77±0.08	0.09±0.009	0.86±0.08
Ethanolic extract (100 µl)	23.55±2.02	0.83±0.05	0.09±0.008	0.88±0.08

The malondialdehyde is an indicator of lipid peroxidation because MDA is one of the fatty acid lipid peroxidation products [164]. The concentration of MDA in the extracts samples is almost the same as the concentration of the MDA in the controle, which indicates that vitamin antioxidants in the extracts had no effect on inhibiting MDA formation. The concentration of vitamin C in our sample extracts was the same or a little less than the control concentration. The concentration of vitamin A of the acetone extract samples was the same or a little less from the control, but the ethanolic extract samples concentration of vitamin A was a very little higher than the control concentration, and the vitamin A concentration increased with increasing the concentration of the extract sample. The E concentration of the 50 µl acetone extract sample was less than the control concentration, while the 100 µl acetone extract sample was more than the control concentration, while the concentration of vitamin E in the ethanolic extracts were both more than the concentration of the control, and the vitamin E concentration increased with increasing the concentration of the extract sample.

The vitamins values (especially vitamin C) are less than they were expected and this can be due to immoderate loss of the vitamins content throughout proceeding the analytical procedures, like drying and storage methods, common errors that result throughout sampling strategies, as well as analysis duration [165, 166].

3.3. Anticancer activity:

The phytochemicals in *Cucurbita pepo* leaves are regarded as anticancer sources, as mentioned in the introduction section. The MTT test is used to determine the effect of our acetone and ethanolic extracts on the cancer cells viability.

The extracts samples showed no significant effect fighting against the hepatocellular carcinoma, as can be seen in figure 3.10. And in table 3.7. The acetone extracts (5, 2.5, 1.25 ppm)

had no effect on decreasing the cancer cells viability, but the 10 ppm acetone extract had 2.5% less cancer cells viability than the negative control, indicating that the activity of the extract sample against the cancer cells increase with the concentration of the extract. The same situation happened with the (5, 2.5, 1.25 ppm) ethanolic extracts, while the 10 ppm extract had 6.2% less cancer cells viability than the negative control.

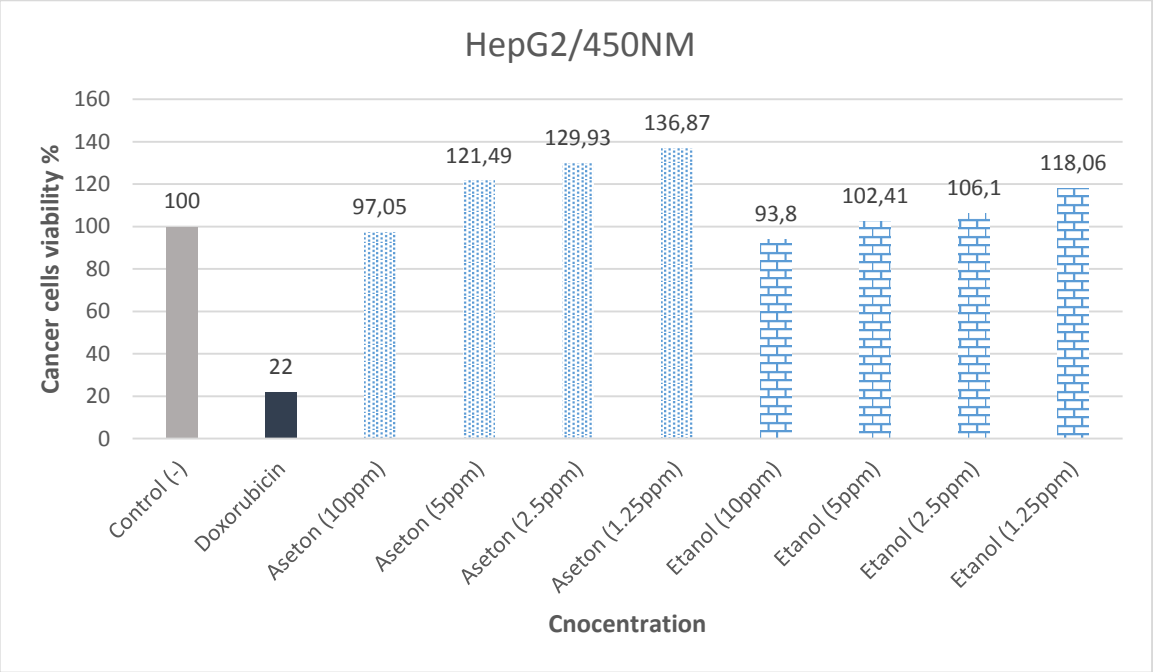

Figure 3.10. The effect of the acetone and ethanolic axtracts on hepatocellular carcinoma.

While the extract samples showed significant effects against human breast cancer as can be seen in figure 3.11.

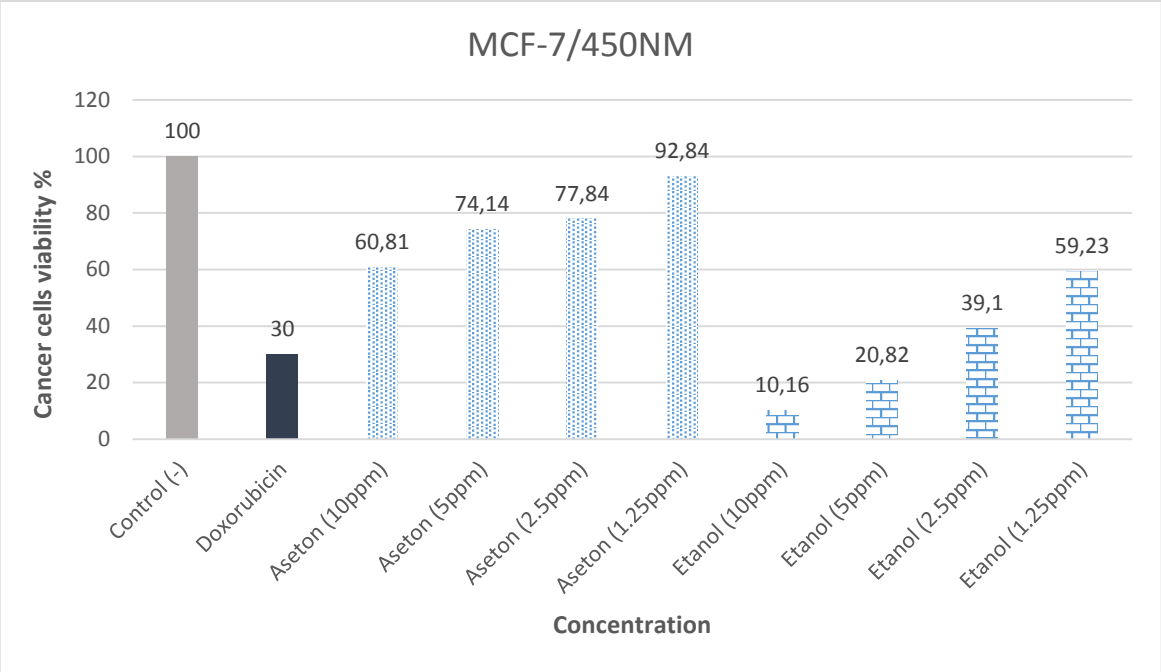

Figure 3.11. The effect of the acetone and ethanolic axtracts on human breast cancer.

The acetone extract at a concentration of 10 ppm had 39.19% less cancer cells viability than the negative control, the acetone extract at a concentration of 5 ppm had 25.86% less cancer cells viability than the negative control, the acetone extract at a concentration of 2.5 ppm had 22.16% less cancer cells viability than the negative control, and the acetone extract at a concentration of 1.25 ppm had 7.16% less cancer cells viability than the negative control. Indicating that the extracts samples activity increases with increasing the extract concentration.

While the ethanolic extract at a concentration of 10 ppm had 19.84 less cancer cells viability than the Doxorubicin which is a breast cancer therapy, the ethanolic extract at a concentration of 5ppm had 9.18 less cancer cells viability than Docorubicin, the ethanolic extract at a concentration of 2.5 ppm had 60.9% less cancer cells viability than the negative control, and the ethanolic extract at a concentration of 1.25 ppm had 40.77% less cells viability than the negative control. Indicating again that the extracts samples activity against the cancer cells increase with increasing the concentration of the samples extracts. And indicationg that the ethanolic sample extract is a potent anti-breast cancer. And also indicating that the phytochemicals responsible for the anti-cancer activities are more dissolvable in the ethanol solvent than the acetone solvent.

Table 3.7. The effect of the acetone and ethanolic extracts samples on hepatocellular carcinoma and human breast cancer.

Compound	Cell lines (Viability %)		Concentration
	MCF-7 cell line	HepG-2 cell line	
Acetone	60.81	97.05	10 ppm
	74.14	121.49	5 ppm
	77.84	129.93	2.5 ppm
	92.84	136.87	1.25 ppm
Ethanol	10.16	93.8	10 ppm
	20.82	102.41	5 ppm
	39.1	106.1	2.5 ppm
	59.23	118.06	1.25 ppm

3.4. Antimicrobial effect of *Cucurbita pepo* leaves:

Cucurbita Pepo leaves can show antimicrobial properties due to the existence of the phytochemicals: alkaloids, saponins, tannins, flavonoids, steroids, and phenolic acids, as mentioned before.

Both acetone and ethanolic extracts of the leaves were effective against the fungus and bacteria, except for the ethanolic extract against the two gram-negative bacteria *E.coli* and *K. pneumonia*, as can be seen in figure 3.12.- 3.13.- 3.14.- 3.15. and figure 3.16. as well as table 3.8.

The diameter of the inhibition zones of the leaves extracts was measured in millimeters, and the (-) refers to no inhibition zone.

Table 3.8. The leaves extracts inhibition zone diameters.

Plant extract	***E. coli***	***K. pneumonia***	***B. megaterium***	***S. aureus***	***C. albicans***
Acetone	10 mm	5 mm	9 mm	8 mm	10 mm
Ethanol	-	-	10 mm	10 mm	10 mm
Control	19 mm	20 mm	25 mm	19 mm	24 mm

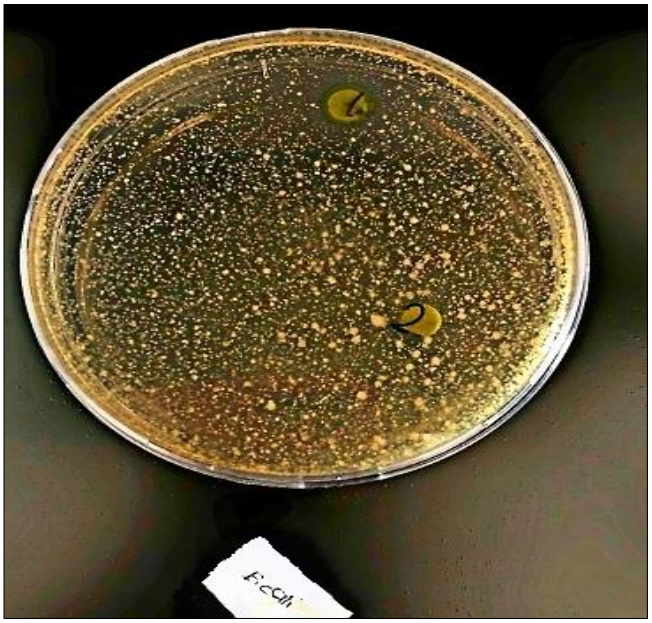

Figure 3.12. The anti-microbial effect of acetone (1) and ethanol (2) extracts on *E. coli* bacteria.

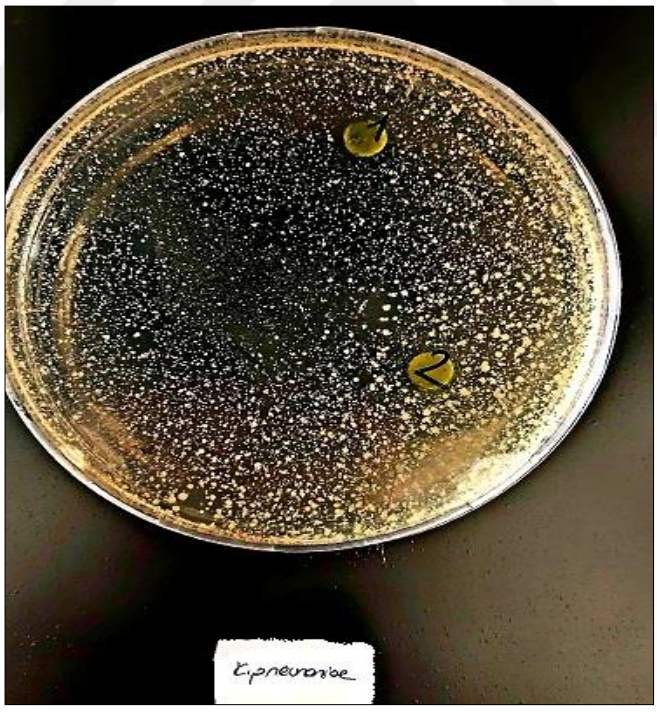

Figure 3.13. The anti-microbial effect of acetone (1) and ethanol (2) extracts on *K. pneumonia* bacteria.

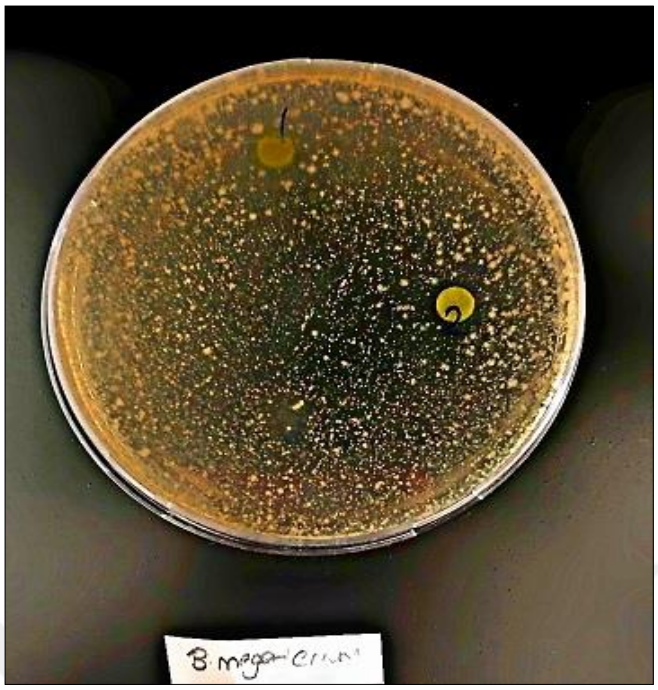

Figure 3.14. The anti-microbial effect of acetone (1) and ethanol (2) extracts on *B. megaterium* bacteria.

Figure 3.15. The anti-microbial effect of acetone (1) and ethanol (2) extracts on *S. aureus* bacteria.

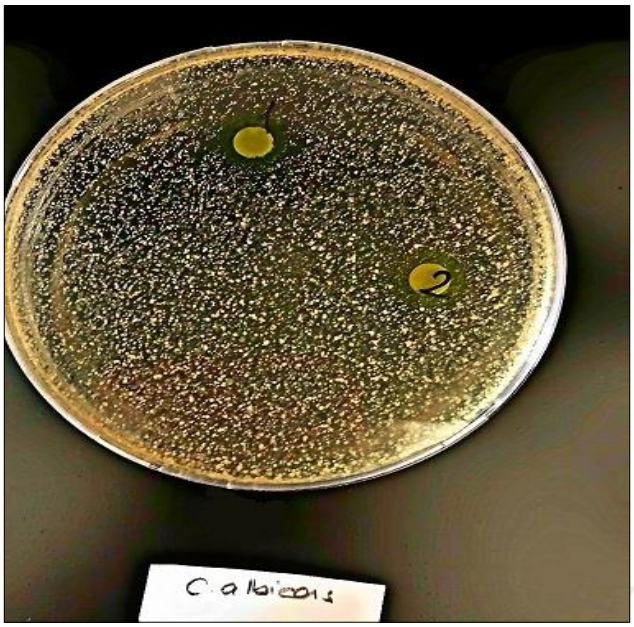

Figure 3.16. The anti-microbial effect of acetone (1) and ethanol (2) extracts on *C. albicans* fungus.

The acetone extract was effective against *E.coli* bacteria, while the ethanolic extract showed no effect; the reason why acetone extract had an effect on *E.coli* while ethanolic extract had no effect can be related to that the phenolic compounds that exist in the leaves are more soluble in the acetone solvent than in the ethanol solvent [167]. So higher concentration of these compounds that exist in the acetone extract causes higher antimicrobial resistance.

While the reason why the ethanolic extract was effective against the gram-positive bacteria and had no effect against the gram-negative ones is due to the gram-negative bacteria outer membrane that has lipopolysaccharide that shields the thin peptidoglycan inner wall, and so the outer membrane is a barrier to the antibacterial constituents, contrasted to the gram-positive bacteria in which the absence of the outer layer and a single membrane structure cause less resistance [168].

And the same case happened with the gram-negative *K. pneumoniae* bacteria, and the ethanolic extract showed no effect, while the acetone extract showed resistance only around one side of the disk. And the diameter was 10 mm, so we divided it by two and wrote 5mm because it was from one side of the disk.

Both extracts were effective against *B. megaterium* bacteria, the acetone extract had a 9mm zone inhibition diameter and the ethanolic extract had a 10 mm diameter. The same case happened with *S. aureus* bacteria; the acetone extract zone inhibition diameter was 9 mm, while the ethanolic zone inhibition diameter was 10 mm. And the last recording was against *C. albicans* fungus, both extracts had a 10 mm zone inhibition diameter.

Some published leaves extract antimicrobial activity articles, agree with our results:

(Dar et al., 2018) recorded that ethanolic extract had no effect on *S.aureus* bacteria [169]. While (Fakoya et al., 2019) agree with our results at similar concentrations of the ethanolic extract [12], but they also recorded increasing the activity when increasing the ethanolic extract concentration, and this quite makes sense because *Cucurbita pepo* leaves extracts are effective against bacteria growth or replication but are dependent on the concentration of the plant extract [154].

And some partially agree with our results: (Dissanayake et al., 2018) recorded that the acetone extract had no effect on *E. coli* bacteria, the same as our results. Still, the extract also had no effect on *S.aureus* bacteria, while our results showed an effect on *S.aureus* bacteria [152].

As well (Oboh et al., 2010) reported inhibition against *E.coli* bacteria with a 4 mm zone diameter, while the other results agree with our result [154].

The difference in the obtained results may be attributable to differences in the microbial strains, the imbuement methods, the bacterial imbuement size, the medium of growing, incubation circumstances, as well as the methods of preparing the extracts. [170].

The antimicrobial activity of the samples can be calculated according to the equation

$$\text{Antifungal (bacterial) activity (\%)} = \frac{Dc - Ds}{Dc} \times 100$$

Where Dc = inhibition diameter of the control, Ds= inhibition diameter of the sample for the acetone extract:

- *E. coli*: Antifungal activity= 47.36%
- *K. pneumonia*: Antifungal activity= 75%
- *B.megaterium*: Antifungal activity= 64%
- *S. aureus*: Antifungal activity= 57.8%
- *C. Albicans*: Antifungal activity= 58.3%

For the ethanolic extract:

- *E. coli: Antifungal activity= zero (NO inhibition)*
- *K. pneumonia*: Antifungal activity= zero (NO inhibition)
- *B.megaterium*: Antifungal activity= 60%
- *S. aureus*: Antifungal activity= 47.36%
- *C. albicans*: Antifungal activity= 58.3% [171].

4. CONCLUSIONS

Through our experiments, we could prove that *Cucurbita pepo* leaves indeed contain significant phytochemicals, and we look forward to valuating these leaves and not to discard them. As they can be used as herbal medicines or folklore medicine, as orderly intakes of this vegetables have the ability to reduce or inhibit several diseases, as mentioned in the introduction part.

Our experimental results showed that the *Cucurbita pepo* leaves acetone and ethanolic extracts had radical scavenging activities against ABTS+•, OH•, and DPPH• radicals, and our experimental results also showed that the leaves extracts had a high total flavonoid, total phenolic content.

Our experimental results for the leaves extracts content of vitamins had only trace amounts of vitamin A and E, but had no vitamin C content. And the vitamins showed no antioxidant effect from vitamins (C, A, and E) of the extracts samples treated with *saccharomyces cerevisiae* yeast and against MDA.

Our experimental results revealed that the acetone and ethanolic leaves extracts samples had no significant effect on hepatocellular carcinoma, while the acetone leaves extracts decreasd breast cancer effects, and the ethanolic extract showed a higher effect decreasing human breast cancer than Doxorubicin which is a breast cancer therapy. And the extracts samples activity increased with increasing the concentration of the extracts samples.

Our experimental results also showed that *Cucurbita pepo* leaves extracts have antibacterial and fungicidal properties, these properties are due to the saponins, tannins, flavonoids, and alkaloids contents of *Cucurbita pepo* leaves that are mentioned in the introduction part.

But for best results, our thesis recommends using high concentration plant extracts because the properties mentioned above are dose-dependent. As we have earlier mentioned in the discussion part.

Furthermore, our experiments showed that the same extracts differently affect different types of bacteria: the ethanolic extract was effective against the gram-positive bacteria but showed no effect gram-negative bacteria due to the dissimilarity in their cell wall structure of the bacteria as we have mentioned in the discussion part.

Aceton is a better solvent than Ethanol for determining the antibacterial activity of the two gram-negative *E.coli* and *K. pneumonia*. And both extracts showed similar effects against the gram-positive bacteria.

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