FIRAT UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
TURKEY

EVALUATION BIOLOGICAL ACTIVITY OF 1,2,4-TRIAZOLE MANNICH BASE

Mahmood Sherzad RAFAAT

Master's Thesis

DEPARTMENT OF CHEMISTRY

Division of Biochemistry

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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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Signature

Prof. Dr. Kürşat Esat ALYAMAÇ
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DECLARATION

I hereby declare that I wrote this Master's Thesis titled “Evaluation Biological Activity of 1,2,4-Triazole Mannich Base” 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.

24 January 2022

Mahmood Sherzad RAFAAT

PREFACE

Every day or season new pathogens and diseases create conflict for human health, so it's important by synthesis and other ways to find novel drugs that have abilities to combat or hinder those defects in the living system. After the first stage, the second thing is the evaluation of their biological activities to clarify capacities, skills, and activities before, during, and after usage of the drug. In this study, we did some biological tests for evaluation beginning activities against bacteria, fungi, and other tests including antioxidants and antitumor assay. For every goal that wants to do, we should predict any impracticable because it is vital to have a previous plan, also in our study, we have some difficulties. To use our compound in the medical field is the most motivated me to work extremely accurately during laboratory thesis work.

"Allah increase me in knowledge". All my attainment of attempted and desired return for Allah, then for Prof. Dr. Mustafa KARATEPE that supervisor my master degree and express my gratitude to his efforts for me, in the depth of my heart I want to thank my parents and our family to continue and help me in every step of my study, I am grateful for Prof. Dr. Metin KOPARIR who gave as that compound that we used for our study and Assoc. Prof. Dr. Serhat KESER who helped us a brilliant advisor in antioxidant evaluation assays and Miss. Shula, also thankful for my university to permitted and qualifier me to study at Firat University, and also for Fen Bilimleri Enstitüsü (Institute).

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Mahmood Sherzad RAFAAT

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ABSTRACT

Evaluation Biological Activity of 1,2,4-Triazole Mannich Base

Mahmood Sherzad RAFAAT

Master's Thesis

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Biological evaluation of a 1,2,4-triazole mannich base that has been synthesized for interesting behavior in the pharmacological area. This study investigated and evaluated multiple biological characters of a derivative triazole compound synthesized by other researchers. Multiple assays were performed like antimicrobial (for bacterial and a fungal), antioxidant, and anticancer to know its ability as an agent versus of those health damage factors. Antimicrobial test managed based on agar disc diffusion, in the antioxidant assays used $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $OH^{\bullet}$ radical scavenging activity methods, used S. cerevisiae yeast cells and treated with the compound to measure vitamins (A, C, E) and MDA inside it and role of the compound as an antioxidant, and measured cytotoxic activity for two different cell lines which are MCF-7 and Hep-G2. The results of this study show that a positive role of a derivative 1,2,4-triazole mannich base as antimicrobial and antiradical assays, like fungal growth inhibition, is more than bacterial, $OH^{\bullet}$ radical scavenging activity is higher than the standard BHT and other radical forms, but didn't exhibit feasibility activity to reduce both cancer cell lines.

Keywords: Antimicrobial, Antioxidant, Antioxidant activity, Biological activity, Triazole

ÖZET

1,2,4-Triazol Mannich Bazının Biyolojik Aktivitesinin Değerlendirilmesi

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1,2,4-triazol manich bazı, farmakolojik alanda sergilediği ilginç davranışlar için sentezlenmiş ve biyolojik olarak değerlendirilmiştir. Bu çalışmada, diğer araştırmacılar tarafından sentezlenen yeni bir triazol bileşiği türevinin biyolojik karakterlerini araştırıldı ve değerlendirildi. Antimikrobiyal (bakteri ve mantar için), antioksidan ve antikanser gibi, sağlığa zarar veren faktörlere karşı bir ajan olarak yeteneğini araştırmak için çoklu testler yapıldı. Agar disk difüzyonuna dayalı olarak antimikrobiyal test yapılmış, antioksidan tahlillerinde $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $OH^{\bullet}$ radikal süpürücü aktiviteleri incelenmiş, *S. cerevisiae* maya hücreleri kullanılmış ve vitaminler (A, C, E) ve MDA düzeylerini ölçmek için bileşik ile muamele edilmiştir ve sitotoksik aktivite için MCF-7 ve Hep-G2 gibi iki farklı hücre dizisi kullanıldı. Bu çalışmanın sonucunda, kullanılan 1,2,4-triazol mannich bazı türevinin antimikrobiyal ve antiradikal testlerinde, mantar büyümesinin inhibisyonun, bakteriyel inhibisyonundan daha fazla olduğu, $OH^{\bullet}$ radikal süpürme aktivitesinin standart BHT'den ve diğer radikallerden daha yüksek olduğu, ancak antitumör aktivitede her iki kanser hücre hattı üzerinde etkili bir aktiviteye sahip olmadığı gözlendi.

Anahtar Kelimeler: Antimikrobiyal, Antioksidan, Antioksidan aktivite, Biyolojik Aktivitesi, Triazol

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SYMBOLS AND ABBREVIATIONS

Symbols

A : Absorbance

wt : Weight

Abbreviations

ABTS : 2,2'-azino-bis(3- ethylbenzthiazoline-6-sulphonic acid)

$ABTS^{\bullet+}$: 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) radical cation

AOA : Antioxidant Activity

CAT : Catalase

DNA : Deoxyribonucleic Acid

DMSO : Dimethyl Sulfoxide

DPPH : 2,2′-diphenylpicrylhydrazyl

$DPPH^{\bullet}$: 2,2-diphenyl-1-picrylhydrazyl radical

$^{1}HNMR$: Proton nuclear magnetic resonance

H_2O_2 : Hydrogen Peroxide

HAT : Hydrogen Atom Transfer

HO : Hydroxyl

$HO^{\bullet}$: Hydroxyl Radical

HPLC : High-Performance Liquid Chromatography

$K_2S_2O_8$: Potassium Persulphate

MDA : Malondialdehyde

MTT : 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

$O_2^{-\bullet}$: Superoxide Anion

RA : Retinoic Acid

RNS : Reactive Nitrogen Species

ROS : Reactive Oxygen Species

SET : Single Electron Transfer

SDH : Succinate Dehydrogenase

WHO : The World Health Organization

^{13}C NMR : Carbon-13 (C13) Nuclear Magnetic Resonance

1. INTRODUCTION

Compounds manner change from one compound to another, the studies have been exhibited importance of an organic compound, that have a lot of significance as a biological activity called heterocyclic compounds. Due to more uses especially in medicine so couldn't be as a normal compound. The structure of vitamins, deoxyribonucleic acid (DNA), coenzymes, ribonucleic acid (RNA), and others contain heterocyclic rings. Classification of aromatic heterocycle compounds into two rings are called five-membered and six-membered rings [1, 2].

Heterocyclic compounds have got in the composition wide range structure with several names. The pharmacological and biological activity of a compound is confirmed by the ability against various pathogenies and their properties like antibacterial, antioxidants, anti-inflammatory, anticancer, and so on. Those activities are displayed in the compound which is called triazole. Triazoles have been grouped into 1,2,3-triazole and 1,2,4-triazole with their derivatives having several significant as well as biological activities such as antimicrobial properties. Medicinal chemistry has been researched on the therapeutic potential of compounds, various pharmacological activities such as antimicrobial, antitumor, and other capabilities have been found in heterocyclic 1,2,4-triazole Mannich bases and their derivatives [3-8].

Free radicals cause damage to the human cells that are the source of some dangerous diseases on the health, so measure the ability to reduce their activities by a method called antioxidant activity. Various assays are done to evaluate antioxidant activities *in vivo* and *in vitro,* the most common *in vitro* methods are 2,2′-diphenylpicrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), due to the experiment requires short time and simplicity. Solid and liquid samples can be evaluated overall antioxidant ability by DPPH assay which is based on hydrogen donors reaction with the stability of DPPH free radical [9, 10].

There are two major groups of bacteria cell envelopes, they are gram-positive cell wall which is surrounded by peptidoglycan layers that contain proteins and teichoic acids, and also they have vital roles in the protection, growth, and interaction of the bacteria and gram-negative bacteria have only one peptidoglycan layer that enclosed via the outer membrane, also this type of cell envelope is more complicated that gram-positive. Also fungal is growth in two different forms which is known as dimorphic, they are developed as yeasts or molds or both forms. The reproduction via mitosis or fusion of two haploid nuclei [11].

Drug discovery has a lot of significance in life to decrease diseases and various health problems that is could be done by testing a new drug or synthesis compounds using the antimicrobial method. Antimicrobial assay including antibacterial and antifungal activities which have a role in the evaluation of novel synthesis compounds by following strains: *Escherichia coli*

(ATTC 25922), *Staphylococcus aureus* (ATCC 25923), *Candida albicans* (ATTC 10231), and so on [12, 13].

Cancer is a major disease and development of cells randomly if compare with normal cells in the body. Side effects of anticancer chemotherapeutic on the patient life one of the reasons that scientists nonstop from synthesizing novel chemotherapeutic as anticancer agents with fewer side effects after treatment [14].

This study aims to evaluate a synthesized triazole Mannich base biological activity to know its ability against microorganisms, radical species, and tumors by several assays according to both procedure fundamentals and investigation by instrumental analysis to achieve primary information about its application in the medicine area as an agent.

2. LITERATURE REVIEW

H.A.M. El-Sherief et al. predict derivatives of the synthesis of acylated 1,2,4-triazole ability against cancer which is caused the death of millions of people each year. According to the results got it was from the compound that synthesized of 1,2,4- triazole scaffold for evaluation against cancer. New compounds synthesized in the study of H.A.M. El-Sherief et al. highest anticancer activity are displayed by a compound compared to other novel compounds against cancer cell growth [15].

Novel 1,2,3-triazole incorporated 1,4-benzothiazinone derivatives are synthesized in that study by M. H. Shaikh et al. the basic factor is to evaluate biological activity. DPPH radical scavenging activity is displayed promising of the phenyl ring having chloro-group, fluoro-and bromo-group at ortho and para position. 1,2,3-triazole synthesized compounds containing benzothiazinone derivatives tested for their cytotoxic activity against the three different human cancer cell lines. Three compounds displayed potential antioxidant activity, so they can be developed as an oral drug due to the new compound's properties like a good drug. The results showed that those compounds have antitubercular, antioxidant, and cytotoxic activity [16].

W. Yan, et al. designed and synthesized more than forty derivatives containing 1,2,3-triazole ring, study efforts to find new succinate dehydrogenase (SDH). SDH has extremely significant in the development of novel fungicides as molecular targets. Antifungal activities *in vitro* were done for the compounds that separated to find the ability against phytopathogenic fungi by mycelia growth inhibition assay caused by display antifungal activities. Biological activities of the 1,2,3-triazole ring compounds have a broad spectrum, including anti-microbial (anti-bacterial, antifungal), and anticancer activities. The derivative compounds evaluated antifungal activities the result showed capacity against significant phytopathogens, including S. sclerotiorum, Gaeumannomyces graminsis, and so forth. The compounds had a different level in antifungal inhibitory activity, so some synthetic compounds exhibited the role of 1,2,3-triazole in the fungicide and permit researchers to develop novel triazoles in the recent and future time [17].

In the study of H. Sadeghpour et al., several methods have been done by spectral (CHN, NMR, and Mass) analyses to determine the structure of synthesized triazole compounds. Broth dilution assay used for evaluation of activity against various fungi of the compound that synthesized for antifungal activities. The assay displayed the brilliant antifungal activity of nitrotriazole derivative compounds against a lot of the fungi, and some derivative compounds have had the highest activity as antifungal activities. The activity one of the synthesized against all fungal but not with Aspergillus species. In general, the antifungal activities of the compounds in this study didn’t exhibit any activity against one of the species fungal called Aspergillus species [18].

Q. Li et al. worked to synthesize new 1,2,3-triazole in the structure of chitosan as derivatives and evaluation antifungal activity *in vitro*. Improve the activity against fungal of the chitosan derivatives, which the most significant thing in that study, they were done by several changes in the structure like triazole with halogens could show antifungal and electron-withdrawing capacity. The FT-IR and 13C NMR spectroscopy measurements were done by every step in the synthesis of derivative compounds. The triazole functional groups in the structure of compounds have a high ability as biological activity against different fungal [19].

Y. Ünver et al. were synthesized novel triazole derivative compounds and evaluated biological activities by various antioxidant methods like DPPH radical scavenging and ferric reducing methods, due to more common for determining antioxidant activity, so those two methods are used and antimicrobial activities. The Mannich bases and the Schiff bases major of the new compounds and nitro substituent carried out on the thiophene ring, which is displayed the ability against the antimicrobial activity. The structure of novel triazole compounds was confirmed in this study by a member of methods that required to define structure new synthesized compounds like H NMR, C NMR, elemental analyses, IR, and mass spectral data.

Antioxidant activity of triazole-thiol among all of the synthesized, exhibit the best activity to decreasing and removing free radicals. According to this study are shown the effect of the reason on the ability or capability compound activities by transforming from the Schiff bases to the Mannich bases observed antioxidant activities decrees against free radical scavengers and converting of substituent type on the thiophene ring structure. The results that were achieved from the study of Y. Ünver et al. all of the new compounds had good antioxidant and antimicrobial activities, based on the composition biological activity increase or decrease and capability of compounds against gram-negative bacteria was in a higher level if compare with gram-positive ones [20].

P.S. Patil, et al. designed and synthesized different triazole derivative compounds and evaluated biological activities especially antibacterial and antifungal activities. Based on this study five compounds have observed and displayed high affinity against both antibacterial and antifungal pathogens after evaluation for their *in vitro* activities. Agar diffusion assay used in the detection of antimicrobial activities. Cytotoxicity of chemical and biological compounds indicates their significance by the known effect on the cells, so one of the most important things of triazole compounds have low cytotoxicity according to the synthesized derivatives of P.S. Patil, et al. [21].

Synthesis and biological ability of several 1,2,4-triazole derivatives have been described in this study as a review. Some researchers more focused on antimicrobial activity of the 1,2,4-triazole compounds, like Ulusoy et al., Ulusoy et al., Kaplancikli et al., and Kidwai et al., and about activities that predicted brilliant activity against pathogens. Another recent synthesis of 1,2,4-triazoles treated to determine antifungal and antibacterial activity could be seen in some studies, as

Liu et al., He et al., and Holla et al., and the results were hopeful in the study. Anticancer activity for new 1,2,4-triazole derivatives withdraw researchers from the synthesis of various triazole derivatives due to some activity of triazoles against particular cancer cells, based on the study of El-Sherief et al. and Ye et al. was displayed [22].

2.1. Heterocyclic compounds

Heterocyclic compounds are significant compounds in medical chemistry, their structures are known as cyclic compounds and are composed of at least one carbon atom and one or more than one different element. Moreover, the atom of elements except for carbon in the heterocyclic compounds are called heteroatom [23]. Heteroatoms are non-carbon atoms are refer to these atoms like nitrogen, sulfur, or oxygen. Generally, two basic classes of heterocycles are non-aromatic and aromatic [24]. Nitrogen has been found in most structures of the heterocyclic compounds as the heteroatom, after that respective oxygen and sulfur [1]. Heterocyclic compounds which contain nitrogen have various significant roles in the low toxicity and biological properties that can be basic in pharmaceutical sciences [25]. Generally, in the name, aromatic heterocycles use '*H*' like italic form to detect the position of an N-hydrogen in the design as clarified in Figure 2.1. They are classified into two special groups known as five- and six-membered rings. Five- and six-membered rings that contain nitrogen are named respectively, by the end with 'ole' and 'ine'. The structure of triazoles always contains three nitrogen and has two tautomers.

Figure 2.1. Structural isomers of triazole compound [26]

Generally, heteroatoms the basic structure of the heterocycles at least have one heteroatom, the most common in their structure that found are N, O, S, B, Se, P, and Si. According to classification, they are grouped into non-aromatic and aromatic heterocycles. Aromatic heterocycles are more useful in the medicinal field if compare with non-aromatic heterocycles. The naming of them could depend on the size of the heterocyclic rings. Heterocycles are grouped as seven-, six-, five-, four-, and three-membered heterocycles and another. Biochemical and heterocycles have an interesting relationship because heterocycles are found in vitamins, DNA, amino acids, and other structures. Heterocycles play a significant role in medicinal chemistry which is the investigation and development of new drugs as therapeutic agents like anti-inflammatory,

anti-tumor, cardiovascular drugs, and other important drugs. Those compounds are known as heterocycle-containing drugs which can find important biological activities [27].

Heterocycles have numerous significant biological functions on human health due to most of the novel heterocycle compounds that are tested which exhibit extremely brilliant roles against a lot of diseases. The activity of their compounds opens a way for new researchers and scientists to follow heterocycles synthesis and use in the pharmaceutical processes [28]. The heterocyclic compounds behave like pharmaceuticals, can be used in numerous fields of medicine, and act as a biological activity like antimicrobial, anticancer, and anti-inflammatory. Five- and six-membered heterocyclic compounds are particularly more used and able in medical processes such as pyrazole [29].

One of the most common and significant compounds that have a lot of medical properties and could be a nascent stage in the synthesis of various drugs that have got the benefit to health in organic chemistry is heterocycles. According to those researchers and synthesized compounds are predicted heterocyclic compounds which contain nitrogen in the structure display more biological activities [30]. Novel heterocycle compounds that have been used in many filed particularly chemotherapy applications, also a synthesis of heterocyclic compounds that have high nitrogen in the structure displayed higher interest in the biological area [31].

2.1.1. Triazole

Triazole was named in 1885 by a scientist which is called Bladin and several triazole derivatives were defined by him. The molecular formula of triazole is $C_2H_3N_3$ and the chemical compound of triazole has got a pair of isomers [23]. Generally, triazoles have two types are 1,2,3-triazole and 1,2,4-triazole, they may have tautomerism [32]. Five-member ring triazole heterocyclic compounds have irritation to skin and eyes and are grouped into 1,2,3-triazole which is solid with melting point equal to 23-25°C and colorless, while the melting point of 1,2,4-triazole equal to 119-121°C that is colorless crystalline solid [27].

Triazole ring has aromatic character, rich with the electron, generally, triazole and its derivatives have a unique structure that is lead them to interact with several of receptors and enzymes, for this reason, have high range activities in the living system, there are many different weak interactions between them like hydrogen bonds, ion-dipole, van der Waals force, and others. Today, triazole compounds use a broad spectrum for the treatment of those patients who are infectious with fungal. Due to low toxicity, harmless, brilliant bioactivity, and other properties that are essential to have in any pharmacological compounds can be also found in triazole. In the beginning, a triazole-antifungal agent like fluconazole has been used as a drug for the treatment of those humans that have infectious diseases via fungal, particularly Candida albicans [33]. Azole groups are significant heterocyclic compounds, they play roles in medical fields and part of the

group which has important biological activities like antifungal and others is triazole. Fluconazole is played antifungal activity roles against candida and other fungal infections and itraconazole the same as fluconazole has a lot of significant characters as an agent against several fungal infections with some side effects both of them are the first generation of five-membered heterocyclic compound which is called triazole [34].

Triazole derivatives have a wide range of applications in the medical area so more important to care synthesis new derivatives and evaluate their capacity to use in pharmacology. One of the types of triazole is 1,2,4-triazoles which can be used in several biological activities like anti-cancer, total antioxidant, antimicrobial, and so on. 1,2,4-triazole derivatives are increased activities in the biological system by linked with mannich base. However, the synthesis of new compounds like sulfur-linked 1,2,4-triazoles has been evaluated and displayed various pharmacological activities [35, 36]. Low toxicity and safe drugs are more interested in researchers to synthesis new therapeutic agents, those properties can be found in the heterocyclic compounds that contain nitrogen have high range applications as medical agents specially triazoles [5].

2.1.2. 1,2,4-triazole

Medicinal chemistry has a significant role in the synthesis of new drugs to use in a wide variety in the biological system as antimicrobial and other activities could be found in those manners in heterocyclic compounds that are used as therapeutic agents called 1,2,4-triazole [37]. The ability of 1,2,4- triazoles in the binding with various biological targets is one of the most significant points to use 1,2,4-triazole compounds in the synthesized novel drugs due to their activity to form a bond like a hydrogen bond and others with biological system [35]. Various drugs are synthesized in the treatment of many pathogens which have a 1,2,4-triazole compound in the structure of those drugs like for cancer diseases used vorozole, while fluconazole is used in the microbial as an antifungal drug, the structure of several triazole-drugs are provided in Figure 2.2.

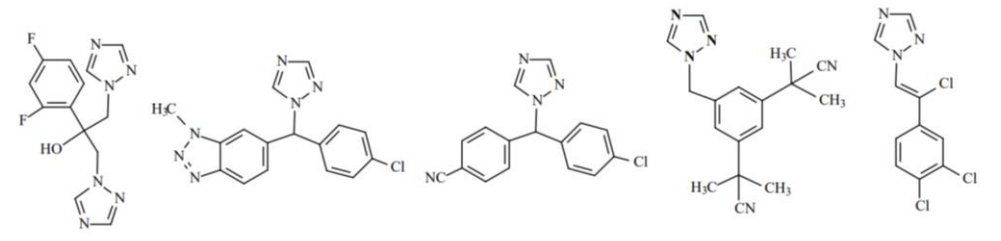


Figure 2.2. Different compound drugs which contain a 1,2,4-triazole ring [38]

1,2,4-triazoles heterocycle compound that is nitrogen-containing biological compound and their application in several biological activities and pharmacological [39, 40]. According to those researches about 1,2,4-triazoles and their derivatives have displayed the importance of 1,2,4-triazoles in the different fields of medicine like biological activities and composition structure of drugs. Antifungal activity of 1,2,4-triazoles and their heterocyclic derivatives have a role in the synthesized several drugs that are used against fungal diseases such as itraconazole, fluconazole, and so on [41, 42]. The 1,2,4-triazole have been utilized in various biological activities as a drug like Fluconazole and Ribavirin used against fungal and viral respectively [20, 43].

2.1.3. Mannich base

Another significant compound that has been used in several pharmacological and biological is mannich bases, however, carbon-carbon bonds are the basis of the formation mannich bases that are known as mannich reactions also called aminomethylation reactions. Generally, mannich reaction is formed by reaction between some compounds which is displayed structure of mannich bases are aldehyde, ketone having α-CH acidic proton/s and primary or secondary amines. Mannich bases in the chemical structure and composition of drugs are affected on the polar function by the rising hydrophilic properties of drugs. Biological activity is the evaluation role of compounds in the biological process especially antimicrobial and other beneficial activities that will be found in the Mannich bases [37, 44]. Mannich bases are composed of the result of three-component by condensation among a hydrogen atom, an aldehyde, and an amine with a substrate. Mannich bases in medicinal chemistry have got multiple advantages with heterocycles like anti-HIV, anticancer, antiviral, anti-inflammatory, antioxidant, and so on [45, 46].

The structure of Mannich bases as three-component from the condensation reaction of more than one acidic hydrogen, an aldehyde component (the most of the time is formaldehyde), and an amine reagent which together forms Mannich bases. Antibacterial activity and some drugs are exhibited in a heterocycle compound and nitrogen-containing which have important biological properties known as 1,2,4-triazole Mannich bases. They have been attention due to before and recent research displayed high ability to use in a wide variety of medical and other fields like antitumor, antimicrobial activities and others will be found in the future studies [41, 47-49]. Between heterocyclic compounds which have nitrogen in the structure, triazole has a brilliant biological activity among them. Furthermore, triazole reaction with mannich bases increases potential activities so is called triazole mannich bases.

In recent studies, a common compound which is carried out in the numerous field of biology and medicine is a five-membered heterocyclic compound, and its nitrogen-containing structure with high activities against several pathogens like antifungal, antibacterial, antiviral, and others could be found in 1,2,4-triazoles Mannich bases [50]. Synthesis of new components which have biological

and pharmacological properties is one of the extremely brilliant works in medicine so, mannich bases have a role in the synthesis of novel biological agent compounds like triazole mannich bases [51]. The Mannich reaction has important properties in the synthesis of novel compounds which is based on used in the drug design [52]. Generally, mannich bases role has been found in the two fields that related to biological activities. According to various studies, mannich bases displayed several interests in medicinal chemistry. Second, increasing of hydrophilic of drugs which is one of the properties of aminomethylation [48].

2.2. Free radicals

Oxygen the basic in the structure of free radicals, while occasionally found nitrogen as free radical both of them reactive oxygen species (ROS) and reactive nitrogen species (RNS) have a powerful ability and rapidly react with the living system by donating their outer electron(s) or sharing their ones with biomolecules. Sometimes, after reacting high efficacy radical with the targets, will form new radicals which are again able to react and damage other targets in the human body [53]. High reactive species are those molecules which contain at least one unpaired electron in the outer orbital are called Free radicals. The most common type of atom radical in the structure of molecular is oxygen and have a brilliant activity against cells and caused cell death, while they are created in the human body [54].

As referred, the number of free radicals in the living organisms determines their effect on the health, so if ROS have in the organisms with high concentrations, caused of cell damage and other diseases. But ROS with low and enough quantities might have some health benefits. A weak or disabled radical that can pass the lipid membrane of the cells is superoxide radical while it could convert to hydrogen peroxide and then to the most reactive radical is hydroxyl radical ($HO^{\bullet}$). The hydroxyl radical can pass through the cell membrane easily and that is generated by the Haber-Weiss reaction and Fenton reaction [55]. Free radicals are unstable chemical species that destroy the balance between oxidative damage and antioxidants. As described HO radicals have extreme activity with biomolecules while generation of $HO^{\bullet}$ is slow. The most important common cations that can be a role in the production of more amount of $HO^{\bullet}$ are Fe^{2+} or Cu^{+}. Figure 2.3 and Figure 2.4 illustrate both Haber-Weiss and Fenton's reactions.

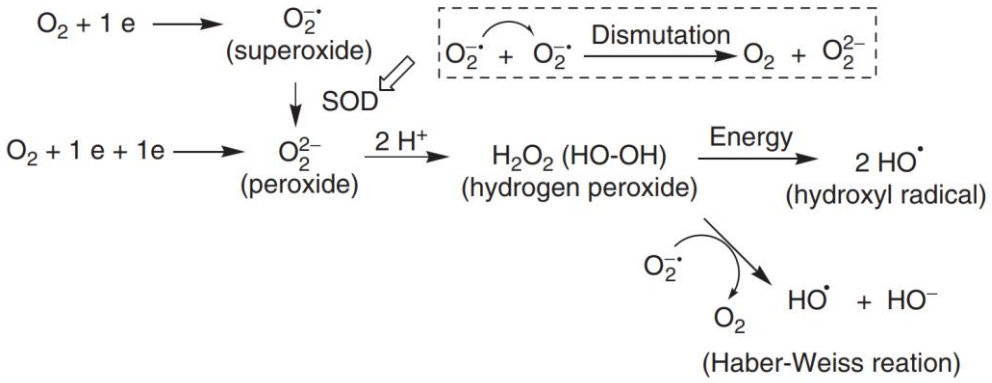


Figure 2.3. Sequence mechanism of oxygen in the formation of radical forms [56]

Generally, there are two sources for the production of free radicals; the first group happens inside our body in various ways as a source like mitochondria and others organisms known as aerobic organisms in the cells. The second way is exogenous that can form radicals in deferent ways such as environmental pollutant agents, radiation, X-rays, and so forth. Both ways affect have high efficiency against cells. HO•, hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{-\bullet}$), and others are examples of reactive oxygen species [57].

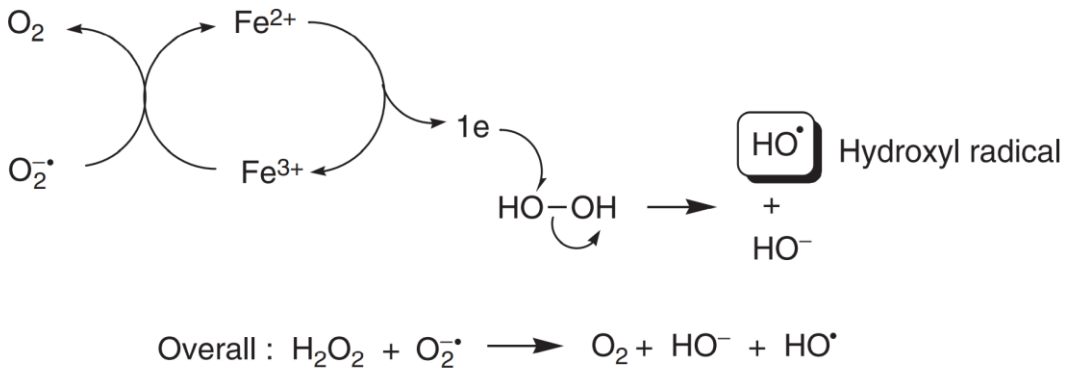


Figure 2.4. The chemical reaction of Fenton for generation HO• [56]

In the living system, cells are synthesized free radicals like ROS with a major level by mitochondria and also may form free radicals due to several enzymes. Oxygen in the body cells participates in numerous reactions to produce ROS, which caused several problems to human health if prepared in high quantity. DNA damage is the most common disease that happened because of the effect of ROS capacity and the development of cancer cells [58]. The human body usually produces ROS in a regular range from particular metabolism because ROS have some essential role to stay body healthy, while ROS causes damage to the biological system by increasing the rate of

ROS formation. The common cell part that has a fundamental role to generate ROS is mitochondria which via the electron transfer system reduce oxygen molecular to $O_2^{-\bullet}$ and then form H_2O_2 [59].

Free radicals have a great ability to react with various biomolecules in the body due to there being a single electron in the valance shell of the molecule, also they attack closer biomolecules to be more stable by removing an electron from stable molecule to their unpaired electron in the valence shell. Generally, there are two common free radicals the most one is oxygen that called ROS, and nitrogen is also known as RNS. Oxidative stress is defined as destroying the balance between antioxidant and oxidant in the cell via several things that are caused by increase oxidants or lack of antioxidants [60]. The capacity of radicals to react with organisms depends on the concentration and which radical is involved in the reaction. Generally, ROS and RNS have been attacked by several biomolecules such as DNA, lipids, proteins, and might others as known aerobic organisms in the living system [61]. Clear, cancer is a dangerous disease that divides in a wide variety in the body and causes of death in many people who have cancer. So, according to more studies evidence that radicals are had a role in DNA damage by generating the initiation stage of carcinogenesis [62].

Free radicals are more reactive molecules and also atoms usually have ROS and RNS and occasionally there are many different types of radicals like reactive sulfur species and other radical species which are effective on the cells and other organisms [63]. However, there are two the most common reactive oxygen species that affect health, like free radicals and molecular species such as $HO^{\bullet}$ and H_2O_2 with different manners in the combat with biomolecules, cells, and so on. Free radicals can be converted from one species to another by accepting or donating an electron [64]. Autoxidation is defined as oxidation of a lot of molecules due to oxygen, so stay this process under the control has been showing extremely significance because can be protected the living system from the several diseases that are come due to free radicals [65].

2.2.1. Oxidative stress

However, increasing the concentration of ROS in the body which causes oxidative stress has a wide variety of side effects to many biomolecules, various cells, and some chronic diseases like proteins, DNA, cancer, cardiovascular and other diseases as shown in Figure 2.5 [57, 66].

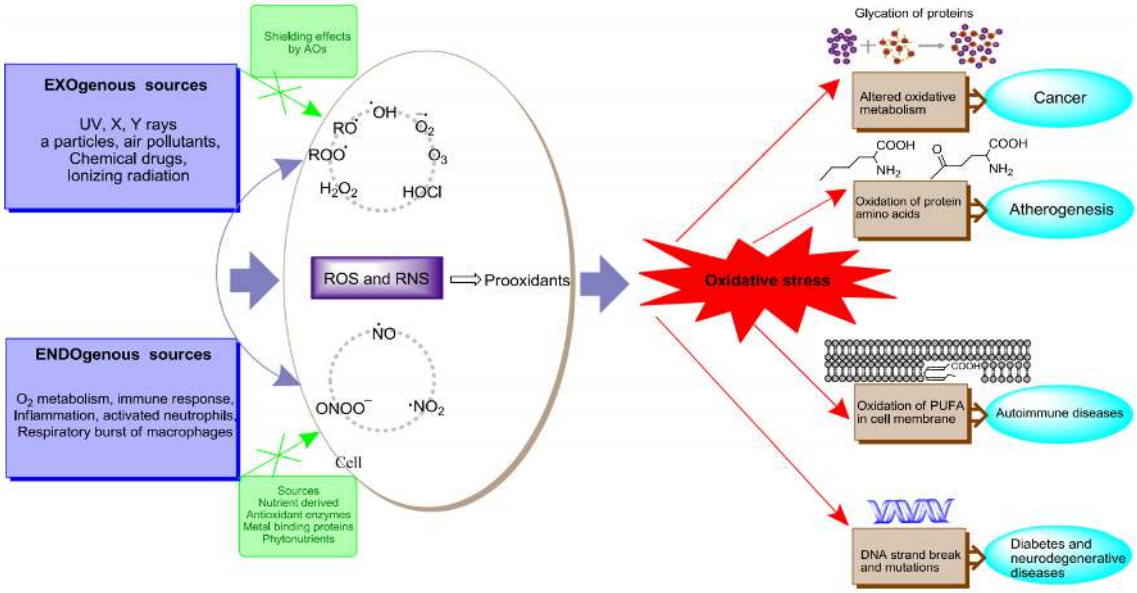


Figure 2.5. The major sources of radical species and their oxidative damages on the biological system [67]

2.3. Antioxidants

Antioxidants are indispensable compounds that require for decreasing radical activity and the initial process in the generation of cancer cells [68]. Antioxidants can be converted dangerous molecules to those molecules that have a low ability to oxidize biomolecules and damage cells by donating an electron to ROS and other radicals that are caused of maintain the body healthy from several disorders and radicals. Also, the antioxidants as other drugs if have been taken in a high amount, can be harmful to the health, like vitamin C have many benefits to our health but if excess in the body has side effects like erosion of dental [69]. Oxidative damage of biomolecules has several side effects on health and it can be removed or made slow by the action of antioxidants which can scavenge radicals. The common antioxidants that require and have many benefits to our health are catalase (CAT), minerals like Se, DNA repair enzymes, vitamin E, carotenoids, and others [70].

Antioxidants combat with free radicals in several ways, one of them is enzymatic antioxidants which are scavenging radicals like reduction of $O_2^{-\bullet}$ into H_2O_2 then the H_2O_2 formed convert to water that is harmless compared to the first composition of radicals by superoxide dismutase (SOD) and catalase or glutathione peroxidase (GPx). Another type of antioxidant that has brilliant roles against numerous health problems, the problems or disorders in the result of increasing ROS and oxidizing biomolecules. The antioxidants such as vitamins C, E, β-carotene, and polyphenols can reduce and delay those agents which can be formed defects in the health [58]. The capacity of antioxidants is high due to their presence at a low level can have roles in the prevention of many diseases like cancer and others by removing ROS, other radical species, and

delaying the oxidation process of the biomolecules. In addition, synthetic compounds that have been used for inhibition or make slow development of cancer cells, are known as antioxidants. About enzymatic antioxidants have significant roles in fighting against ROS like catalase [71]. Many studies clarify the importance of enzymatic, natural, and synthetic antioxidants (as displayed in Figure 2.6) to remain bodily healthy by combatting with free radicals, which is done by several ways like neutralization or scavenging radicals and delay oxidative damage [72].

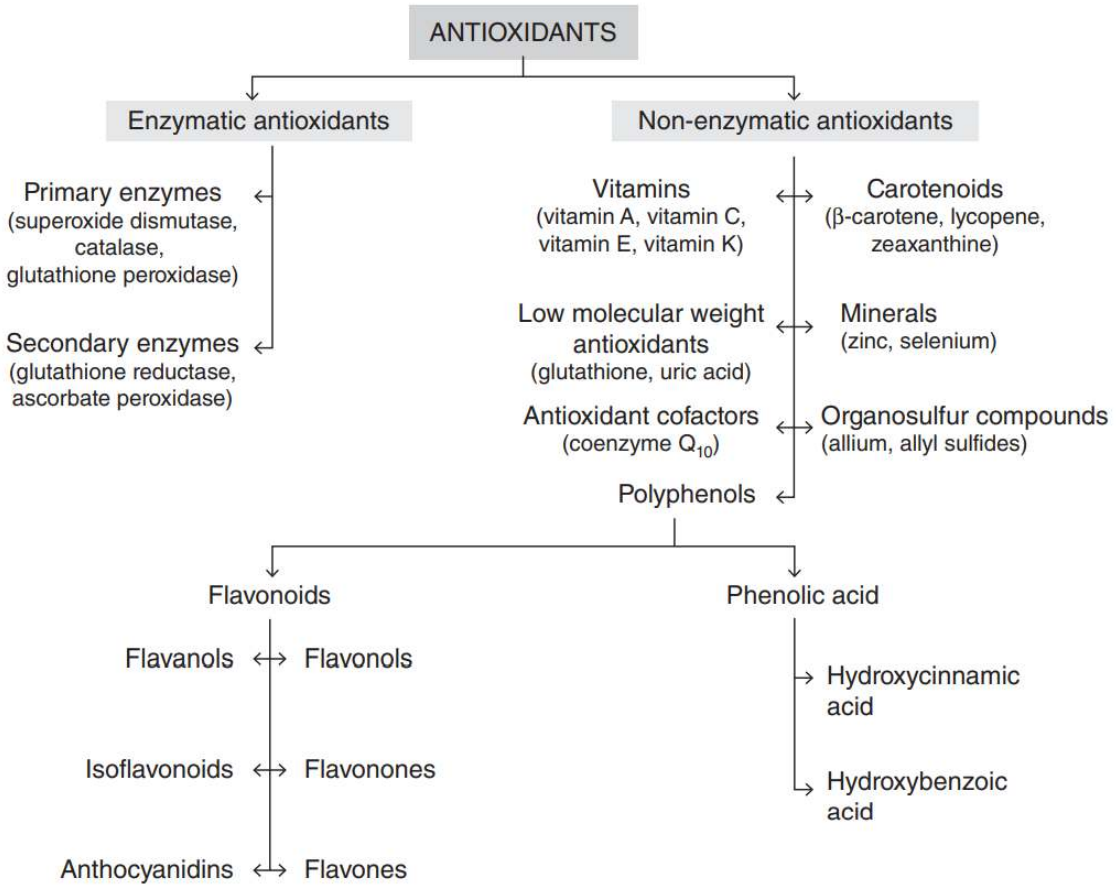


Figure 2.6. The most common antioxidants that can be discovered in plants [73]

Generally, a lot of studies displayed antioxidants can be found in a high range inside the diet that has brilliant ability against oxidative stress like β-carotene, phenolic compounds, and others are vital for remain the body's healthy by inhibiting or decreasing free radical capacity (Figure 2.7 shows the role of antioxidants against oxidative diseases) [74]. Many factors have essential roles in the detection of antioxidants activities like chemical structure, reactive groups, antioxidant ratio, and other reasons that have been linked with antioxidants [75].

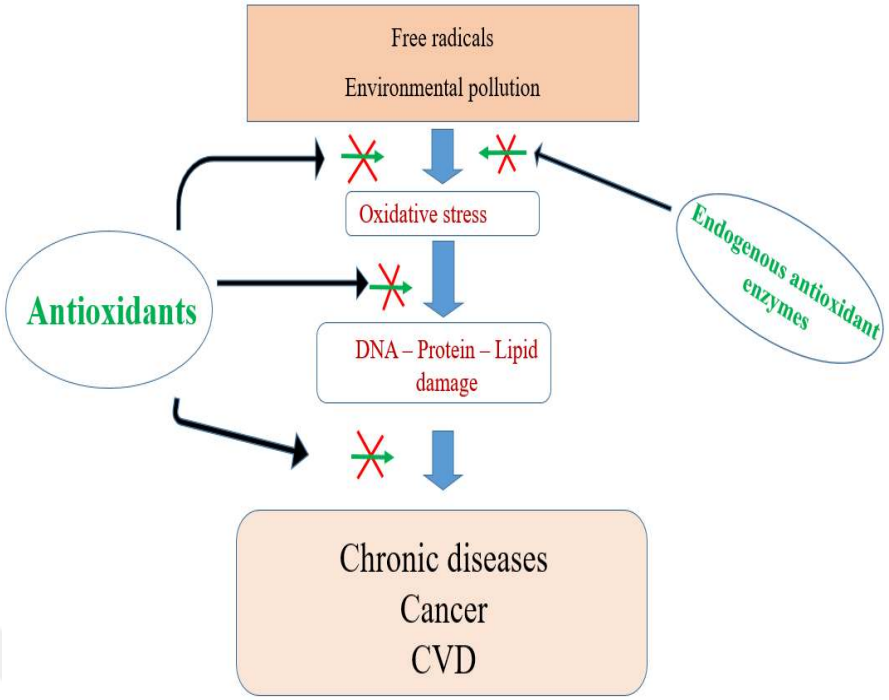


Figure 2.7. The essential function of antioxidants versus of radical damage species

Many defects in human health like carcinogenesis and others have been happened due to a process that will be occurred as carbon-centered radicals is known as the peroxidative process. Conversely, antioxidants have been vital features in reducing or stopping this process that have a role in the increased oxidative stress, Figure 2.8 illustrated both peroxidative processes that form carbon-centered radicals (R•) and peroxyl radicals (ROO•) and prevention of peroxidative process by antioxidant actions. Also, radical species will be formed by antioxidants, this is taking place during the action of antioxidants, so they have pro-oxidant manners. However, the low tendency of antioxidants for the generation of species is significant, while it is related to concentrations, stage of oxidation, and other conditions.

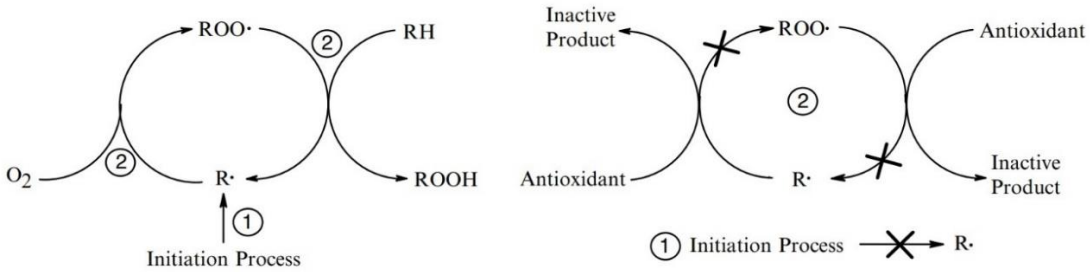


Figure 2.8. Critical presence of antioxidants in the peroxidative reaction mechanism [76]

With the increasing amount of radicals in the body, for remaining body healthy can depend on the antioxidants due to their power against free radicals. Reaction among particular antioxidants and oxidants at a constant rate is known as antioxidant activity. For evaluation antioxidant activity *in vivo* and *in vitro* are developed by measurement of effect free radicals on the biomarkers and plasma lipid peroxidation level. According to this study, there are around 29 methods for evaluation antioxidant capacity, 10 of them are *in vivo*, and others that 19 are *in vitro* methods. All methods have a vital role in the determination of capacity. Scavenging and preserving biomolecules from the side effects of free radicals are produced by the action of a powerful antioxidant is called polyphenolic compounds [77]. Antioxidant activity is usually determined by single electron or hydrogen transfer or both assays, they are the basis of antioxidant reaction. However, the ability to remove radicals is known as antioxidant capacity [78].

2.3.1. Vitamin A

The human body to stay in a healthy state require vitamin A which can dissolve in lipids as well defined as retinol, and generally can attain it to the body via external sources like nutrients because production in the body cannot be carried out. Initially, McCollum and Davis in 1913 discovered vitamin A before all other vitamins which have an affinity to lipids or soluble on it. Additionally, vitamin A as an agent has multiple significant roles for human health like increasing potential of health against disorders or pathogens, keeping the health of the eyes, and giving strength to the bones, thus amount this vitamin more or lower than the request value caused of various diseases [79, 80]. Heart disease has a high cause of death in the world, so antioxidants like vitamin A reduce the effect of oxidative damages particularly upon heart disorders [81]. Figure 2.9 is shown the structure of three constitutes of vitamin A present which are retinoic acid (RA), retinol, and retinal, which are called retinoids, the first one is more powerful if compare with others in living organisms. Also, all-trans-RA with 9-cis-RA are both of them the most common compounds derived from RA [82, 83].

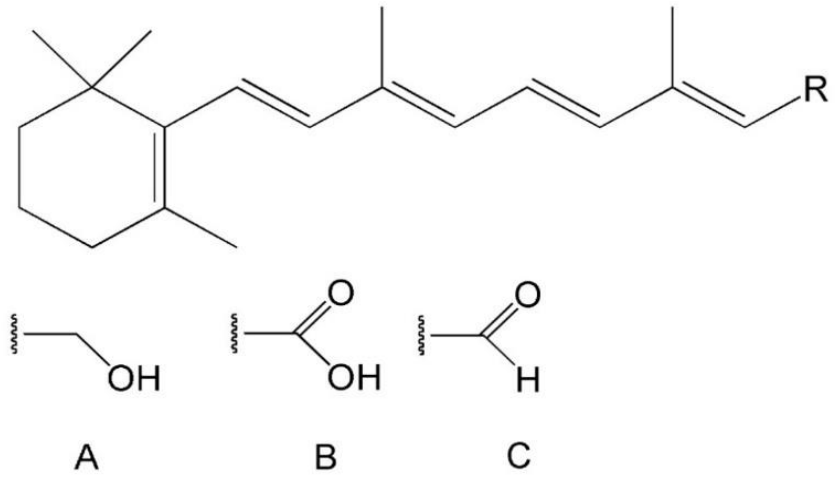


Figure 2.9. Retinoids structure, where if R is a kind of A, B, C defined respectively, retinol, retinoic acid, retinaldehyde [84]

Furthermore, β-carotene is generated by several plants, after reaching into the gastrointestinal tract in the human body partially convert to retinol forms, then collect and set aside for future use as retinyl esters (also explained in Figure 2.10) [85, 86]. Pass of β-carotene to retinol over some metabolisms then produce RA inside the organisms, it does not remain in the liver [87]. When the nutrients of vitamin A arrive in the digestive system later absorb by those cells that line the inner surface of the intestine then alter to retinyl esters, and the liver is an area for collection[88]. The liver contains a certain amount of vitamin A but with vary composition which is known as esterified retinol, while if the quantity of vitamin A increased in the body after taking a specific range of it as dose for a very long time during a particular disorder, the hepatotoxicity may occur [89].

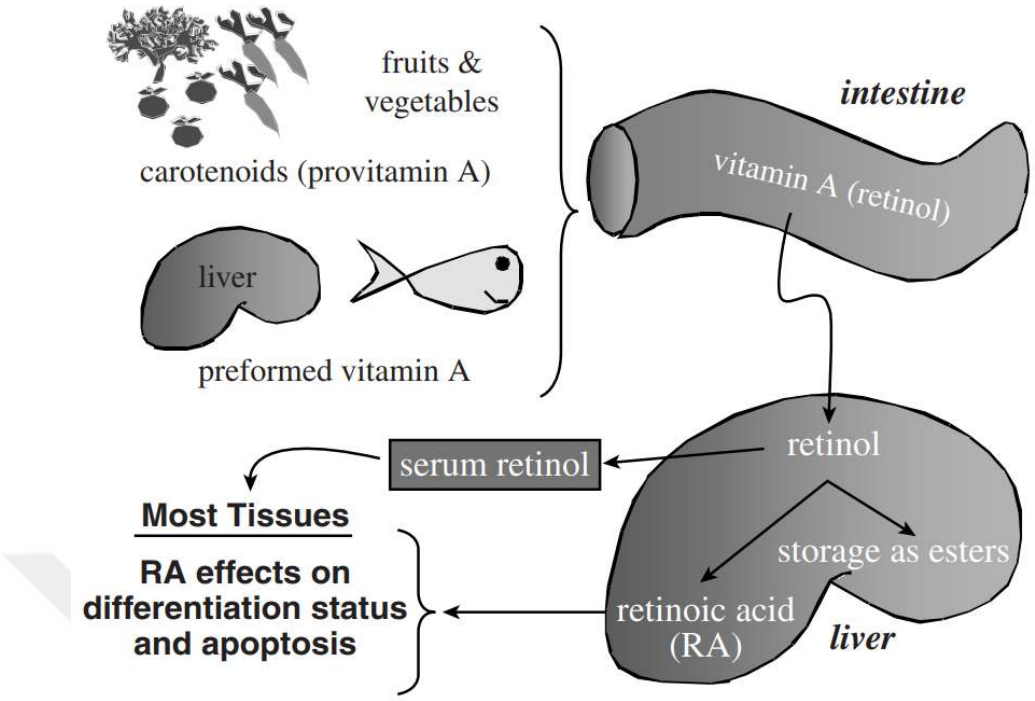


Figure 2.10. The schematic diagram of vitamin A during travel through organisms [90]

Carotenoids are defined as hydrocarbon compounds that involve various chemical structures like α-, β-carotenes, and so on, while the beta form is frequent abundance than others. When β-carotene reaching to the gut wall due to β-carotene-15,15'-oxygenase (BCO1) in consequence yield retinal, then retinal reductase make alternation over retinal to retinol [91]. As well, for transporting the vitamin A form to the organs based on the requirement in the blood via specific protein carried out which is called retinol-binding protein [92]. Carotenoids can be discovered in high concentration in many deep-green herbals and those plants that have orange or yellow color [93]. Most dietary foods which contain a major source of vitamin A forms consist of the β-carotene, retinyl esters, and retinol, then followed by a series of metabolic reactions in the body, as illuminated in Figure 2.11.

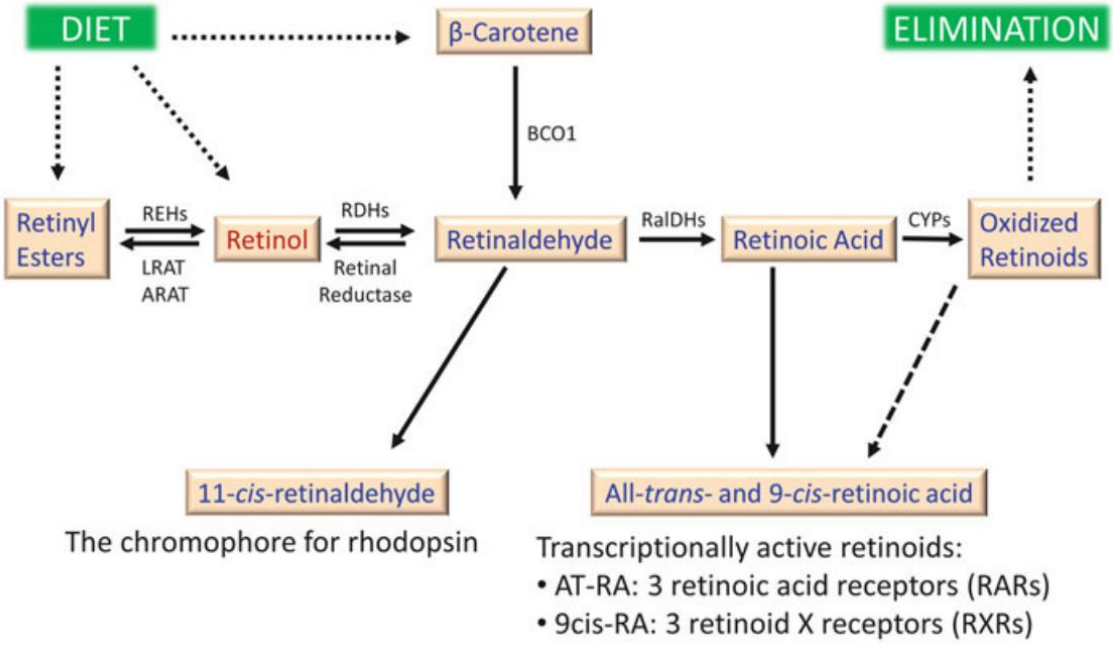


Figure 2.11. The general metabolic pathways of vitamin A from sources into individual tissues. Where LRAT, lecithin retinol acyltransferase; ARAT, acyl-CoA: retinol acyltransferase; RDHs, retinol dehydrogenases; REHs, retinyl ester hydrolases; RalDHs, retinaldehyde dehydrogenases; CYP, cytochrome P450 [94]

2.3.2. Vitamin C

Another description of vitamin C is called ascorbic acid or ascorbate, which could discovery in a high amount in diet and plants, also isn't tending to combine with lipids, and it has an antioxidant character which led to protective health from multiple disorders [85]. In 1932, biochemist Albert Szent-Györgyi found vitamin C, which predicts it has a high capacity in the treatment of multiple health defects. Various agents affect the pharmacological functions of vitamin C that may happen during metabolisms, life habits, and environmental situations [95]. Living systems in the human body need vitamin C as an important compound for many factors that directly relate to human health, directly by the gastrointestinal system is absorbed, and it has a solubility in water. Additionally, the only way can get it from the diet is because it is impossible to generate vitamin C inside the body, while it is created from glucose via the gulonolactone oxidase enzyme which takes place only in animals. When redox metabolism accrues in vitamin C (as explained in Figure **2.12**) by donating a single electron to create dehydroascorbate, the reaction can be inverted in both steps (ascorbate free radical and dehydroascorbate) respectively, by NADH-dependent reductase and glutathione, and ascorbate free radical inert free radical.

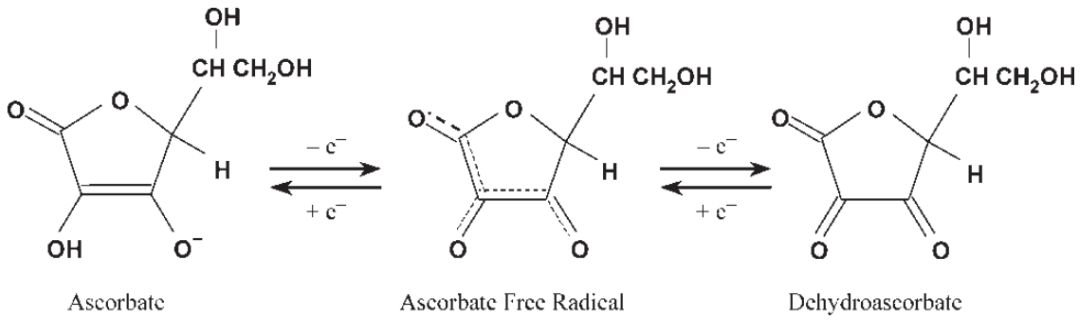


Figure 2.12. Redox reactions occur over vitamin C. When the ascorbate free radical yield, amid those oxygen atoms that have dashed line a single electron is delocalized, then rise to dehydroascorbate [96]

Furthermore, vitamin C is defined as a powerful and useful component for the body, has a certain disposition towards increased immune system, as an antioxidant to eliminate radical species, and various functions. Otherwise, defect in the quantity of ascorbic acid are caused by numerous diseases like scurvy [97]. Due to vitamin C having an affinity for water so, those radical species in the aqueous media will be delayed by the antioxidant power of vitamin C before reach into lipid peroxidation [73]. Some study centers that cancer research confirmed many cancer groups can be decreased their effects on the body by antioxidant like vitamin C, which also caused of keep away biological system from influencing oxidative stress which is responsible for many diseases [90]. Moreover, vitamin C can protect biological organisms from radical species in aqueous media due to its ability is high in this media, and also in lipid media has been taken place via reproduction of vitamin E which is tending to combine with lipids, as Figure 2.13 is explained by chemical structure [98].

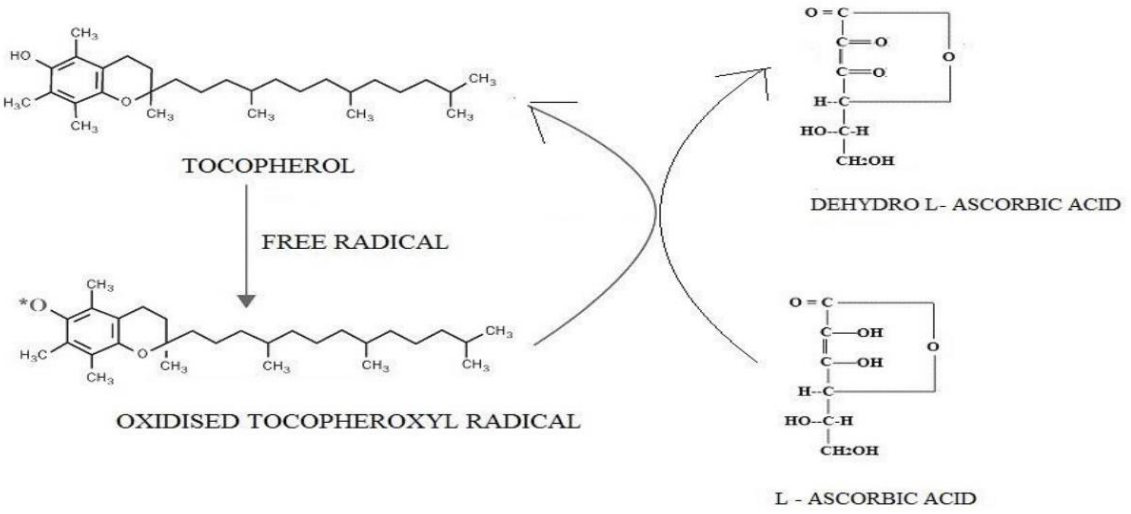


Figure 2.13. Vitamin C act as an antioxidant for regeneration of vitamin E from radical composition after reaction with radical species [99]

2.3.3. Vitamin E

Both radical atom forms which are consist of ROS/RNS particularly, HO˙ and peroxynitrite can be involved in various defects in the health so, to diminish their amount, vitamin E can be seen of a considerable amount as antiradical, however, has and provides numerous benefits to health, over of them raise immune system talent. Solubility of vitamin E in the lipid area is high, presents many tissues in the body, compares other radical species that have extreme intensity for peroxy-radicals, and introduces to keep membranes of a living system [100, 101]. Furthermore, the structure of vitamin E constitute of eight dissimilar forms which divided into two compound groups including tocopherols and tocotrienols, and both of them have alpha (α), beta (β), gamma (γ), and delta (δ) structures (whole structure forms demonstrated in Figure 2.14). Inside the body alone α-tocopherol can be classified as vitamin E while remaining compounds don't exhibit the consistent conditions *in vivo* [102]. HO group on α-tocopherol has a brave antiradical manner, that caused of or addition ability of vitamin E as an antioxidant [103]. The two structure groups of vitamin E own different tails, tocotrienols, and tocopherols respectively, have another definition called isoprenoid and phytyl side chains which are composed of conjugated unsaturated bonds and single bonds. The nutrients of vitamin E are kept and collected in the liver subsequently arrive and then absorb by way of intestine cell walls, according to the necessary tissues liver supply the vitamin to them.

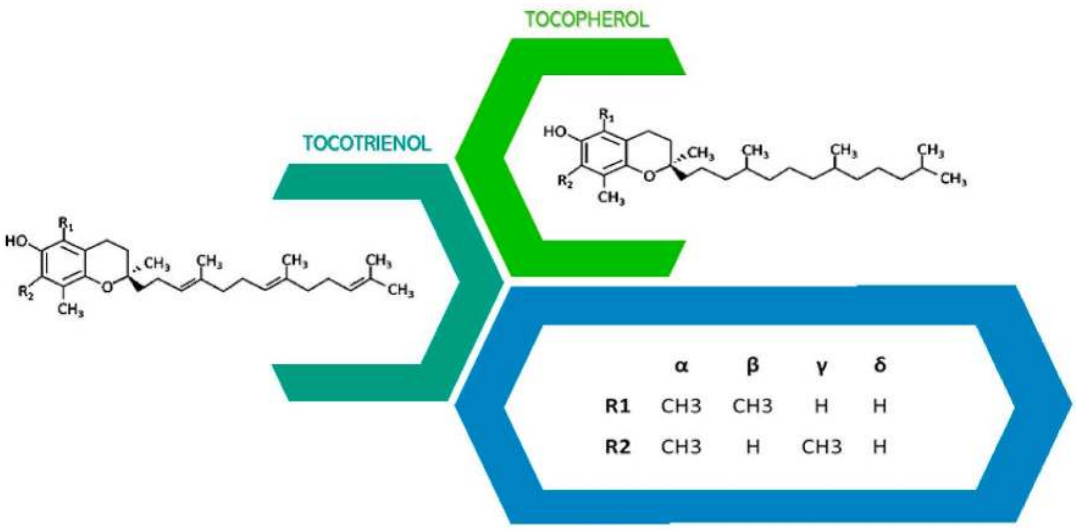


Figure 2.14. Chemical structure of tocopherols and tocotrienols [104]

Fruits and vegetables contain a high value of vitamin E, also it is highly sensitive to light [85]. When present vitamin E (α-tocopherol), decrease oxidants that are responsible for many diseases in humans due to the capability of α-tocopherol by the limited activity of peroxyl-radicals that is a significant factor for maintaining cell membranes especially, polyunsaturated fatty acids.

Tocopheroxyl radical (which is fixed and doesn't have the ability for reproduction radicals (harmful ones)) is generated by the reaction between both radical species and α-tocopherol, then the formed radical can react again with radicals especially, peroxyl radical [105, 106]. In 1922, the rats had predicted and found vitamin E as major molecules by Evans and Bishop, many decades later the studies illustrated also it can act as a useful component inside the body. The only way humans can get the vitamin E is diet, mainly γ-tocopherol present in the diet then α-tocopherol, but *in vivo* α-tocopherol has a greater interest if compare γ-tocopherol with other forms. Afterward, vitamin E (VE-OH) react with peroxyl radicals ($LOO^{\bullet}$), thus tocopherol radical (VE-$O^{\bullet}$) yield then via vitamin C (VC-H) regenerate vitamin E by donating H-atom [107, 108]. As explained in the below reactions.

$$ROO^{\bullet} + VE - OH \longrightarrow ROOH + VE - O^{\bullet}$$

$$VE - O^{\bullet} + VC - H = VE - OH + VC^{\bullet}$$

2.4. Antioxidant activity

The ability of antioxidants on the cancer cells displayed significant of those supplements on the health and another role they have also a power to reduce side effects of some chemotherapies that used during cancer treatment that is enhanced to take antioxidants in a necessary quantity [109]. In recent years, antioxidants have been used in different fields of life on the earth like medical, commercial products, and so on [110]. The assess power for destroying and removing free radicals is known as antioxidant activity (AOA). Unfortunately, for estimation AOA require known antioxidant influence on the area of oxidation not by a straight way, also can be detected by numerous methods [111].

For evaluation AOA there are several assays usually, at least two assays in the process and each of them displays diverse results, that may come from those differences between assays like various procedures in the application of each one, the inner and outer environment, and so forth [20]. The compounds that role to delay or/and destroy ROS and RNS which cause of protection living system and able to exhibit as AOA. Also, each method has particular states and unlikeness properties if compare with others (Table 2.1 displayed characters of two *in vitro* assays) like in reaction terms and mechanisms, also in the results [112]. According to several researchers, AOA assays are divided into major parts like AOA assays composed both *in vivo* and *in vitro* by Niki [63]. AOA is divided into *in vitro* and *in vivo* methods which are composed of 20 and 10 assays respectively. AOA can apply in two method groups first *in vitro* methods to increase evaluation quality, the best choice is to use numerous assays in the AOA, and each assay has particular behavior. While *in vivo* methods is generally rely on utilizing animal organs or blood during the

assay [113]. Decrease and increase of AOA would alter based on the concentration, structure, and surrounding factors [114].

Table 2.1. Description of both ABTS and DPPH advantages with disadvantages [115]

Assays	Advantages	Disadvantages
ABTS	Can be done for both organic and aqueous phase	Alternation happen under the influence of light and temperature
	Stable to pH	
DPPH	Low-priced and uncomplicated	Dissolve in organic phases
	Fast to arrive the steady-state	Alternation happen under the influence of pH

In addition, there are six different colors in the visible spectrum region, the range of wavelength starts from 400 to 750 nm as explained in Figure 2.15. Also, it is important to know knowledge about it due to the absorbance in colored assays display complementary color like DPPH radical appears as deep purple while absorbing light at 517 nm, however, can be seen the similar action in ABTS assay.

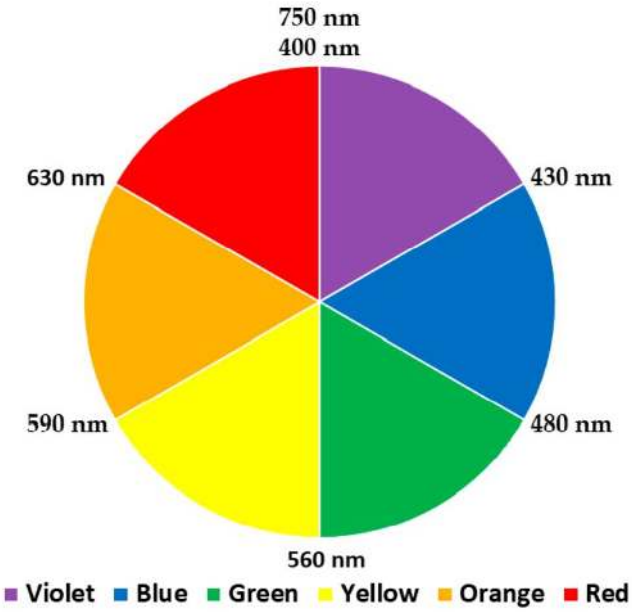


Figure 2.15. The color circle is an abstract illustrative organization of color hues around a circle with their absorbance [116]

Generally, for indication AOA, three analytical methods can be used which include spectroscopic techniques, electrochemical techniques, and chromatographic techniques [117].

Nevertheless, another significant should consider choosing a suitable method in the antioxidant evaluation process [118]. The single electron transfer (SET) and hydrogen atom transfer (HAT) are the main classes of the AOA methods, which include numerous assays such as the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) and so far [54]. If comparing DPPH assay with other ones, the DPPH assay has better advantages like without more difficulty in the process, quick and other features [72].

Nowadays, in the measurement of antioxidant activity-specific techniques used which is defined as spectrophotometric, they are fast and appropriate techniques, and their operation depends on the reaction of SET or HAT from the antioxidants with colored radical or other radical species. To dominate antioxidants over radical species have to pass into two chemical reactions which are HAT or SET, both reactions explained the behavior and mechanism against ROS in the below [119, 120]. When radical species react with antioxidants based on the SET assay, the radical is reduced and the alternation happens upon the color of a solution via antioxidant ability, they can determine the capacity value of the antioxidants. The rank of the solution color is altered based on the AOA range. While in HAT-based methods, the antioxidant reaction caused of appease radical capacity and remove them via transfer H-atom to radicals, after that can measure the antioxidant power [121, 122]. Table 2.2 is explained some AOA assays and both SET- and HAT-based assays pass through dissimilar reaction mechanisms with different speeds, both assays explained by Figure 2.16 and Figure 2.17.

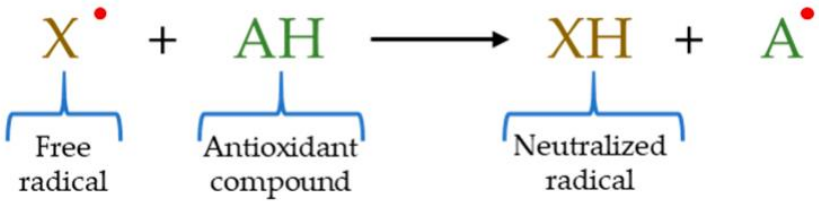


Figure 2.16. The chemical reaction of antioxidants with radicals based on HAT

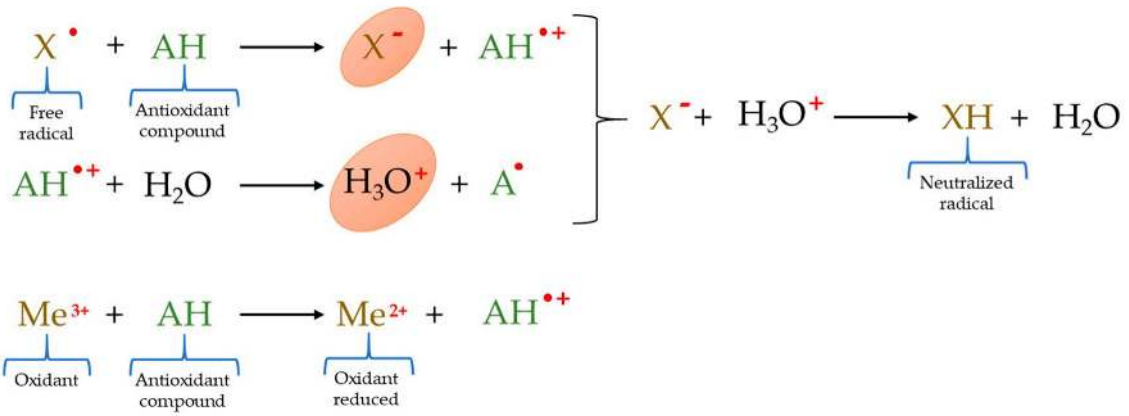


Figure 2.17. The chemical reaction of antioxidants with radicals based on SET [123]

Table 2.2. Several characters of some AOA assays [124]

Methods	HAT/SET principal	Radical/Chromophore	Wavelength (λ)	Rate of pH
DPPH	SET-based assay	DPPH$^{\bullet}$	515 nm	7-7.4
Trolox equivalent antioxidant activity (TEAC)	SET-based assay	ABTS$^{\bullet+}$	734 nm	7.4
Thiobarbituric acid reactive substances (TBARS)	SET-based assay	MDA-TBA Adduct	532 nm	2
β-carotene bleaching assay	HAT-based assay	Peroxyl radicals (ROO$^{\bullet}$)	470 nm	5.5-7.5
Hydroxyl radical averting capacity (HORAC)	HAT-based assay	HO$^{\bullet}$(p-hydroxybenzoic acid) fluorescein	λ_{ex}= 488 nm λ_{em}= 515 nm	Phosphate buffer

2.4.1. DPPH scavenging activity assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is one of the interesting spectrophotometric methods employed for AOA [20]. In the beginning, the color of stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) is purple, during reaction with antioxidants its color alter to yellow, and the alternation takes place based on the ability of radical species scavenger compounds as approved by the Brand-Williams method [9, 53]. DPPH$^{\bullet}$ was defined by several authors, also a reaction of antioxidants with DPPH stoichiometry was explained by Brand-Williams with a member of researchers [125]. The reaction occurs among an antioxidant with a DPPH radical (a radical on the nitrogen) after mixing them, also the color of the solution before the antioxidant is

purple, while the antioxidant interaction with it the color is converted to yellow[121]. After adding the antioxidant to the DPPH methanolic solution, the color is changed and antioxidants concentration has a direct influence on the color of the solution, due to those electrons that accept from the antioxidants to $DPPH^{\bullet}$ form [126]. For indicating and measurement antioxidant activity from the DPPH method (which is established upon the spectrophotometric) which determine via those DPPH radicals that stayed after reaction of antioxidant with $DPPH^{\bullet}$ [127]. To indicate the power of novel drug against free radicals, this assay can be chosen as a suitable assay, also both below reactions are exhibited reactions of $DPPH^{\bullet}$ with (AO-H) antioxidants and ($R^{\bullet}$) radical forms[72, 128].

$$DPPH^{\bullet} + AO - H \longrightarrow DPPH - H + A^{\bullet}$$

$$DPPH^{\bullet} + R^{\bullet} \longrightarrow DPPH - R$$

The reaction of DPPH radical passes through two types of reaction which is include hydrogen atom is slow while electron transfer is quick, so SET has power and influence over HAT when interacting with antioxidants. The surrounding environment has a direct effect on the DPPH reaction due to its extreme sensitivity. The solvent that utilizes in the DPPH mechanisms reaction has direct affinity on it, like the HAT mechanism happening often if water s placed into the area of reaction, but in presence of methanol (generally used in the reaction), the SET mechanism is dominant over HAT because bind hydrogen atoms very powerful (as shown in Figure 2.18) [129]. The desired time for reaction and the antioxidant concentration is contrarily proportional with the signal intensity of $DPPH^{\bullet}$. It was recommended as a SET reaction assay by Foti with fellow workers [78]. The below equations in Table 2.3 illustrate both SET and HAT reaction mechanisms of $DPPH^{\bullet}$ with (Ar-OH) [130].

Table 2.3. SET and HAT reaction mechanisms

SET mechanisms	HAT mechanisms
$Ar - OH \rightleftarrows Ar - O^{-} + H^{+}$	$Ar - OH + DPPH^{\bullet} \longrightarrow Ar - O^{\bullet} + DPPH - H$
$Ar - O^{-} + DPPH^{\bullet} \longrightarrow Ar - O^{\bullet} + DPPH^{-}$	
$DPPH^{-} + H^{+} \longrightarrow DPPH - H$	

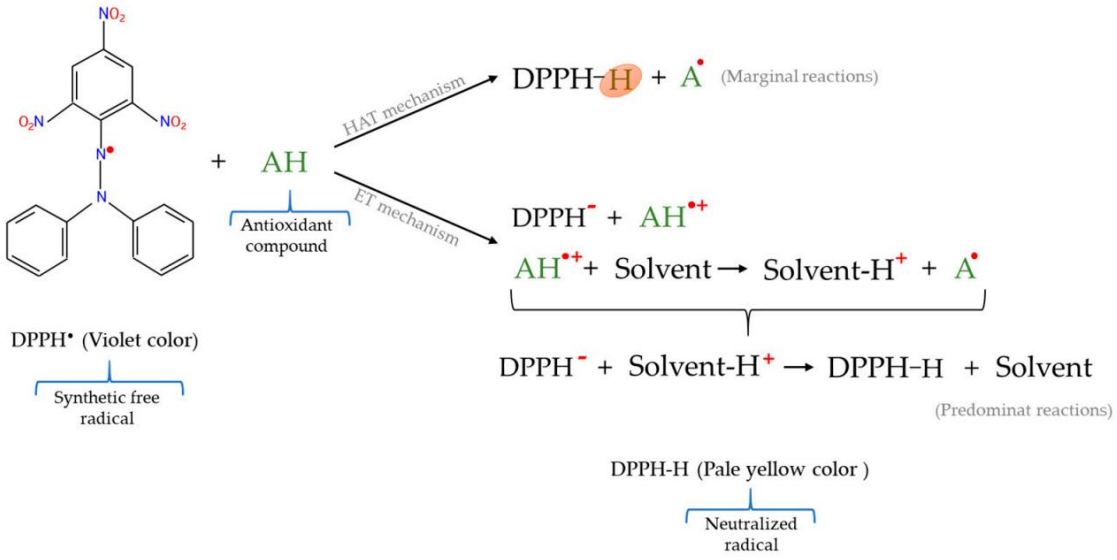


Figure 2.18. AOA according to DPPH• assay by the chemical reaction mechanism [123]

Around 1920, Goldschmidt and Renn found the DPPH radical. The antioxidant can accept an electron or a hydrogen atom to neutralize the radical and alter it into a reducing form. Furthermore, the DPPH assay has several interesting points like simple, inexpensive, very fast method which can react a lot of antioxidants in a short time, and so on. Otherwise, DPPH radicals don't affinity or dissolve in water, during the analysis necessary to far away from the light, and care for reaction with those radicals that exist inside the solution [116]. Generally, the absorbance of DPPH radical (DPPH•) is 517 nm, while the absorption is reduced after passing through a reduction via antioxidant or radicals, and the percent of antioxidant capacity was calculated via the equation [131]:

$$Radical\ scavenging\ activity\ (\%) = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100 \qquad (1)$$

where A_{Sample} is the absorbance of DPPH in the presence of the required compound while $A_{Control}$ is the absorbance of the initial concentration of the DPPH.

2.4.2. ABTS radical cation decolorization

$ABTS^{\bullet+}$ [2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)] radical cation is one of the significant assays that can be used for AOA for several sample solutions like pure synthesize substances, also for those antioxidants that have an affinity for water and organic [132]. Re et al. and Miller et al. developed ABTS [2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)] assay, and its absorbance equal to 734 nm [78]. ABTS afterward loss an electron from the nitrogen atom yield the ABTS cation radical ($ABTS^{\bullet+}$). However, numerous different ways have been used in this process, one of them potassium persulphate ($K_2S_2O_8$) which has an oxidation role over ABTS (the

chemical reaction structure of $K_2S_2O_8$ with ABTS displayed in Figure 2.19) [110, 133]. Before the reaction of $ABTS^{\bullet+}$ with antioxidants, the color of $ABTS^{\bullet+}$ solution is blue-green and then yields colorless solution due to reduction into ABTS (antioxidant with $ABTS^{\bullet+}$ reaction illustrated in Figure 2.20) [130]. Two reactions have a role in the generation of $ABTS^{\bullet+}$, they include enzyme reactions which are quick and simple, otherwise, chemical reactions need lots of time usually more than 16 h, as described by Cano and co-workers. Additionally, several benefits of this assay can predict like not complex method, speed, organic and aqueous solvents can be used as dissolve media of $ABTS^{\bullet+}$, and many other advantages [134, 135].

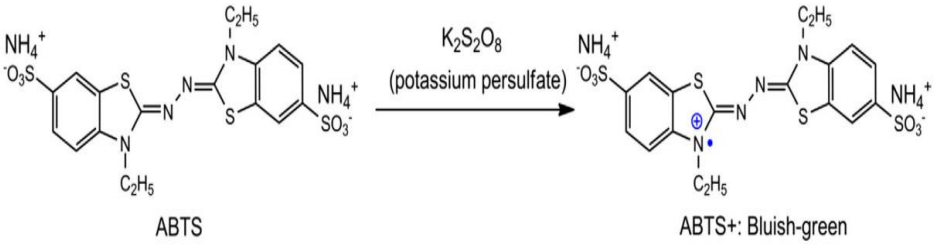


Figure 2.19. Generation $ABTS^{\bullet+}$ from ABTS via potassium persulphate [121]

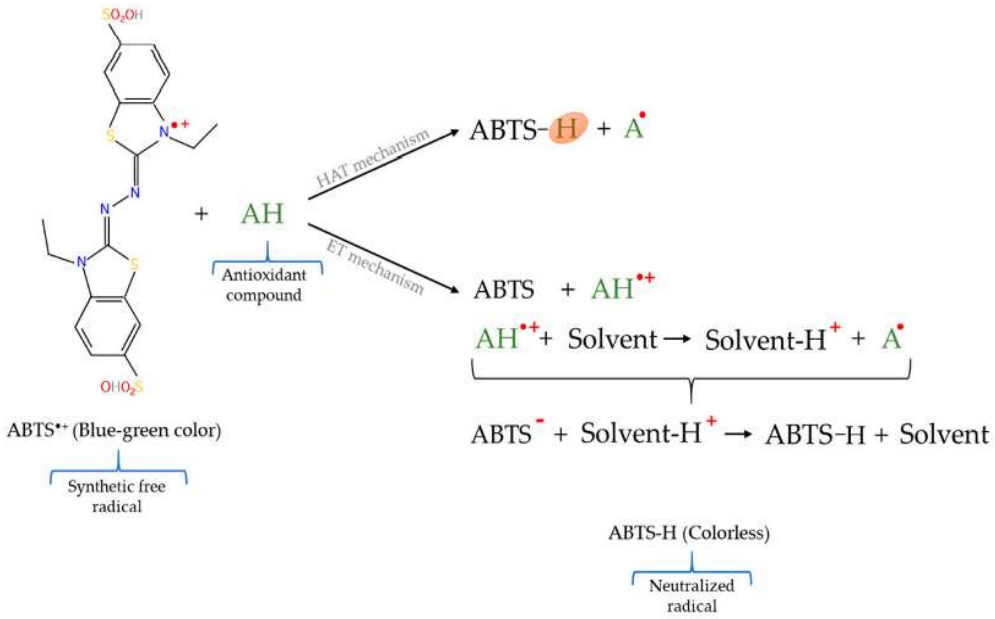


Figure 2.20. Antioxidant role in the neutralization of the radical [123]

2.4.3. Hydroxyl radical scavenging activity

Radical species have high efficiency against the living system, particularly $HO^{\bullet}$ has extremely power to interact with polyunsaturated fatty acids and the result is caused of a defect in

the cells [54]. The HO• assay scavenger is defined by Halliwell et al [136]. Generally, in the body HO• has been generated by a defect in the electron transition to oxygen (O_2) with the effect of transition metal ions, and at the third reduction reaction of O_2 is yield. Also, deoxyribose has been widely used in the hydroxyl radical scavenging capacity assay which is a particular antiradical activity assay for HO• [137]. In hydroxyl radical scavenging activity assay, focus on the HO• characters in production until scavenging, so removing HO• can be estimated value due to deoxyribose (which degrade sugar and discern the amount of HO•) of compounds and also foods. The cyclic furan of deoxyribose is broken and created malondialdehyde (MDA) after being attacked by HO•, then thiobarbituric acid (TBA) with the formed MDA reacts. And read its absorbance at 532nm, the rate proportional with the quantity of HO•, and otherwise with antioxidants [138, 139].

2.5. Antimicrobial activity

One of the antimicrobial assays that have been used since 1940 until now for numerous particular microorganisms is called the agar disk diffusion method. Also, several characters lead researchers to apply the agar disk-diffusion method in their studies via the following properties: huge spectrum of microorganisms can be tested, lack of complexity, require a lower price, and get results clarified. However, antimicrobial assays have been played considerable roles in the development of agents for pathogens. In the agar disk-diffusion methods at the end of the process, by measuring the diameter of inhibition growth zones that take place upon the microorganisms if the compound has the ability as an antimicrobial agent [12]. In this study, several bacterial and fungal strains were used like Escherichia coli ATCC25322, Bacillus megaterium DSM32, Klebsiella pneumoniae ATCC700603, Staphylococcus aureus ATCC25923, and C. albicans FMC17 is fungal [131].

Microbiology is a study of microscopic organisms and agents that usually cannot be seen by the eye. Microorganisms play role in huge areas such as food, medicine, ecology, biology, and so on. Also, they can be caused by several infectious diseases or pathogens that is depending on the microbe species. There are numerous microorganisms on our planet like bacteria, fungi, and other living systems that have both sides for the human body helpful and harmful, they are summarized in Table 2.4. Microorganisms have a size that cannot be seen without a microscope like bacteria, viruses, and fungi that could be able for making pathogens in humans. Bacteria don't have a nucleus and are surrounded by a cell wall, and fungi possess a clear nucleus, but viruses generally don't have a cell structure. Also antimicrobial capacity is one of the brilliant tests that have been used in the drug discovery and ability against pathogens [12, 34, 140, 141].

Table 2.4. Lots of microorganism groups an agent that responsible for numerous pathogens [142]

Microorganisms	Infectious organisms
Bacteria	Gram-positive: S. aureus, Corynebacterium, Peptostreptococcus, Clostridium perfringens, and so on. Gram-negative: Moraxella, Enterobacteriaceae (E. coli, Klebsiella, and Salmonella), Fusobacterium, and so forth.
Fungi	Candida, Aspergillus, Mucor, Histoplasma, and Coccidioides.
Viruses	Hepatitis A, B, C, D, E, severe acute respiratory syndrome (SARS) virus, and others.

2.5.1. Antibacterial activity

Microbial has been living on the earth plane for a long time especially bacteria that is the first form of life around 3 billion years ago on the earth. Bacteria can grow in various environments and has been dispersed in all places of the Earth such as the North Pole, in the depths of the oceans, and so forth. A wet and warm environment makes a brilliant area for increasing the growth of bacteria and also they can become adjusted inside and on the human body [143]. Each bacteria species have a different character in their shapes like spherical (coccus), rods, and spirals also they have several composition structures, mobility, and other properties [140]. In bacteriology, it is significant to detect those bacteria that are caused by infectious diseases in human health and defect in animals and plants, because identification and management increasing tendency to control the recognized bacteria [144]. Cell envelopes are defined as the wall that surrounded bacteria cell (it have two kinds of cell wall as described their manners in Table 2.5) and protective bacteria intact from the external environment, there are three groups for composition their structure include; polysaccharides, lipids, and proteins [145].

Table 2.5. Classified cell envelopes of bacterial with their behaviors [146]

Gram-Positive bacteria	Gram-negative bacteria
The cytoplasmic membrane is present in the internal.	The cytoplasmic membrane is present in the internal.
They have a thick peptidoglycan layer and surrounded inner layer.	They have a thin peptidoglycan layer and are contained between two layers.
They don't have an outer membrane and have both wall teichoic acids (WTAs) and lipoteichoic acids (LTAs) in the composition while having lipopolysaccharides (LPS) is absent.	The outer membrane surrounded the peptidoglycan layer and contains lipopolysaccharides (LPS), but both wall teichoic acids (WTAs) and lipoteichoic acids (LTAs) isn't present.
While the periplasmic space between the inner and peptidoglycan layer.	Also, there is a space among outer and inner membranes called periplasm that is much greater volume than gram-positive bacteria.

There are diverse shapes of bacteria, also they have a few micrometers in length, and they have a symbiotic and parasitic character from the duration of their life depending on the bacteria

species. The science that deals with bacteria include the identification and characterization of bacterial kinds that study known as bacteriology, while a bacteriologist is a person who works in the field of bacteria.

Staphylococcus aureus is defined as gram-positive bacteria with a coccus shape. S. aureus has play role in several pathogenic skins and soft tissue infections that occur from the superficial of the patient such as abscesses [147, 148]. The Staphylococcus species are small in size with a hardy structure, there are more than 50 Staphylococcus species that are caused by skin diseases in humans and animals also they are present everywhere. The most common species are Staphylococcus aureus which is caused by some pathogen especially in the skin, animals that accompany humans may be the major reason for the transfer of S. aureus to human [149].

Escherichia coli (E. coli) is defined as a group of bacteria that can be found in the water and soil, however, it lives on humans, plants, and animals, also E. coli can live in several environments. Generally, E. coli are the cause of many pathogens in the humans that are infected with this bacterium. E. coli is a gram-negative bacteria and a tiny organism that doesn't exist a nuclear membrane which is called prokaryote. In 1885, the first German scientist who recognized E. coli is Theodor Escherich, who found in the stool specimens of some babies who have various defects like stomach pain, diarrhea, and other health problem from the result of inflammation of the intestine [150].

2.5.2. Antifungal activity

Fungal species have various sizes and shapes, they caused many infections also called mycoses [151]. There are many types of fungal such as yeasts, rusts, molds, and mushrooms, some fungal-like yeasts have a role in many pathogenic in humans, those infections that take place in humans due to fungal are defined as mycotic, the most common mycotic infections that occur in the skin, nails, and others defects in the human health, while much other fungal cause of several diseases in the plant, unfortunately, the number of cases that infected with fungal are going to increase with higher potential [152]. When the ability of immune defenses reduce fungal species get a high capacity for many pathogens. From 50,000 fungal species or higher, approximately 300 species cause mycotic infections. Among Candida species, the most species that cause mycotic infections are Candida albicans [153].

Yeast a major fungi group with cell length, width, and size are 20–50 μm, 1–10 μm, and 2–3 μm respectively, that can increase or divide via budding. Candida albicans that defined as dimorphic fungus include: budding yeast and hyphal, it can often live on the mouth, gastrointestinal, and also skin of the human called candidiasis when damage to the tissues or make a defect in the health, such as to cause of "thrush" in babies. 150 Candida species have been identified so far, while some of the species caused of pathogens in human approximately 15 Candida species, that

include: Candida albicans, Candida parapsilosis, and so on. The most common diseases that occur due to Candida species are skin and nail pathogens like intertrigo [154-156].

2.6. Cancer

The most common disease in the world is a cardiovascular disease after that, cancer comes with high spread, growing abnormally, and is extremely powerful inside the patient's body. So, knowns as a dangerous problem on the health. Many therapies have been developed to combat cancer, while so far not having any treatment to solve cancer diseases completely and without side effects [15]. In 2018, 9.5 million people who patients with cancer were death approximately equal %50 of all cases. However, cancer is the second cause of death around the world. Proliferation and angiogenesis are the basic knowledge of cancer, continuous replication of cancer cells leads to tumor generation or development if replication uncontrollable is called proliferation. Despite; the process which increases the formation of novel blood vessels due to cancer cells and many health problems known as angiogenesis [157, 158].

Cancer cells are developed in the body by different steps, starting from initiation, and promotion, then progression as a process to generation of cancer is known as carcinogenesis. Many reasons help and lead to carcinogenesis, they are including ultraviolet, smoking, viruses (like liver cancer due to hepatitis B, C, and other cancers by infection with several viruses), bacteria, chronic diseases, quality of diets, ROS, and so forth [159, 160]. Free radicals have been a major role in the increasing cancer cells, due to their ability to defect on the DNA that is caused by oxidative damage of cells [57]. Cancer is a disorder that happened in the genetic and can be spread to every tissue in the body [161].

A period of time or poor cell health, the cells will have been dead that is called apoptosis, while if the death of the cells isn't normal, that is caused by any defects in the health like neoplasms and others, but patients with cancer have lower apoptosis in the cancer cells if compare with control cells [162]. Diagnosis for detection of cancer cells is extremely vital because determining the cancer cells in the beginning stage in the body help to treat in the early time compare those patients who have been distributed cancer in lots of tissues. Due to new knowledge approved less than 50% of people might possess at least one cancer form without feeling in their lifespan [163]. According to several predictions, may remain cancer a huge problem for human health until the unknown time in the future. The diagnosed cancer patients, exhibited prostate cancer in men while breast cancer in women has a high range if compare to other cancer groups, except skin cancer. The process which is caused by the generation of tumors known as tumorigenesis is multistep to develop a tumor and spread inside the body of patients. And it is important to determine the tumor in which step of progression due to help scientists to use the right therapy for it because in each step behave of tumor and body resistance are different [164].

Abnormal growth of cells may clarify a chronic disease that is the cause of generation tumor or cells respectively solid or liquid form structure. Many treatments have been used for cancer disorders depending on the group and stage of diagnosis, the main treatments include surgery, chemotherapy, and so forth. However, the chemotherapy makes some defects in the health of a patient who used during the treatment, while necessary of chemotherapy for relieving cancer and/or tumor caused of usage and neglect their side effects such as kidney, and other disorders. There are many methods have that are the DNA-therapy interaction, that have brilliant roles in the synthesis process of novel drugs like nuclear magnetic resonance, spectrophotometric methods, DNA-footprinting, and so on. There are many drugs used in the cancer treatment area and they are grouped as mechanism action of therapies. Chemotherapy had shared in the medical field in the 1940s with high capacity, many drugs have been started to grow as an agent to combat cancer [165]. In recent years, studies focus on the evaluation of the stage or structure of patient's tumor then they select suitable therapy for them, while in the past time scientists discover and develop drugs to use for those patients who have an infection with cancer, and determine the respond of patients for one as illustrated in Figure 2.21.

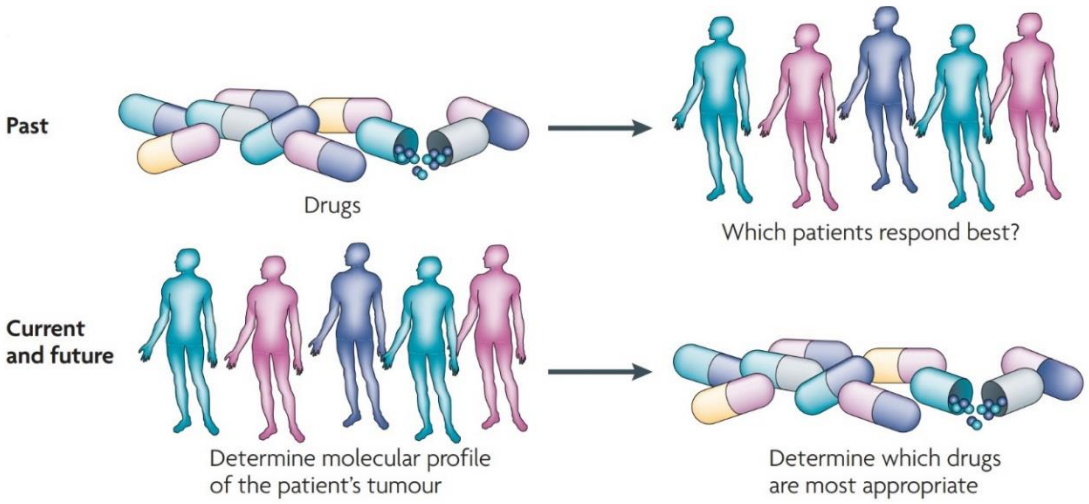


Figure 2.21. Treatment of tumor patients via medicine between past and recent years [166]

Generally, cancer has various group approximately 100 or higher, each of them have different properties and treatments, by evaluation type and stage of cancer can be treated better. There are several cancer therapies but one of them is the most common for cancer treatment, it acts as reduce growth or kill cancer cells is called chemotherapy [167]. That is clarified chemotherapy has got high disadvantages for the body of patients after treatment, so natural drugs have displayed a good treatment for combat with cancer and many therapies are created for treatment from plants. And the World Health Organization (WHO) predicts the number of patients increases in the future

time and also WHO illustrated one-thirds of patients maintain their life by far away from factors caused of rising carcinogenesis [168]. The environment that is surrounding human life greatly affects the population health like a defect in diets, poison materials on the space, and others which support the body for many diseases like cancer known as awful disease. So far don't have any therapy that can reduce cancer cells and without side effects after treatment [169].

2.7. Medicinal chemistry

Medicinal chemistry is more focused on the synthesis of new drugs so, in the past, the studies worked in a high range on the plant for drug generation while in recent years, many researchers especially chemists synthesized novel drugs from chemical reaction without neglecting natural sources as a first way in the pharmaceuticals. Also work as a team with various disciplines like chemists, toxicologists, pharmacologists, and other fields give extreme energy in the design, development, and test of new drugs. Generation and development of new compounds that have behavior like drug candidates will be clarified by medicinal chemistry which is followed by some steps for drug development from natural sources, chemical formation, and others via identification, preparation, improve capacity, reduce side effects, and development then use as a clinical agent. Nowadays, there are many ways to obtain the drug, while the most common way for the generation of drugs by synthetic like Paracetamol and some other methods like natural sources from plants and so forth. Drugs are composed of numerous chemicals, if the drug cause side effects on the body which is called poison, while if the drug prevents or reduces disease, the drug is medicine. Generally, chemicals have both toxic and medicine features, but increase or decrease one of the properties change with the person that uses and conditions of use during the treatment[170].

There are several ways to develop drugs, hence the development process requires more time approximately 8 years, it is impossible to predict usage as medicine, and the process is complicated, also most of the compounds will be failed to use in the clinical area during drug development [171]. In each year lots of compounds or molecules approximately millions are arranged while cannot say all of them are drug because every molecule have to exhibit the same drug characteristics after that it will know as a drug [152]. Generally, there are no drugs to say it is free from side effects for the body of the user, also if taking drugs in a high amount more than required causes of poison and also death. After discovering novel compounds that have similar properties as drugs, that is not all the work in drug development, also it is necessary to know drug functions and suitable in the body and as a drug [172].

At least two atoms held together by chemical bonds to form the smallest part of the substance are known as a molecule. Also, functional groups are a significant part of molecules, due to determine physical, chemical, and reactivity properties of molecules, they often form as the result of the assembly of atoms. Ammonium, carboxylate, and hetero-atoms like nitrogen are the most

common and powerful functional groups in drugs. Drugs pass through three phases, the first stage is the pharmaceutical phase which is defined as a beginning process that starts from the path by which a drug, fluid, or other substance is taken into the body until it is absorbed inside the intestinal wall. The second is the pharmacokinetic phase in this phase the period of drugs that starts from absorption of drugs in the body until they reach every organ and target organ inside the body, in some minutes drugs are transported into each organ via the bloodstream. In the end, drugs reach a point are known as the pharmacodynamic phase which is then displayed the effect of the drug on the organ, all phases are shown in Figure 2.22. Drug molecules in the body can interact with various biomolecules, cell nucleus, and other molecules. The aqueous medium is a center for all biological reactions, this is maybe due to water contributing as a major part of the body and all living organisms.

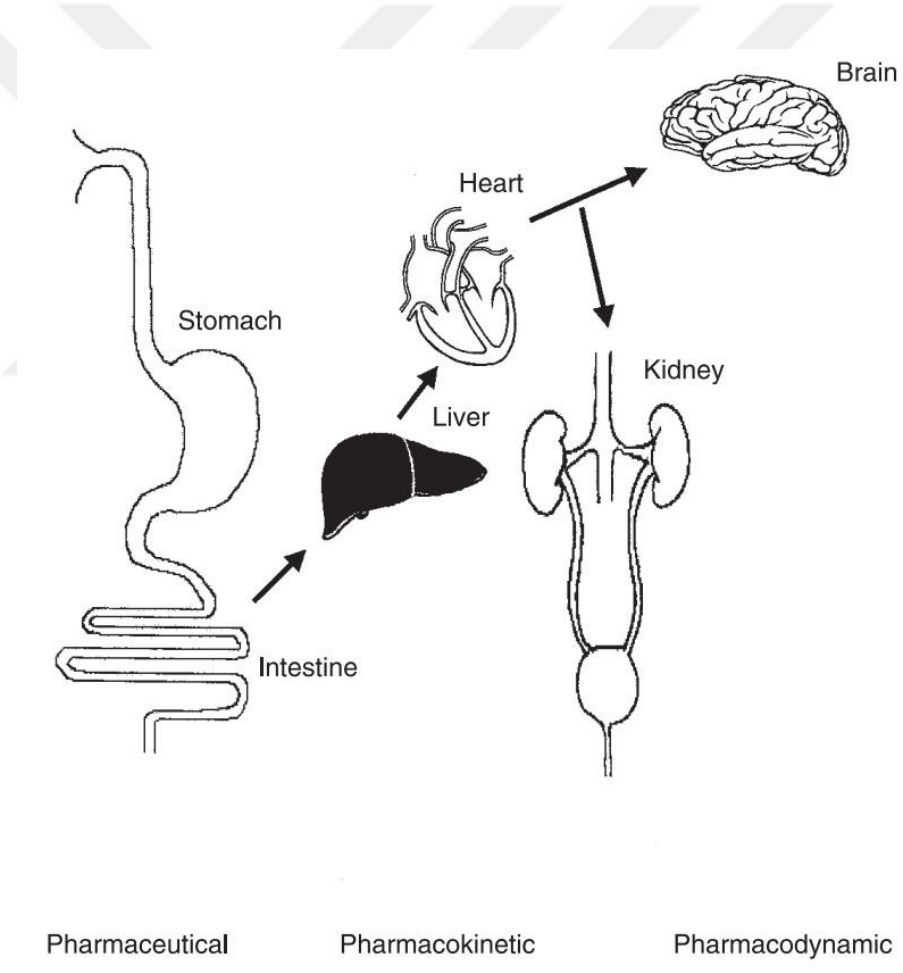


Figure 2.22. Three steps take place when drugs pass through the body which initiates when reached into the body until excrete via a particular organism [152]

One of the significant sciences that have vital roles in the synthesis and development of many anticancer therapies is chemistry. Detection of new drug molecules that have properties like pharmaceutical, isn't easy because should know ''druggability'' of drug or convert them to it, also

it is required to get information about reactivity and solubility drugs that have been helped in the understanding metabolism action. Due to toxicity of chemotherapies have been made many health problems for cancer patients, so many studies attempt to discover and develop other therapies that are caused to fight and kill cancer cells, while healthy cells stay safe from the toxicity of anticancer drugs [173]. Figure 2.23 clarifies phytochemicals like antioxidants and anti-inflammatory have brilliant activities in the protection of health, radical species quenching, and also in the treatment, especially those phytochemicals that have been a generation from the plants they may possess low side effects.

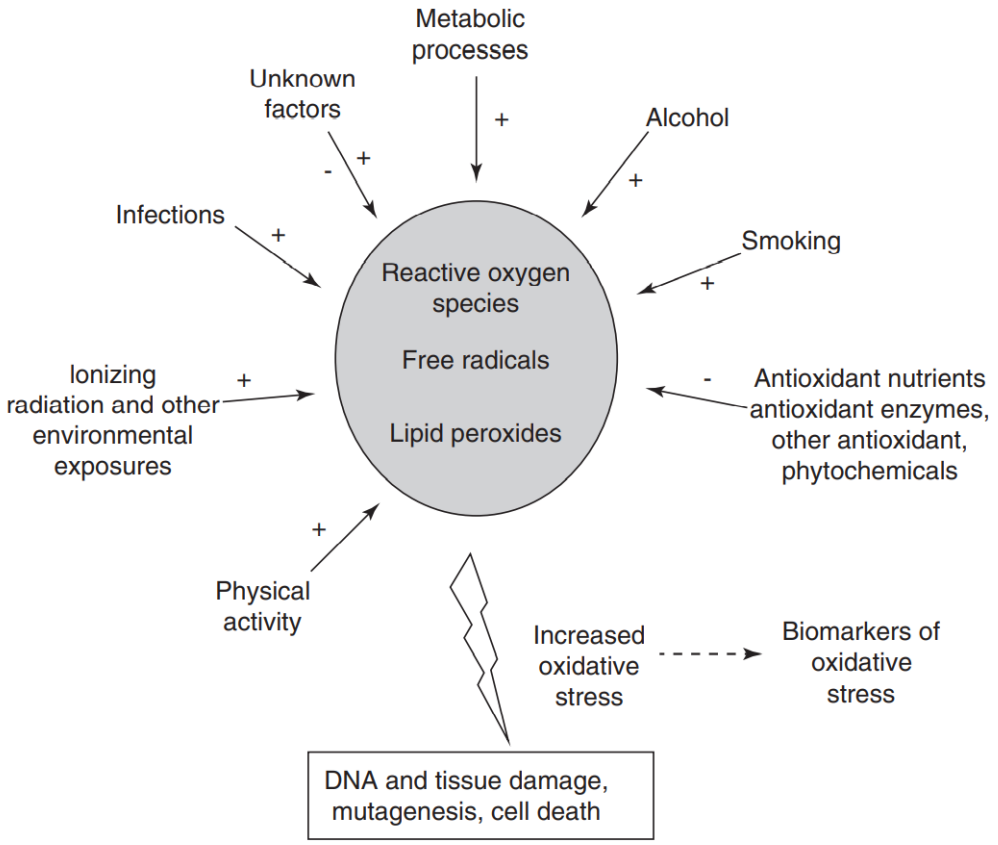


Figure 2.23. Advantages of phytochemicals for human health [174]

Antibiotics are chemical substances that are used in the treatment of many infectious disorders, they have been produced from a wide spread of microorganisms like fungi. Antibiotics have been used in the medical field in a high range due to their role in reducing those diseases caused by microorganisms and each antibiotic may display diverse properties in their action. Penicillin is an antibiotic that has an inhibition role in the synthesis of the bacterial cell wall [151]. Antibiotic is a substance that is often formed or derived from several organisms like fungi and bacteria, antibiotics can decay or inhibit microorganisms from their normal growth. The increasing

amount of antibiotic dosage when pathogen gets resistance for the drug then the antibiotic may reduce the growth of pathogen as well as the cause of some defects in the host [131, 175].

In recent studies, researchers are more propensity to synthesis new organic compounds, while if compared with the past can see natural sources widespread in the drug fields. And many compounds that have been discovered evaluated for their biological activities by testing in various ways for exhibition pharmacological character. To discover and choose a substance that has behaves like medicine, requires more tests to evaluate their properties like effectiveness, safe during usage, and others, while approximately for confirming a substance that can be suitable for medicine at least hundreds of compounds have been tested. Chemical materials are basic drugs and they have character against several disorders in humans and others because of their ability to interact with the biological system. The concentration of a drug in the body determines its activity, also the main reasons that are linked with the drug concentration are the pharmacokinetic and the pharmacodynamics phases are demonstrated in Figure 2.24.

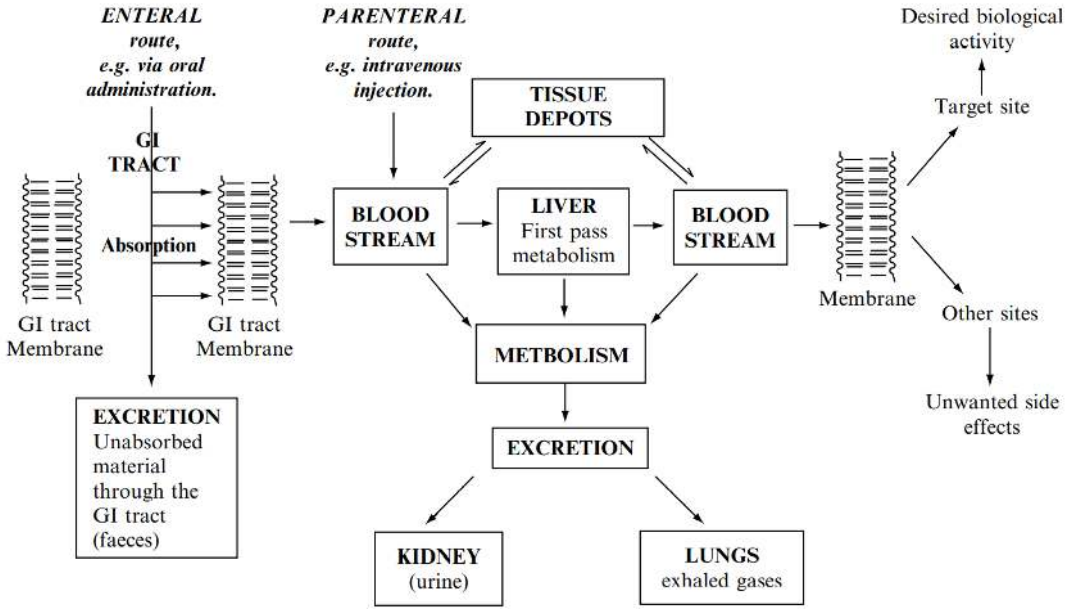


Figure 2.24. The schematic process of absorption, dispersal, metabolism, and transfer of drugs to the target location [172]

2.7.1. Azole drugs

Azoles are five-member synthetic compounds and have been a great role in the treatment of mycoses of human patients with fungal, so it is known as an antifungal. There are two main groups of azoles via the number of nitrogen atoms are triazole and imidazole, respectively they have three and two nitrogen atoms in the structure and the ability of triazole higher than imidazole against fungal infections (both structures displayed in Figure 2.25). The triazoles consist of fluconazole,

isavuconazole, itraconazole, posaconazole, and voriconazole, while the imidazoles include ketoconazole, miconazole, and clotrimazole and other azoles currently under the development. Also, antifungal of azole drugs have a brilliant activity in the inhibition of the synthesis of ergosterol from lanosterol, because ergosterol is essential for fungal cell membrane function, Figure 2.26 displayed the inhibition mechanism [176, 177].

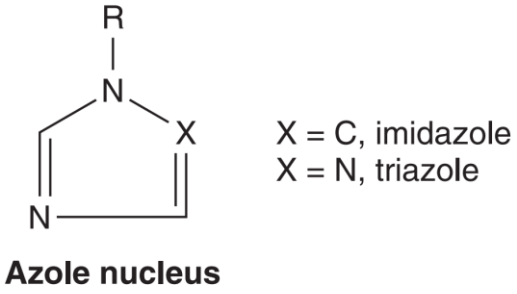


Figure 2.25. The major azole forms include: triazole and imidazole [177]

In 1985, Richardson with coworkers described a novel azoles compound that has many biological manners like therapeutic conditions, fewer side effects, and others, while it is used as an agent against fungal and it is defined as fluconazole. And its chemical formula is 2-(2,4-difluorophenyl)-1,3-bis(1H-1,2,4-triazol-1-yl)-propan-2-ol and $C_{13}H_{12}F_2N_6O$ and it is polar and hydrophilic. Fluconazole can combat with or prevent several species of the Candida, including Candida albicans and so forth, but not against all fungi. Generally, fluconazole is taken by the mouth and transfer inside the body intravenously, also gastric pH doesn't reduce its action and is finally excreted from the body by the renal if it remains a higher amount [178].

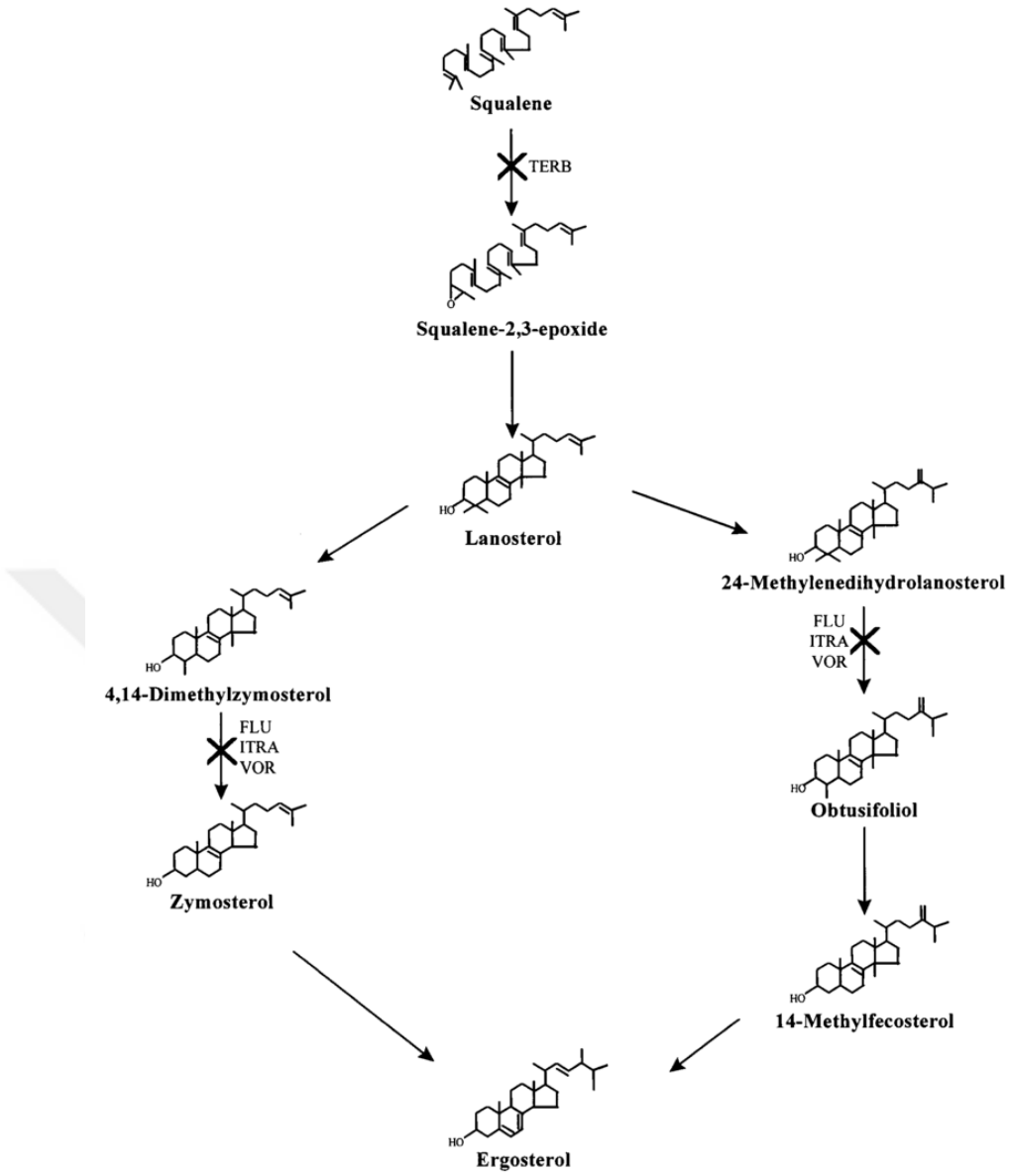


Figure 2.26. The potency of antifungal agents to prohibit formation ergosterol during its biosynthetic pathway, the agents including TERB, terbinafine; FLU, fluconazole; ITRA, itraconazole; VOR, voriconazole [179]

Itraconazole is defined as a group of triazole drugs that are used in the treatment of some type of fungal infections that cause some defection on the human health like onychomycosis, so it has antifungal capacity. Gastric pH and food have been affected in the drug absorption, also if it is taken with rifamycins the bioavailability level of the drug decrease. Without done alternation in the composition of the itraconazole dose is removed from the body by urine with the extremely low quantity [180].

Generally, azole drugs have a good interaction inside the body with different organisms like the liver and others during mechanisms due to several actions like interference with transport proteins, pH level, and so on. During those recent years number of humans, that patient via fungal

infections going to be increased. In the past time, just amphotericin B is used as a useful antifungal but it also has to behave toxically until near decades azoles replaced as brilliant and low toxicity drug. So, both of them have been used and had a role against mycoses [181].

3. MATERIAL AND METHOD

To clarify or make clear how this study is done, whole the process is explained in this chapter. The analytical techniques, instruments, procedure of assays, and with many actual pictures are provided and proved in this thesis.

3.1. Instrumental analysis

In this study, numerous instruments that used for evaluation biological activity (antimicrobial activity, AOA, determine vitamin A, C, E, MDA, anticancer activity) of the triazole Mannich base. The major instruments that operated and applied in this study, present in considered forms in (Figure 3.1, Figure 3.2, Figure 3.3, Figure 3.4, Figure 3.5, Figure 3.6, Figure 3.7, Figure 3.8, Figure 3.9, Figure 3.10, Figure 3.11).

Figure 3.1. A pH meter instrument that used for measurement concentration hydrogen-ion in a particular solution to achieve its pH value (during AOA used)

Figure 3.2. HPLC instrument that used for investigation vitamin A, C, E respectively at 326nm, 254nm, 296nm

Figure 3.3. UV-visible spectrophotometry instrument that used for evaluation AOA at various absorbance

Figure 3.4. DEN-1, McFarland Densitometers instrument that used for the measurement concentration of both bacterial and yeast cells (during the antimicrobial activity used)

Figure 3.5. Centrifuge instrument that used for separating particles from a solution by applying centrifugal force

Figure 3.6. A sensitive balance instrument that used to weigh numerous compounds with extremely accuracy

Figure 3.7. NMR instrument that used to identify the chemical structure of the compound

Figure 3.8. The laboratory hood that used during work on the evaluation anticancer to maintain from external contaminants

Figure 3.9. Elisa device that used for detect capacity of our compound against cancer cell lines

Figure 3.10. CO_2 incubator used to keep atmosphere of the medium and culture of the cells

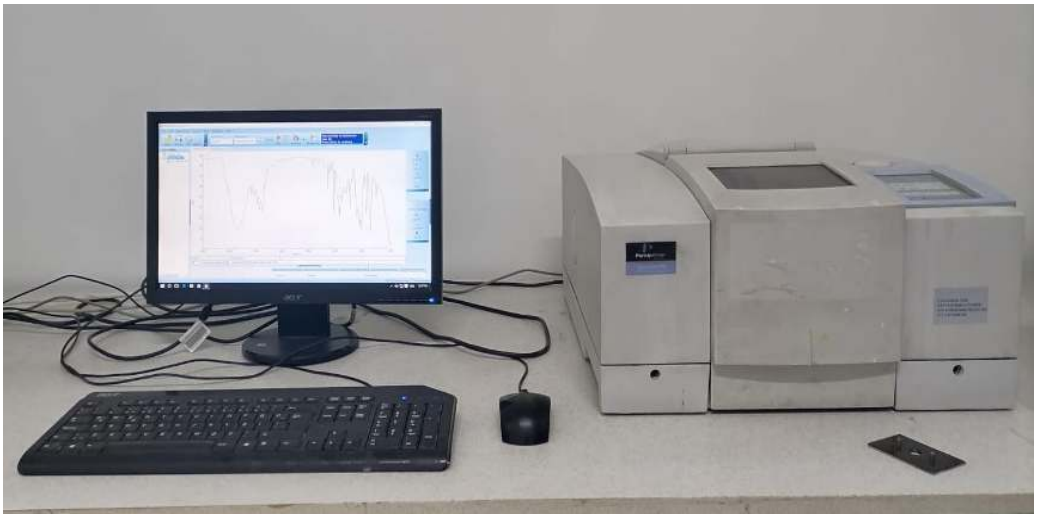


Figure 3.11. FT-IR Spectrometer instrument that used in this study

3.1.1. High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is defined as a type of column chromatography that separates the substances in a specific mixture under high pressure, also it is supplied a lot of knowledge about the analytes like identification of them [182]. HPLC instrument has been a wide range application in numerous fields, also identify more than one substance in a short time and extremely efficient separations, that is displayed its significance in the science and industry. HPLC also consists of the liquid mobile phase and solid or liquid stationary phase, both phases their solvent are immiscible when the mobile phase passed through the stationary phase that is generally had inside the column [183, 184]. High-performance liquid chromatography (HPLC) device that is performed in several analytical methods and it is composed of several major parts that are including solvent reservoirs, pump, injector, column, detector, and data acquisition and control system, Figure 3.12 displayed all parts of the instrument. The column is considered to be of an extreme degree of interest among other parts of HPLC that are created from steel tubes and their longs are 5–25 cm. In the synthesis of new chemical compounds that have certain pharmaceutical character, HPLC is used and carried out as a specialized instrument in the whole of drug stages [185-187].

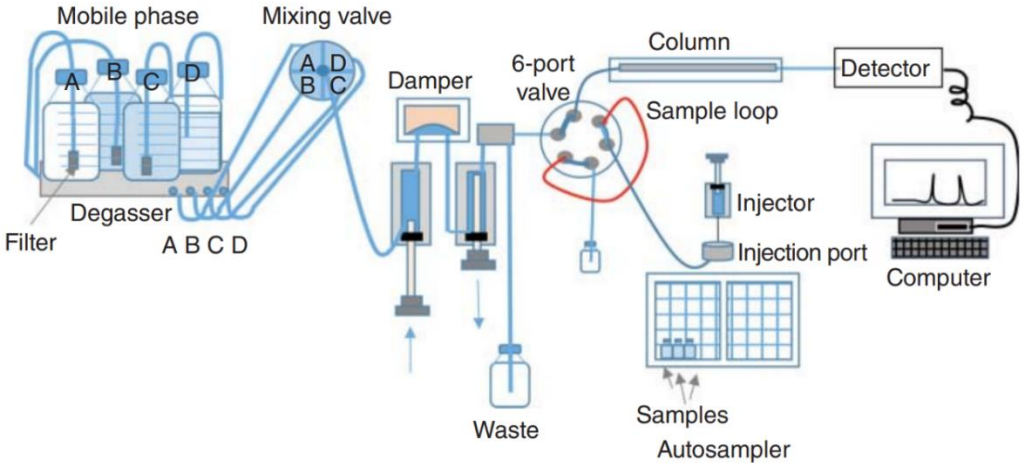


Figure 3.12. Principal of HPLC instrument [183]

3.1.2. UV-Visible Spectrophotometry

The first scientist made known that sunlight after going through a prism (defined as a clear and triangular device) which can diverse the white light into constituent colors was identified by Isaac Newton. Approximately, thirteen decades later by Herschel and co-workers ascertained two forms of light that are impossible to see by eyes which are called infrared and ultraviolet light (as explained whole ranges in Figure 3.13). Then across one-half century was estimated that the wavelength of a piece in the component is unique at absorption and emission light which was explained by Kirchoff. Furthermore, the rate of light from a medium into another is varied rely on the density of the area, the bending that took place during this period can be measured by a refractive index which is indirectly proportional to the light speed travels [188].

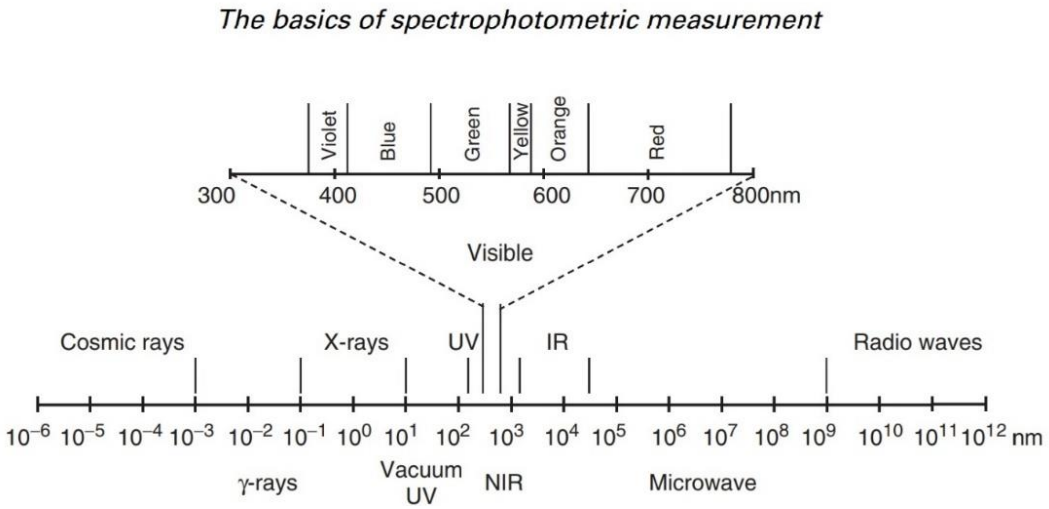


Figure 3.13. The electromagnetic range [189]

Also, the interaction among substances (sample) with light (radiation), and investigation of various request components can be done due to mighty and influential instrumental called spectroscopy method. For evaluation, several drug compounds can be obtained benefit from Ultraviolet-Visible spectrophotometry (Figure 3.14 is shown a composition diagram of it), the method of working by absorption of one of the radiations by test solution. The advantages that can cause motivation to apply this method are accuracy, done though the sample quantity is smidgen or careful with the number of substances, and inexpensive technique. The perfect meaning for spectroscopy that obtained this name from Latin in both spectron and skopien words respectively, express as spirit and looking into the world [190-192].

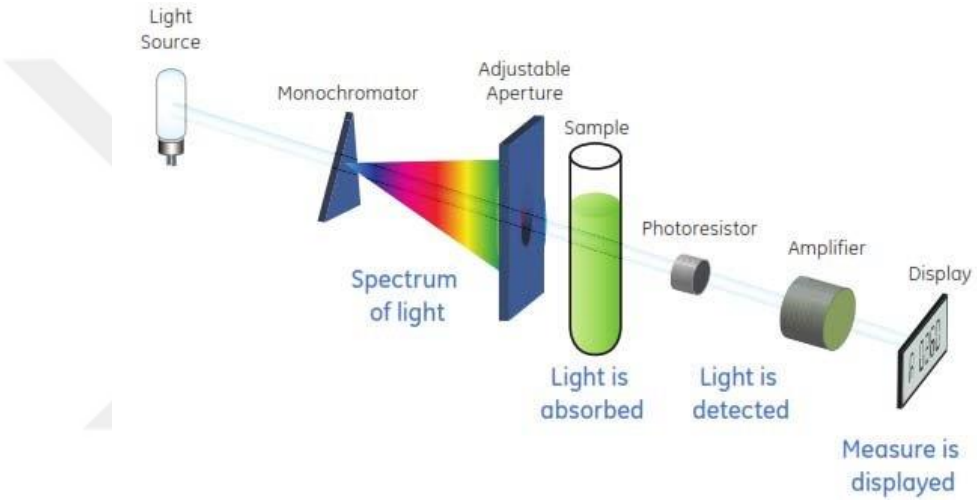


Figure 3.14. Schematic diagram of UV-visible spectrophotometry [193]

3.2. Sample collection

The 1,2,4-triazole Mannich base synthesized compound that used in this study is called 2,6-Bis(4-phenyl-5-thioxo-4,5-dihydro-1,2,4-triazol-3-yl)-pyridine. It was acquired from (Prof. Dr. Metin KOPARIR), in the Chemistry department at Firat University. For determination chemical structure of the compound in this study, managed by operation FT-IR and NMR instrument on it (^{13}C-NMR (The attached proton test (ATP)) and ^{1}H-NMR), as can be seen in Figure 3.15, Figure 3.16, and Figure 3.17 respectively. And in Figure 3.18 shows the chemical structure of the compound.

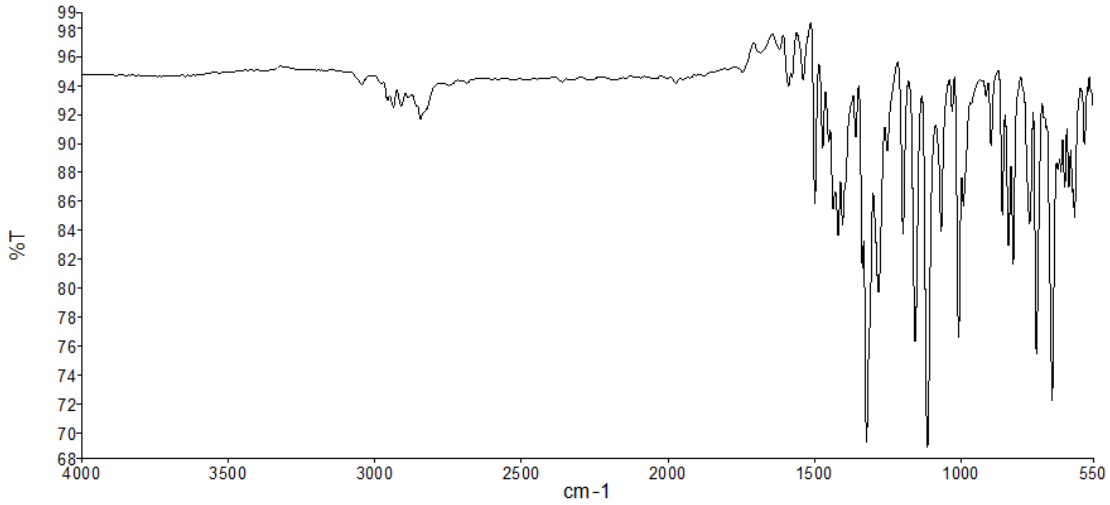


Figure 3.15. Our compound FT-IR spectrum

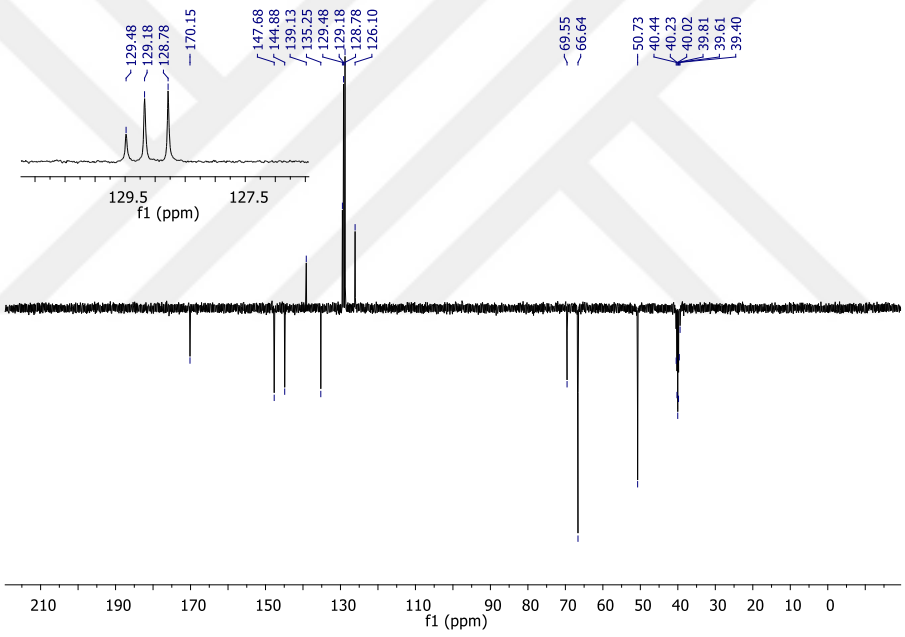


Figure 3.16. The ^{13}C-NMR spectrum of the triazole Mannich base

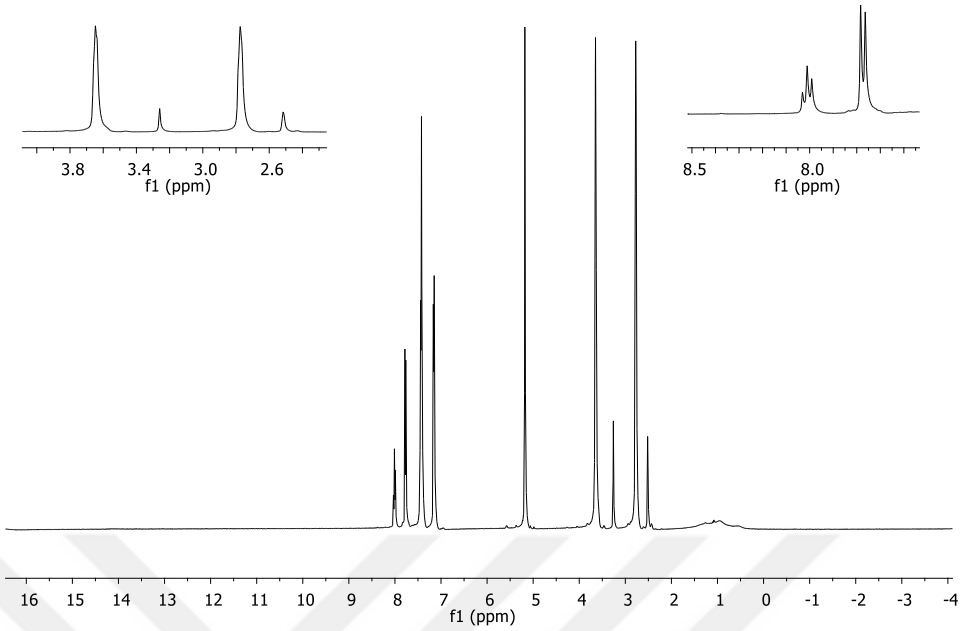
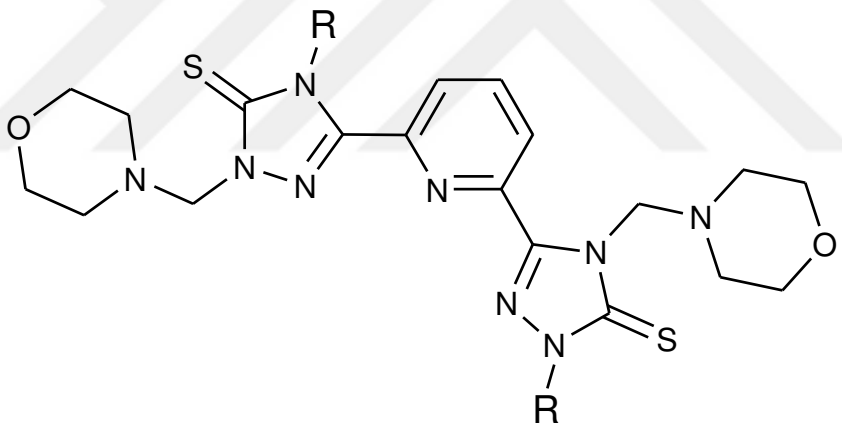


Figure 3.17. The ^{1}H-NMR spectrum of the triazole Mannich base



R:Phenyl

Figure 3.18. The structure of the synthesized triazole Mannich base that used in this study

3.2.1. Sample preparation

In 10 ml of the volumetric flask was dissolved 0.03135g of the triazole compound (its molecular weight is 627g/mol) by dimethyl sulfoxide (DMSO) with a concentration of 5000µmol, according to the equation for determination of weight:

$$wt(g) = \frac{5000\mu mol}{l}\frac{1l}{1000ml} \times 10ml \times \frac{1mol}{10^{6}\mu mol}\frac{627g}{1mol} = 0.03135g\ /\ 10ml \qquad (2)$$

3.2.2. Materials

Lots of chemicals and reagents used in the total procedure of methods, that describe them in the below based on especially to each assay as follows:

- Sample preparation; Dimethyl Sulfoxide
- Antimicrobial activity; Müeller Hinton Agar and Sabouraud dextrose agar applied for bacterial and fungal respectively,
- DPPH radical activity; DPPH, Methanol
- $ABTS^{\bullet+}$ activity; ABTS, $K_2S_2O_8$, Ethanol
- HO radical activity; Deoxyribose, Phosphate buffer (NaH_2PO_4), Ferric chloride, H_2O_2, Ethylenediaminetetraacetic acid, Ascorbic acid, Trichloroacetic acid, Thiobarbituric acid
- Vitamin C; Saccharomyces cerevisiae, $HClO_4$, Distilled Water, mobile phase
- Vitamin E; Saccharomyces cerevisiae, Solution of (H_2SO_4, Ethanol), Distilled Water, n-hexane, Nitrogen gas, (methanol, acetonitrile, chloroform)
- Antitumor activity; MCF-7 and Hep-G2 cell lines, MTT reagent, DMSO

3.3. Methods

Each method or assay has a particular behavior that makes it different from another one, hence it is important to have previous knowledge about the usage of chemicals, devices, and also requirement apparatus for the assays.

3.3.1. Antimicrobial methods and procedures

For evaluation antimicrobial activity of a synthesized compound that used in this study apply based on the agar disk diffusion method. Also, at Microbiology Laboratory of the Biological department came by microorganisms (bacterial and fungal), then carried out for both assays. Four strains of bacterial applied in this study (*Bacillus megaterium*, *Escherichia coli*, *Staphylococcus aureus, Klebsiella pneumoniae*) for antibacterial activity of the synthesized compound, they were grown in Nutrient Broth (NB, Difco) and incubated at 35 ± 1°C for 24 hours that achieved them at the Biological department (Microbiology laboratory) of Firat University.

Then, via McFarland densitometers instrument measured concentration of bacterial at a rate of 1% (10^6 bacteria/ml) and inoculated inside Müeller Hinton Agar (three-times). Shaking them well, later prepared four sterile Petri dishes whose diameter is composed of 9 cm and poured 25 ml of the microorganisms into ones, and is dispersed from the medium homogeneously. Also, 100 µl of the compound that combined (impregnated) with several 6 mm diameter discs, and each of them was placed into a particular bacterial Petri dish. Afterward, they were retained for approximately 1.5 h at 4°C (present in Figure 3.19). At the end of the process at 37 ± 1°C for 24 hours the

inoculated Petri cell-culture dishes were incubated. Streptomycin sulfate 10 µg / disc as a control compound was used for bacterial strains and negative control in this study employed dimethyl sulfoxide (DMSO). The inhibition area happened on the bacterial Petri plates around the disc, due to the activity of the compound and for measurement their activity was evaluated in millimeter (mm).

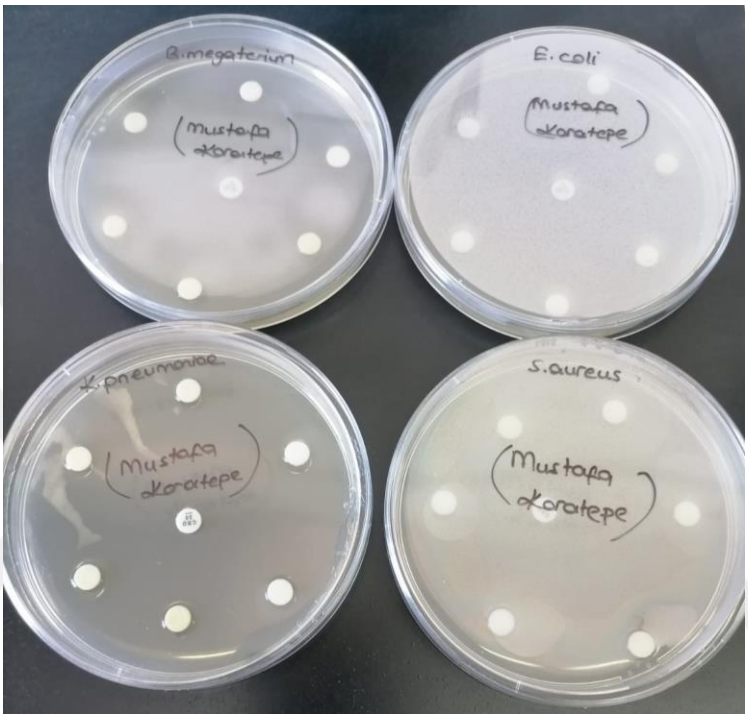


Figure 3.19. Bacterial strains of synthesized triazole

Another microorganism is yeast, for a synthesized compound evaluated antifungal activity against Candida albicans, so yeast strain that was grown (inoculated) in Malt Extract Broth (Difco), during 48 hours at 25 ± 1°C incubated, and which was done by the Biological department (Microbiology laboratory). After that, the Sabouraud dextrose agar was made ready for inoculating the culture yeast from broth into agar with a rate of 1% (10^4 yeast/ml) and shaking it well. After shaking, poured 25 ml from the prepared agar yeast into a sterile petri dish that spread it homogeneously inside the cell-culture dish that diameter is 9 cm. The disc which is 6 mm of its diameter, was submerged in the 100 µl synthesized compound, then added to the yeast media in the Petri plate, and stored at 4°C nearly for 2 hours (as an exhibit in Figure 3.20). Then, for 72 hours at 25 ± 1°C, the yeast was kept in the inoculated petri dish. Nystatin 30 µg/disc as a control disc was used for yeast strain and compared with the synthesized compound and for negative control in this study used DMSO. The inhibition area happened on the yeast Petri plates around the disc, due to the activity of the compound and for measurement its activity was evaluated in mm [194].

Figure 3.20. The fungal strain of synthesized triazole

3.3.2. Antiradical assays and procedures

Antioxidant activity has a vital role in the evaluation of the biological activity of various new synthesized compounds, by known antioxidant activity lead to getting information about the function and pharmaceutical properties of the compounds. The aforementioned heterocyclic compounds like triazoles have antioxidant activity in some fields to decrease radicals, however, 1,2,4-triazole Mannich bases have significant biological properties so many studies try to synthesis new derivatives of triazoles [136].

In the evaluation methodology of DPPH, 100 µl, 250 µl, 500 µl of synthesized compound (5000 µM) was added to another prepared 4.0 ml methanolic solution of DPPH$^{\bullet}$ (25 mg/l). The mixture is made ready, afterward shaken the mixture well, at room temperature stored in the darkness for 30 min. During the stored time the mixture color changed from deep purple to yellow color, as can be seen in Figure 3.21. Then, at 517 nm measured the absorbance of the mixture was via a spectrophotometer, to determine its activity, also used methanol as a blank [116].

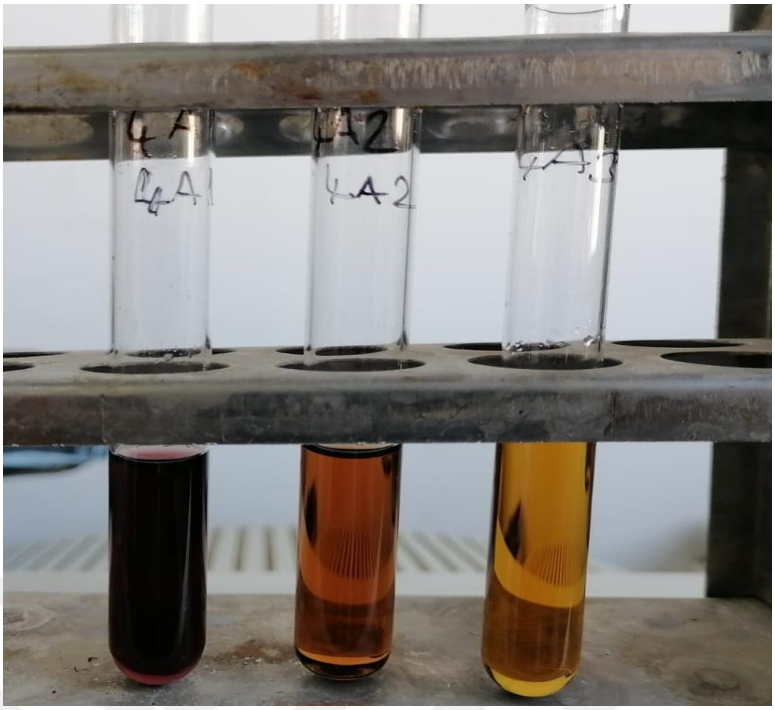


Figure 3.21. DPPH radical after reacted with triazole compound as an antioxidant agent. Tubes from left to right added 100 µl, 250 µl, 500 µl of compound

This assay was carried out according to scavenge of radical cations of ABTS ($ABTS^{\bullet+}$) antioxidant which can donate an electron. However, $K_2S_2O_8$ works as an oxidizing agent that was 2.45 mM from $K_2S_2O_8$ reacted with 2 mM ABTS, the role of action to produce $ABTS^{\bullet+}$, also the mixture was retained in a dark place for 16 h, at room temperature. After passing the required time, the color of the solution changed to blue-green, the solution of $ABTS^{\bullet+}$ was diluted in ethanol (the solution color is shown in Figure 3.22) until an absorbance of 1.520 ± 0.025 was obtained at 734 nm. The 100 µl, 250 µl, 500 µl synthesized compound had prepared (5000 µM), poured 3 ml from the last solution and blended (the color became colorless as shown in Figure 3.23). And the formed solution was again maintained while this time for 30 min. Then, was assessed the value of absorbance at 734 nm, before sample tests reading by the device, phosphate buffer poured in a cuvette as a blank for assay [116].

Figure 3.22. Solution of both ABTS and $K_2S_2O_8$, after stored for a request time, also it diluted with ethanol

Figure 3.23. The solution color changed to colorless after adding triazole compound on them with different amounts from left to right respectively, 100 µl, 250 µl, 500 µl

To evaluate hydroxyl radical scavenging activity, mixed 500 µl of 3.6 mM deoxyribose, 20 mM phosphate buffer ($NaH_2PO_4.2H_2O$-NaOH) which pH equal to 7.4, 200 µl of 100 mM ferric chloride ($FeCl_3$), 1.0 mM H_2O_2 (100 µl), 104 mM Ethylenediaminetetraacetic acid (EDTA) (200

µl), also (5000 µM) the solution of synthesized compound (100 µl, 250 µl, 500 µl), and the last one before vortexed the mixture poured 100 µl of aqueous ascorbic acid to the pre-mixed reaction mixture (1.0 mM). Then, the sample solution was mixed vigorously, and incubated for 1 h at 37 °C. After passing through a request time the sample was mixed with 1 ml of trichloroacetic acid (2.8% w/v) and 1 ml of thiobarbituric acid (1.0% w/v), that vortexed and heated at 50 °C for 30 min in a water bath, and then cooled the sample solution at room temperature for recording the absorbance at 532 nm, the procedure explained in Figure 3.24 with different values and modifications, for this assay used different blank that called is n.hexane [195].

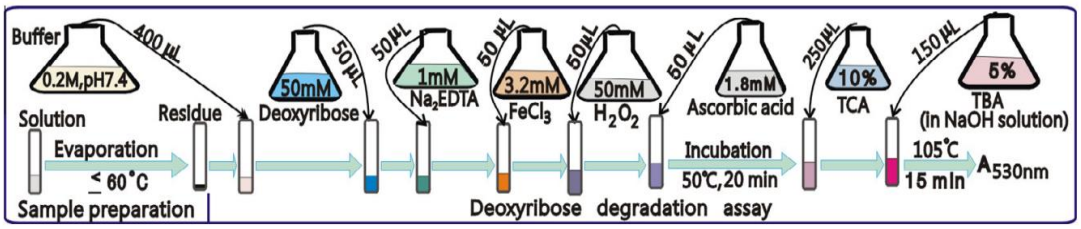


Figure 3.24. The procedure of hydroxyl radical scavenging activity [139]

3.3.3. Investigation of *Saccharomyces cerevisiae*

This method used a type of yeast called Saccharomyces cerevisiae cells, before starting the tests the yeast was prepared in Microbiology Laboratory, and the ratio of the yeast was 10^6 cells/ml after incubated for 2 days at 25 °C in Malt Extract Broth for inoculation. In addition, the analysis process was done via HPLC (Shimadzu, Japan model) with a column (A Shimadzu Shim-pack VP-ODS column, 150 mm length and 4.6 mm), however, Table 3.1 demonstrates the basic information about the process.

Table 3.1. General information about the operation of the device

Subject	**Wavelength** (nm)	**Injection volume** (µl)	**Column temperature** (°C)	**Detector (monitored)**	**Flow rate** (mL/min)
Vitamin A	326 nm	50 µl	30°C	UV absorbance	1.0
Vitamin E	296 nm	50 µl	30°C	UV absorbance	1.0
Vitamin C	245 nm	20 µl	30°C	UV absorbance	1.0
MDA	254nm	20 µl	30°C	UV absorbance	1.0

Vitamin C was determined in the yeast cell by the HPLC and the same method had done for MDA analysis at 245 nm and 254 nm respectively. The first step, added 2ml of the yeast to the tubes, afterward 50 µl and 100 µl of the agent (the 1,2,4-triazole compound (5000 µM)) inserted to the tubes respectively and DMSO as a control (100 µl), then vortexed the tubes at least 10 times

and was stored in a freezing point. Second step, the tubes at room temperature via a syringe were agitated and vortexed, poured 2 ml of perchloric acid (0.5 M) and 0.5 ml from distilled water to the tubes and vortexed. In the third step, for10 min at a speed of 6000 rpm centrifuged, after that precipitation was produced due to the lysis of the cell. In the fourth step, 20 µl of the liquid layer over the residue obtained after it had centrifuged was taken each of them according to the request rate for analysis by HPLC. In addition, Weighed 0.05 g and 0.38 g of mono-potassium phosphate (KH_2PO_4) and heptane sulfonic acid respectively, after that dissolved by methanol (125 ml) and added distilled water (375 ml) in a volumetric flask with 350 µl of triethylamine ($C_6H_{15}N$) and the pH equal to 3.6 after arranged via H_3PO_4 (phosphoric acid) as mobile phase.

Both lipid-soluble vitamins A and E in the yeast cell were analyzed by HPLC at 326 nm and 296 nm respectively, however, both vitamin groups passed from the same process. In the first stage, poured 2 ml of the yeast into two tubes after that added 50 µl and 100 µl of the compound (5000 µM) that dissolved in DMSO, and vortexed more than 10 times then froze it and used DMSO (100 µl) as a control. In the later stage, added 2 ml of ethanol that contained sulfuric acid (H_2SO_4) at 1% and 1 ml of distilled water then vortexed by each addition step before that agitated by a syringe 4 times. In the third stage, inserted n-hexane (300 µl) and shook then centrifuged each tube for 4 min at 6000 rpm, afterward the upper layer formed in the solution composed of n-hexane and lipophilic vitamins and moved this layer to other tubes, and this stage was repeated once more. Via nitrogen gas, the n-hexane became vapor and stayed the desires in the tube, and it was dissolved by 100 µl of the mobile phase and last stage, injected 50µl of the solution for analysis by HPLC, about the mobile phase, consisted of methanol/acetonitrile/chloroform with various ranges 47:42:11, v/v.

3.3.4. Anticancer and cytotoxicity assay

Both Michigan Cancer Foundation-7 (MCF-7) are used for breast cancer and Liver Hepatocellular Carcinoma (Hep-G2 (as shown in Figure 3.25)) was achieved by Biotechnology Institute at Ankara University, also they are human cell lines.

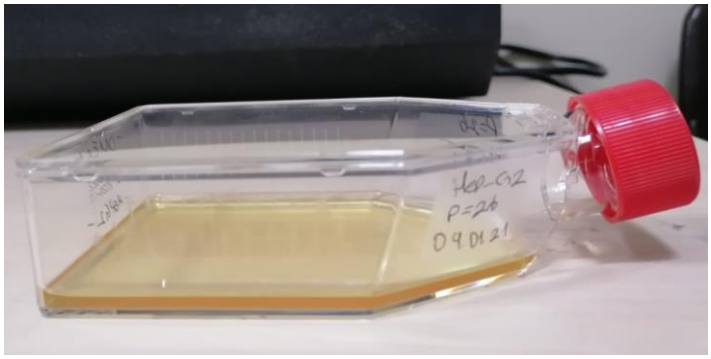


Figure 3.25. The Hep-G2 cell line that used in this assay

The cell lines developed in DMEM are an abbreviation for Dulbecco's Modified Eagle Medium (1% of both L-Glutamine and Penicillin-Streptomycin, 10% FBS (Fetal Bovine Serum)) under 5% CO_2 atmosphere in 25cm² flasks at 37˚C. The flask surface was covered when the cell lines were cultured in it, subsequently, that washed the flask by sterile Phosphate buffered saline (PBS) solution (5 ml) to take away the medium in it, incubated the flask for 2 min at 37˚C in an oven with 5% CO_2 when added 1 ml of Trypsin-EDTA on it. Afterward, differentiated the cells from the flask, the reagent became inert with 5 ml of medium and for 5 min with a speed 1200 rpm was centrifuged, then isolated the supernatant from the cells. In the first row added the medium as a blank which was free from the cells at 37˚C with 5% CO_2 atmospheric for 24 hours incubated. Whole the process of this assay was done at the Medical School of Firat University. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method that was used for investigation cytotoxicity or cell proliferation (anticancer) characters of the compound.

Both cells lines (2×10^4 cells) separately were placed and cultured in 96-well plates for 24 h 5% CO_2. Afterward incubated, 100µl of the sample compounds had added 12 times on the 96-well plates with different concentrations (5000µM, 2500µM, 1250µM, 650µM), at 37˚C with 5% CO_2 atmospheric for 3 days was incubated. After that, eliminated the media from the cell, and added the stock MTT which prepared in neutral pH solution (sterile phosphate buffer) is equal to 7.2 then poured 20µl of MTT (5mg/ml) solution and in a dark place (5% CO_2) for 4 hours at 37˚C was incubated. Formazan crystals that were produced after incubation, then with 100µl of DMSO dissolved and remained for 10 min in a dark area. For determination potent of control and compare with of the compound activity used Doxorubicin (2.5 ng/ml) as a standard in the third row, while negative control was the medium in the second row, and the assay based on MTT method. The results were recorded and obtained via enzyme-linked immunosorbent assay (ELISA) plate reader instrument at 450 nm, then calculated the percent values of the compound at different concentrations after comparing both absorbance (*A*) of sample and control, as can be seen in the below equation:

$$Percentage\ (\%)\ of\ the\ compound\ = \frac{A_{sample}}{A_{Control(-)}} \times 100 \qquad (3)$$

4. RESULTS AND DISCUSSION

According to those results observed in this study, several positive information or outcome were obtained about the skill and power of triazoles particularly the synthesized compound that was used. The below sections explain the activity of the agent for reducing or stopping ability those factors that used because they are responsible for many disorders or damage and make a defect in health.

4.1. Antimicrobial activity

Antimicrobial results that were obtained of five different organisms based on the disk diffusion method, observed various inhibition zone on the Petri dishes while two bacteria have similar values after measuring the values via millimeter by a ruler. Figure 4.1 and Figure 4.2 show the inhibition area of bacterial species and fungi. In the fungal (*C. albicans*) area potential of the compound that is used is highest compared to other organisms or bacterial species. However, the bacterial inhibition of *S. aureus* is more than *E. coli*, and it is more than both *B. megaterium* and *K. pneumoniae*. Some other studies confirmed that the derivatives of 1,2,4-triazole have influence potential against several microorganisms and the growth inhibition convert that is depending on the structure and concentration of the compound. As can be seen in that study [42], the compound (3a) otherwise our compound was more effective for bacterial than C. albicans, so it is more clarified that the main factors effect on the antibiotic properties of the compound is constitution structure of it and the groups that linked to triazole, and microorganism behaviors like resistance. Also, it is extremely significant to know, which factors affected higher on the compound ability against microbes. The factors alter among the species of microorganisms, hence, it was tried to predict more carefully about the results and reasons by the available information.

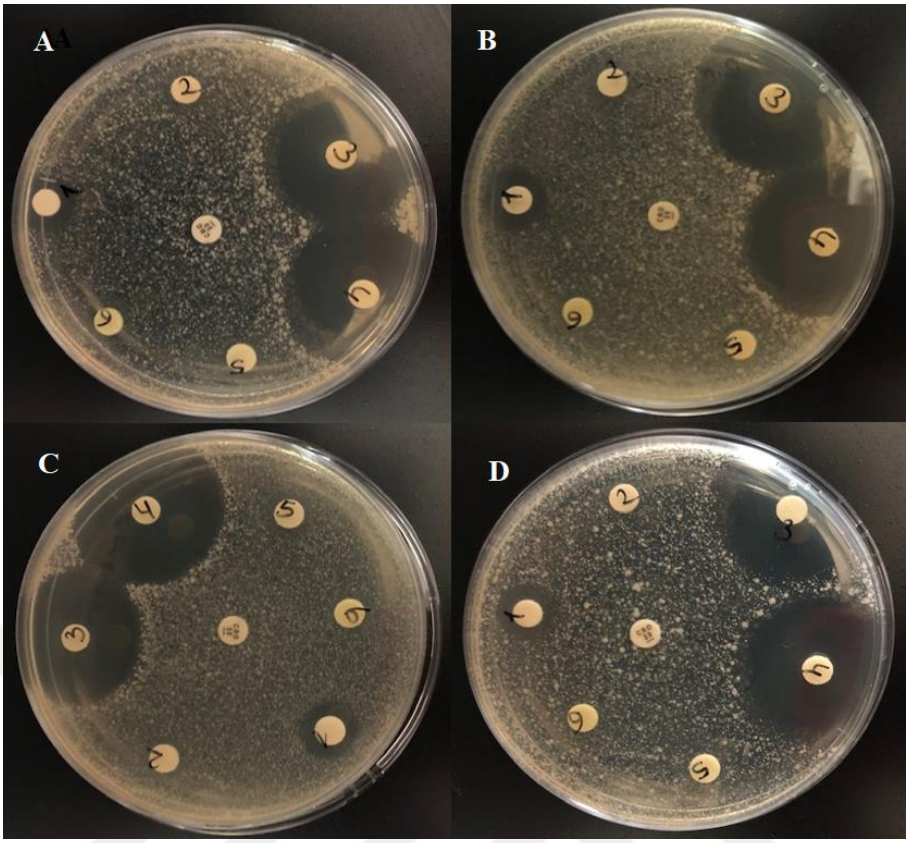


Figure 4.1. Bacterial strains after incubated for 24 h with the sample compound, number 1 on the Petri dishes is the test sample. A, *E. coli*; B, *B. megaterium*; C, *K. pneumoniae*; D, *S. aureus*

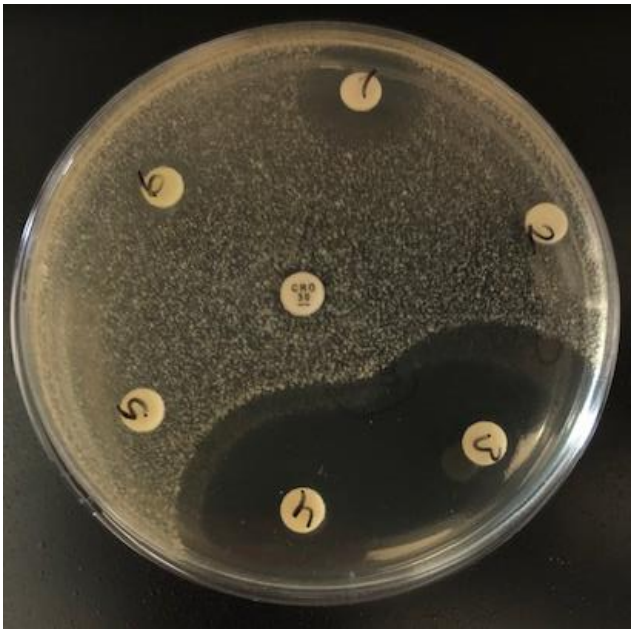


Figure 4.2. Fungal strain after incubated for 72 h with the sample compound, number 1 on the Petri dishes is the test sample, for *C. albicans*

Figure 4.3 illustrates the force of the compound and the control to prevent bacterial growth usually, different between species, one of the major reasons is the structural arrangement of the cell wall, particularly among positive and negative cell wall, phenyl group that attached to fourth position (the third nitrogen atom) on the derived 1,2,4-triazole mannich base and other groups, and complicated chemical structure of the compound. *S. aureus* (gram-positive) has the lowest resistance as a target for the agent, the reason may be via doesn't possess an outer membrane as a resistance, so it could be caused of the inhibition growth of the bacteria. Also, efflux pumps are another factor that exists in both groups of cell walls, leading to reducing the influence of the agent on it by emitting the agent from inside to outside of the bacterial. Additionally, the rate of the agent that could pass through the bacterial membrane by the potent of the agent or that path leads to transfer of the agent, which is dissimilar between bacterial species.

Though the activity of the agent as an antifungal that is used is good, but lower than the control approximately 20 percent (as demonstrate in Figure 4.3). The phenyl group and other groups like morpholine (N-methylmorpholine) that attached to the 1,2,4-triazole may be increased potent against the *C. albicans*. However, some other factors are related to the chemical constituent of the compound and the fungal feature maintained from external agents. The nitrogen atom of 1,2,4-triazole can bind with the enzyme responsible in the ergosterol biosynthesis and then delay or prevent the action of the enzyme in the reaction process and make defect in the cell cycle of the fungal, because ergosterol has significant role in the arrangement of fungal functions and the power of the cell membrane directly proportional with ergosterol value.

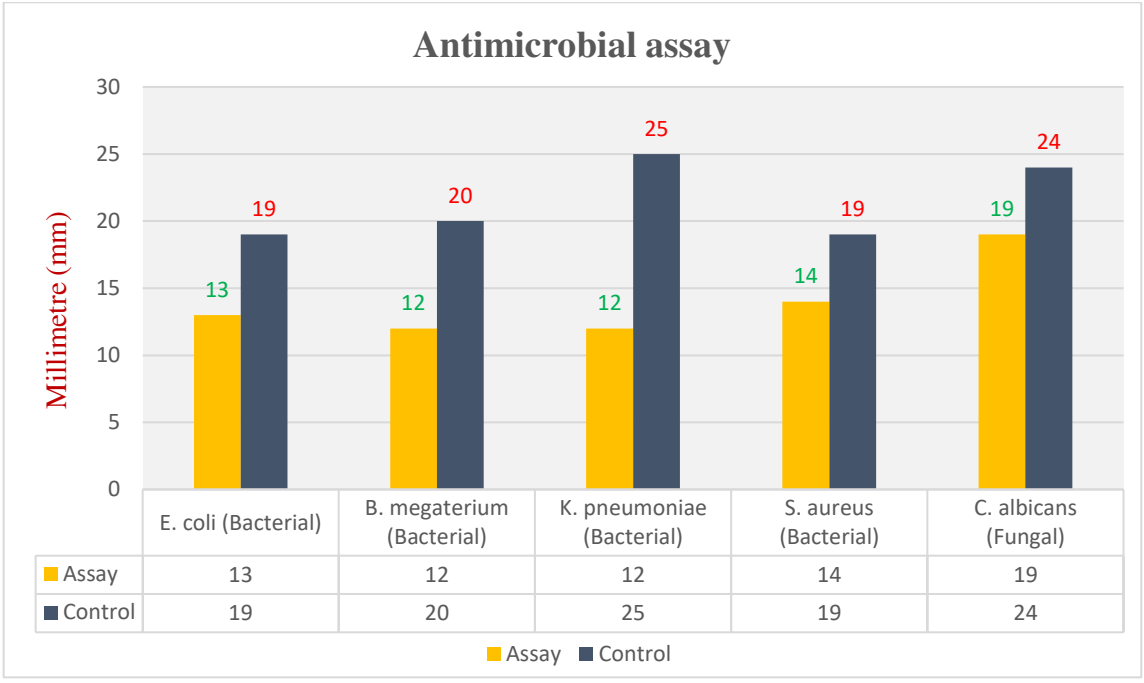


Figure 4.3. The inhibitory rate of the triazole compound against microbe culture

4.2. Antiradical activity

The results obtained from the antiradical process are displayed a great capacity for quenching radical species. The 1,2,4-triazole derivative compound increase its activity when the amount of the compound increased, originally, 100 µl of the compound was added to the solution, then increased its ratio multiple by 2.5 and 5, observed the absorbance was decreasing gradually, otherwise antiradical power increased with decreasing absorbance. Because the remaining radical species reduce with rising concentration or amount of antiradical agent in the solution, also the color intensity is depleted ($DPPH^{\bullet}$) or become colorless ($ABTS^{\bullet+}$) depending on the activity of the agent. In each range scavenging of $DPPH^{\bullet}$ is lower than both species, while $HO^{\bullet}$ quenching is higher than both others.

Absorbance and scavenging of radical species percent of all antiradical activity methods can be explained respectively, in Table 4.1 and Table 4.2, for sample compounds at different values, control, and tert-butyl hydroxyl toluene (BHT) as a standard antioxidant, its absorbance, and ratio of scavenging of radical species achieved from the advisor of this experiment. In addition, converting the values from absorbance to percent via the equation (1.

Table 4.1. Absorbance of antiradical activity

Sample Tests	Control	100 µl	250 µl	500 µl	BHT (500 µl)
Absorbance of $DPPH^{\bullet}$	3.135	2.960	0.894	0.527	0.131
Absorbance of $ABTS^{\bullet+}$	1.519	0.531	0.400	0.100	0.050
Absorbance of $HO^{\bullet}$	1.555	0.540	0.255	0.059	0.087

Table 4.2. Scavenging activity of radical species (%)

Sample Tests	100 µl	250 µl	500 µl	BHT (500 µl)
% of $DPPH^{\bullet}$ scavenging activity	5.58%	71.48%	83.19%	95.82%
% of $ABTS^{\bullet+}$ scavenging activity	65.04%	73.67%	93.42%	96.71%
% of $HO^{\bullet}$ scavenging activity	65.27%	83.60%	96.21%	94.405%

4.2.1. DPPH scavenging activity assay

This study verified that with the increasing amount of the test compound, the concentration of $DPPH^{\bullet}$ that reduced was raised by the agent that acted as antiradical scavenging, Figure 4.4 illustrates that the absorbance in this study is inversely proportional with the amount of the compounds. The color degradation of the solution increased with the amount of agent, due to the reduced higher quantity of $DPPH^{\bullet}$ in the solution, thus varying to high stable form which is not

present radical behavior. However, the structure of both the radical and the agent with solvent directly have influence on chemical reaction rate. The majority medium reaction is electron transfer because hydrogens form strong H-bonding with methanol solvent that is a lower ratio of donating hydrogen to the radical. About the first or the lowest amount of the test compound (Figure 4.5 illustrates the scavenging activity of all compounds) has an extremely low activity to quench DPPH• (5.58%) and also two other values have low potent compared to other assays, these are may be via several factors like structure and composition of the agent, the radical on the nitrogen atom of DPPH• remain for a long time and they cannot produce dimerize form, the single electron that located in the center of DPPH• so the huge antioxidant structure difficulty reaches to react with it so in the lowest amount of the agent that used approximately 5 percent of radicals reacted with it, and other factors may be responsible for agent activity. In addition, several studies like [196] approved that in the presence of 1,2,4-triazole in the composition of compounds (such as 6d) ability to remove DPPH• is high and their activity changed with alternation in both concentration and the groups in the structural composition.

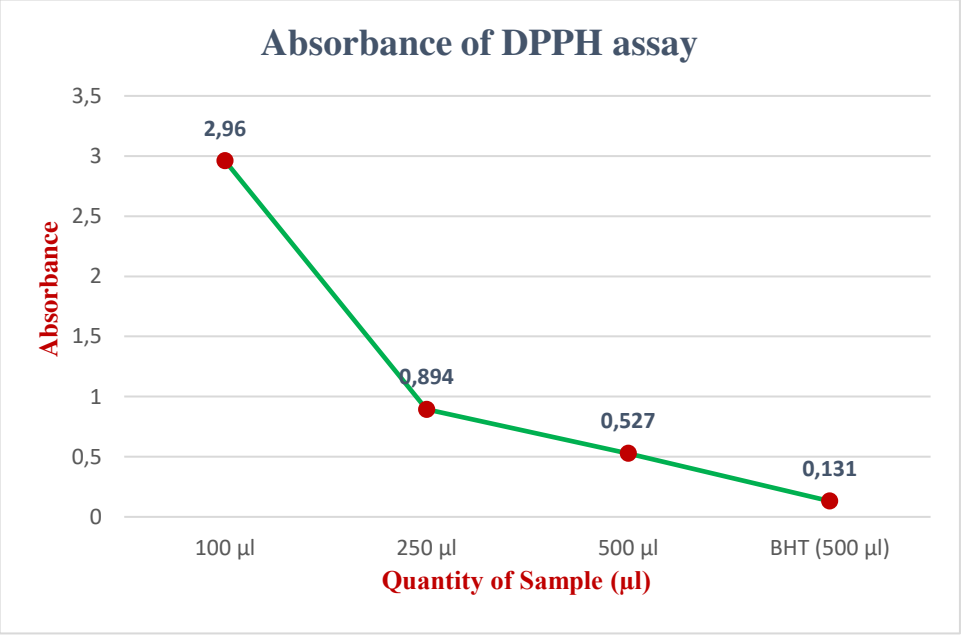


Figure 4.4. The absorbance of test samples (DPPH•)

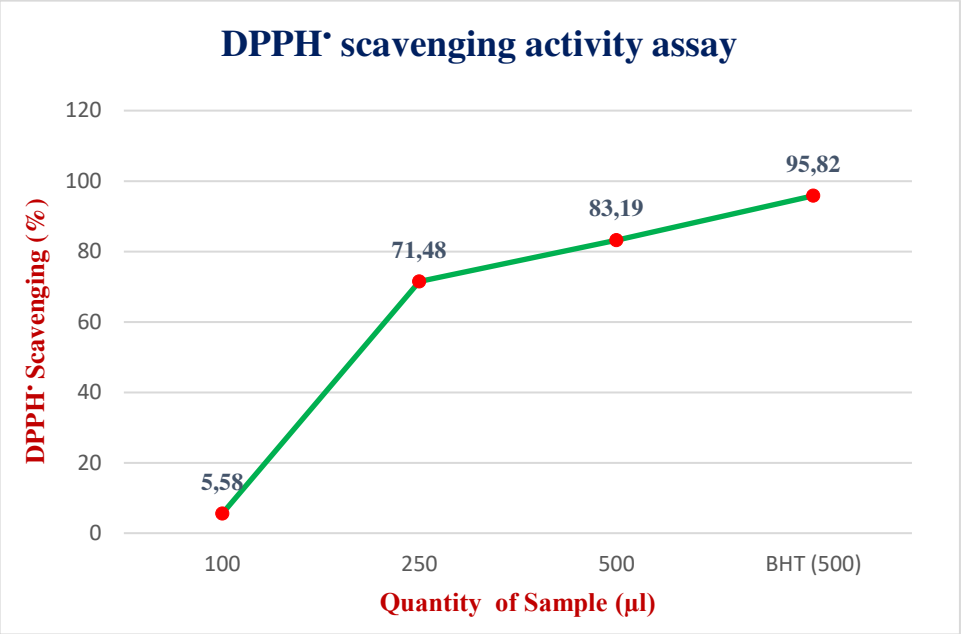


Figure 4.5. The capacity of the test compound to scavenge DPPH•, with different amount

4.2.2. ABTS radical cation decolorization assay

About the result of this method, at the different amounts, the compound has potential as an agent to remove the radical cations via reduction process, then the solutions became colorless because of the stable ABTS yield by antiradical effort. The cation of the $ABTS^{\bullet+}$ assists in the closeness of both particular antioxidants and the radicals. For oxidation ABTS to $ABTS^{\bullet+}$ the reaction media requires a powerful agent for the generation of $ABTS^{\bullet+}$. In addition, can predict by presence small amount of the agent high scavenging can be seen in Figure 4.7. Also, Figure 4.6 demonstrates absorbance decreased gradually when the value of the agent in the reaction mechanism (solution) raised, due to the concentration of the $ABTS^{\bullet+}$ in the solution was fall. Also in [197], researchers determined the capacity of 6c compound against $ABTS^{\bullet+}$ as a radical species, the phenyl group attached to the triazole compound has an excellent antiradical activity, however with modification in the group the activity was changed.

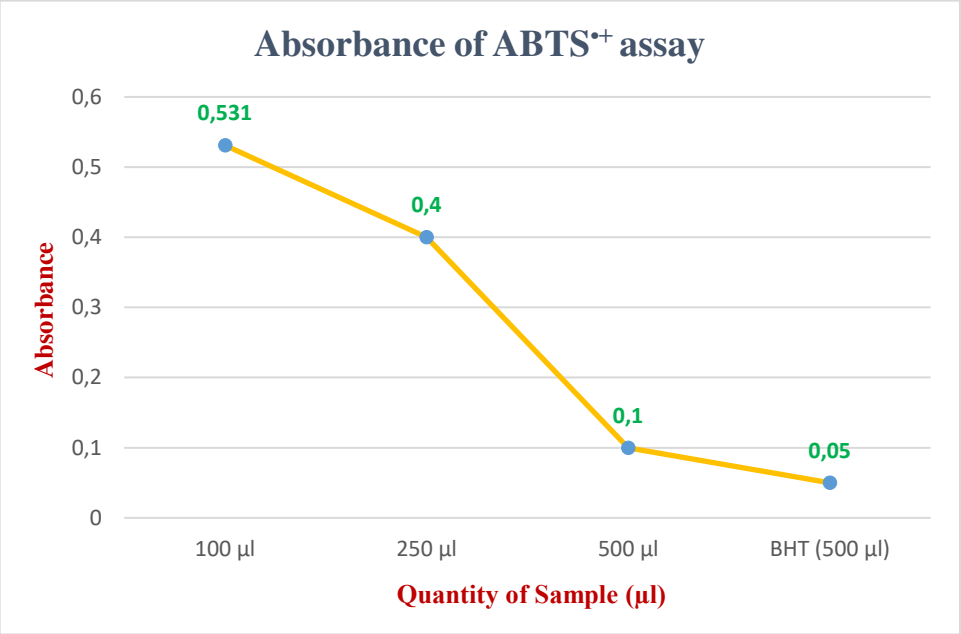


Figure 4.6. The absorbance of test samples ($ABTS^{\bullet+}$)

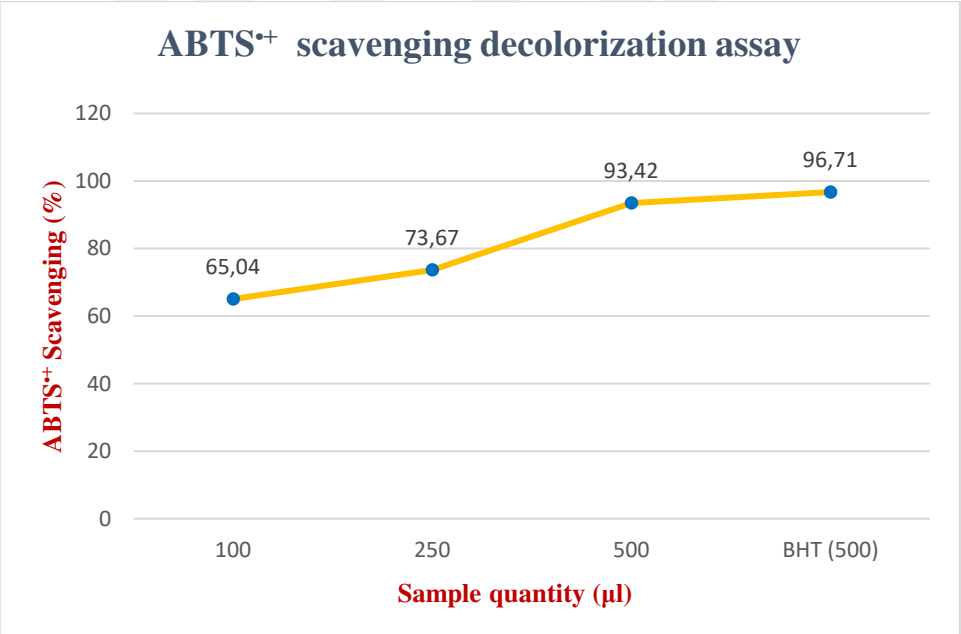


Figure 4.7. The capacity of the test compound to scavenge $ABTS^{\bullet+}$, with different amount

4.2.3. Hydroxyl radical scavenging activity assay

However, the result of this test that approved the potential of the agent at three different amounts, compare other assays that were done before HO radicals were scavenged more than other radical species, the reasons may be via reactivity of $HO^{\bullet}$ or the agent power to contribute an electron to $HO^{\bullet}$. Various reagents were used during this procedure, each of them having pertained function. Figure 4.8 and Figure 4.9 explain the relationship between the number of samples with both

absorbance and radical scavenging. One of the significant reactions in this method is HO• formation by reaction between H_2O_2 and Fe^{2+} with EDTA. The critical points to describe this method are HO• because the antioxidants or agents deal with a radical form that can be damaged with high energy to the body heath and the ability of the agent is higher than the standard BHT against the radicals at the same amount. Like our compound, the L2 compound in the study [136] possesses higher activity than BHT to inhibit the HO radical in the media, for that reason the compound derivatives may also have a good AOA if apply *in vivo* against radicals.

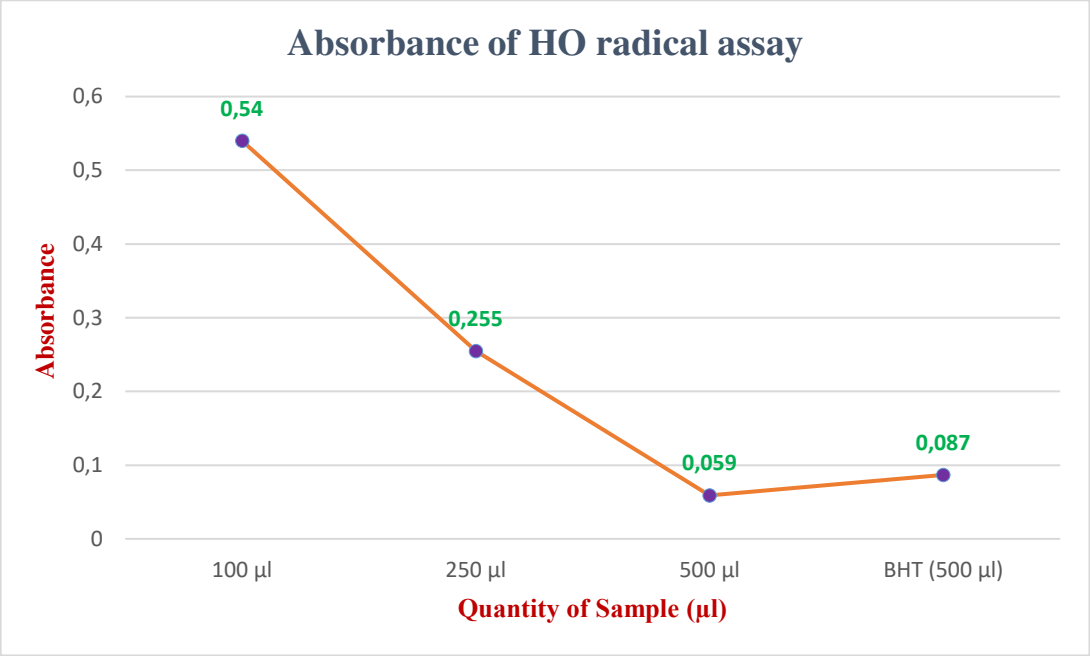


Figure 4.8. The absorbance of test samples (HO•)

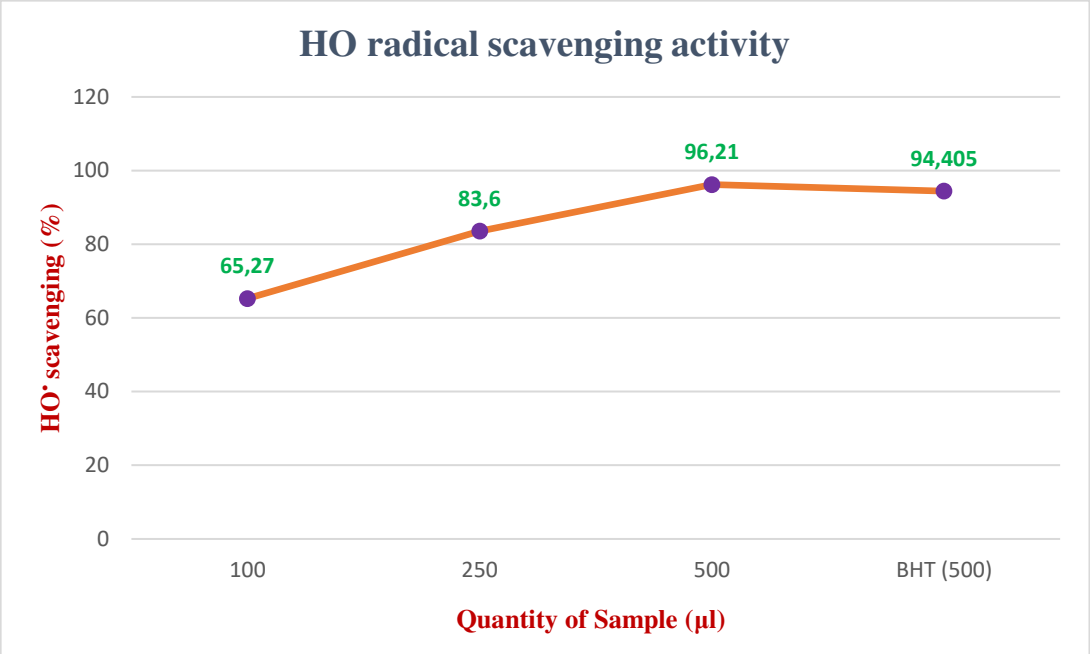


Figure 4.9. The capacity of the test compound to scavenge HO•, with different amount

4.3. Measurement of *Saccharomyces cerevisiae*

Our goal from this assay evaluates the targets range in the yeast and antioxidant potent of the sample compound. First, about vitamin A when the yeast was treated with 50 µl of the test sample, the amount of the vitamin is lower than the control, some factors might be the effect on it the most common when extracted into n-hexane layer may remain a portion of vitamin in the solution and other factors, however, the concentration level increased with the increasing amount of the sample. The second one is the range of vitamin C, E increased directly with the amount of the sample and both vitamins have significant characters as an antioxidant. The last target is MDA, the value reduced when added 100 µl of the compound, also it is clarified MDA has a significant role in the increased oxidative damage in the human body, for this reason decreasing MDA value determines the role of our compound as an antioxidant agent. Table 4.3 shows the amount of vitamin A, E, C with MDA in the *S. cerevisiae* cell after being treated with two different amounts of the sample triazole compound and the DMSO as a control. Where, $A_{(mg/l)}$ is the value achieved as a result of analysis by HPLC device in both vitamins and MDA, $B_{(mg)}$ is the intermediate value before the last value, and parts per million (ppm) is the concentration of targets in the yeast cell.

Calculation of the vitamin A and E concentration (ppm) equations are given as:

$$A_{(\frac{mg}{l})} = Result\left(\frac{mg}{l}\right) \times \frac{1l}{1000ml} \times \frac{1ml}{1000\mu l} \times solvent\ volume\ (\mu l) \qquad (4)$$

$$ppm = \frac{A_{mg}}{sample\ quantity\ (g)} \times \frac{1000g}{1kg} \quad (5)$$

Calculation of the vitamin C and MDA concentration () equations are given as:

$$A_{(\frac{mg}{l})} = Result\left(\frac{mg}{l}\right) \times \frac{1l}{1000ml} \times \frac{1ml}{1000\mu l} \times injection\ volume\ (\mu l) \quad (6)$$

$$B_{(mg)} = \frac{A_{mg}}{injection\ volume\ (\mu l)} \times total\ volume\ of\ reagents\ (\mu l) \quad (7)$$

$$ppm = \frac{B_{mg}}{sample\ quantity\ (g)} \times \frac{1000g}{1kg} \quad (8)$$

Table 4.3. Vitamins and MDA values in the yeast cell

Group	Vitamin A/(ppm)	Vitamin E/(ppm)	Vitamin C/(ppm)	MDA/(ppm)
Sample 50µl	0.07±0.015	0.81±0.07	0.86±0.06	23.67±0.92
Sample 100µl	0.09±0.002	0.89±0.04*	0.91±0.05	18.17±0.65*
Control	0.08±0.009	0.79±0.06	0.82±0.02	23.12±0.26
*p < 0.05 means statistically was significant				

4.4. Anticancer activity

The sample compound at different concentrations (5000µM, 2500µM, 1250µM, 625µM) treated *in vitro* with the cancer cell lines of Hep-G2, according to the MTT method that used for measurement cytotoxicity of the agent on the liver cancer cell line. The cell viability of the compound increased instead of decreased, so based on our result the 1,2,4-triazole derivative compound doesn't have cytotoxicity to reduce the Hep-G2 cell line (as shown in Figure 4.10), while if see the results from one concentration to another are the difference and it may be in appropriate concentration and structure composition exhibit cytotoxicity effect, based the consequence of some other studies that demonstrated the triazole derivatives may own potential against Hep-G2 cell line which are depending on the composition of compounds, ability to interact with biomolecules, dose. While, based on the study [198] the compound of 3d that has phenyl group which is directly attached to forth situation and it is more powerful than other substituents, if compare our compound it didn't exhibit any influence on the cell line. Therefore, the structure of the compound, type of substituent and its character on the compound, and ability to interact with the target are the major factors to determine the effect on the cell viability and cytotoxicity of the compound.

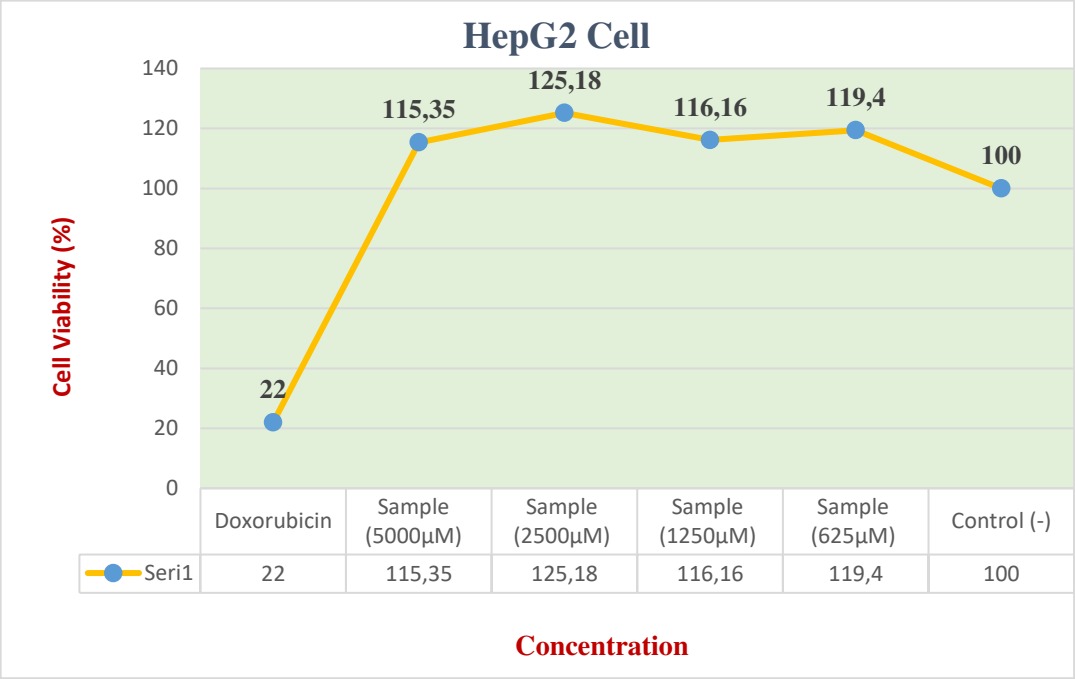


Figure 4.10. Potential of sample compound at several amounts against Liver cancer cell line

Various concentration (5000µM, 2500µM, 1250µM, 625µM) of synthesized compound was used for measurement its cytotoxicity against a group of human cancer which is called MCF-7 cell line by MTT assay. The results modified that are depended on the concentration used during the process, for example, the highest cytotoxic potential at 1250 µM of the agent to reduce cell proliferation in MCF-7 as shown in Figure 4.11, but if compare with doxorubicin it is low active. In addition, the structure of the compound particularly the ability to interact with the cell, the resistance of the cell to external sources, cell behavior, and other factors influence the usage as a drug in the field of chemotherapy. Otherwise, the 1,2,4-triazole derivatives in some other studies considered have potential against cancer specifically cell line of MCF-7, however, the activity converted from excellent to low depended on compositional structure, those groups that connected, and their position on it like in [199].

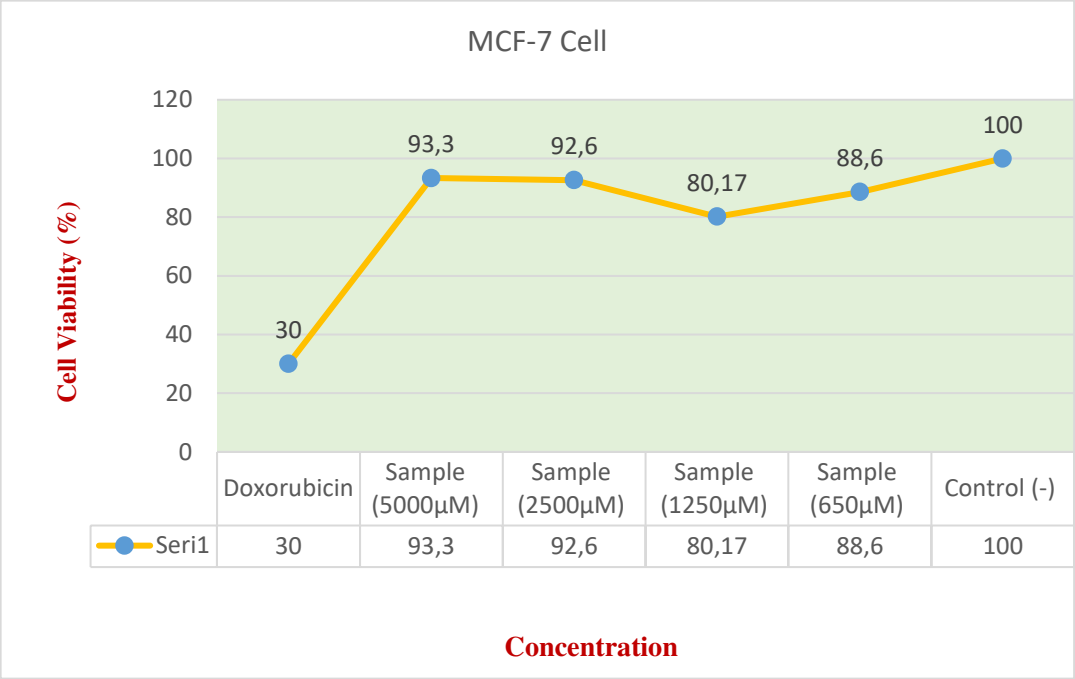


Figure 4.11. Potential of sample compound at several amounts against Breast cancer cell line

5. CONCLUSIONS

The power of the 1,2,4-triazole derivative may be influenced by the constituent of the compound or opposite reasons that are used on them or both parts. So, it is important to persist investigation for this agent so that know more and more about its ability and development in structure if required to increase power and usage as a drug for pathogens or any diseases that become defects in the wealth of the body. This study concluded, it is a good compound that may use against microbes specific fungal and based on the microbe species activity to inhibit growth was modified. About the scavenging of the radicals, all three species displayed activity, while HO radicals were undergone more scavenging compare to other species by the compound. Furthermore, our compound that was used had a significant role in depleting MDA and raising vitamin levels in the medium, thus it may act as an important antioxidant both *in vivo* and outside of the living system. However, it didn't have any significant cytotoxicity against both cell lines like in Hep-G2, but it has very low activity in MCF-7 and that was clarified every cancer cell species have particular characters for this reason activity of the 1,2,4-triazole compound altered based on the cell line manner. The structure composition of the 1,2,4-triazole has significant value because the 1,2,4-triazole that contain in other structure compounds increased capacity against several diseases until usage as a drug in markets, otherwise, may some other derivatives don't have benefit for life.

RECOMMENDATIONS

Several methods were used in this study to know and achieve biological information of the synthesized compound. It is recommended to investigate antiviral, different methods and techniques that didn't use before, and *in vivo* assays to determine it is ability inside the body.

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