# THE NEW MELATONIN DERIVATIVE, ITS SOME BIOLOGICAL ACTIVITIES AND HPLC APPLICATION

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**MSc Thesis**

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**May- 2019**

**REPUBLIC OF TURKEY**
**FIRAT UNIVERSITY**
**THE INSTITUTE OF NATURAL AND APPLIED SCIENCES**

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**Submitted Date: 22/04/2019**

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**MASTER THESIS**

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**Submitted Date: 22.04.2019**

**Examination Date: 20.05.2019**

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**May -2019**

## ACKNOWLEDGEMENT

All thanks and appreciation to my supervisor's *Prof. Dr. Mustafa KaratePe* for all his efforts to complete this work. I appreciate and thank the efforts of *Prof. Dr. Metin Koparir* for all the support and assistance he has provided in the organic part. I am very grateful to all the lab workers at IR and NMR including PhD students *Mustafa Ersin Pekdemir* and *Fatih Biryan*. Thanks to *Prof. Dr. Sevda Kırbağ* and her assistant PhD student *Şule Inci* for the biological part. All appreciation and thanks for the information and effort he has provided *Doç. Dr. Serhat Keser* in antioxidant part thanks for everyone who provided information or any assistance to complete this work and special thanks for PhD student *Ibrahim Nazem Qader* for all help especially in academic writing. Thanks to all the employees at Firat University. All thanks and appreciation to my family for their support and encouragement. The financial support of the Management Unit of Scientific Research Projects of Firat University (FUBAP) is gratefully acknowledge (project number FF.18.25).

**Srwa Hashim Mohammed Al-Nabawi**

**May-2019**

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## SUMMARY

## THE NEW MELATONIN DERIVATIVES ITS SOME BIOLOGICAL ACTIVITES AND HPLC APPLICATION

In this study, it is aimed to synthesize a new derivative of melatonin and evaluate the biological properties of this new derivative as well as to develop a new method for analyzing HPLC. In the first step, the new derivative of this compound was prepared with benzyl chloride. The purity control of the obtained derivative was made by means of melting point and the chemical structure was confirmed by IR, $^{1}H$ NMR and $^{13}C$-NMR spectral analyses and results with elemental analysis were supported. In the second step, antimicrobial and antioxidant properties of synthesized melatonin derivative were investigated using different microorganism and antioxidant methods. In the last step, the method for the analysis of the new derivative in biological sample by HPLC-UV was developed.



**Keywords:** Melatonin derivative; Antioxidant activity; antimicrobial activity; HPLC-UV**.**

# ÖZET

YENİ MELATONİN TÜREVİ, BAZI BİYOLOJİK AKTİVİTELERİ VE HPLC UYGULAMALARI

Bu çalışmada, yeni melatonin türevi sentezlendi ve bu bileşiğin biyolojik özellikleri ile birlikte HPLC-UV ile analizi için metot geliştirme amaçlandı. İlk olarak, benzil klorür ile bileşiğin yeni türevi sentezlendi.

Yeni türevin saflığı erime noktası ile, yapısı ise IR, $^{1}$H NMR and $^{13}$C-NMR ve elemental analiz teknikleri ile aydınlatıldı. Ikinci olarak, antimikrobiyal ve antioksidan özellikleri farklı mikroorganizmalar ve antioksidan yöntemler kullanılarak belirlendi. Üçüncü olarak, yeni bileşiğin HPLC-UV ile analizi için metot geliştirildi.



**Anahtar Kelimeler:** Melatonin türevi, Antioksidan aktivite, Antimikrobiyal aktivite, HPLC-UV

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## ABBREVIATIONS

| | |
|---|---|
| ATP | Adenosine triphosphate |
| DPPH | 1,1-diphenyl-2-picrylhydrazyl |
| ABTS | 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) |
| FRAP | Ferric reducing ability of plasma |
| ORAC | Oxygen Radical Absorbance Capacity |
| HORAC | Hydroxyl Radical Antioxidant Capacity |
| TRAP | total reactive antioxidant potential |
| CUPRAC | Cupric reducing antioxidant capacity |
| HPLC | High performance liquid chromatography |
| MMA | Methyl methacrylate |
| PMMA$^{\bullet}$ | Poly methyl methacrylate radical |
| AIBN | 2,2'-azobis (isobutyronitrile) |
| BPO | Benzoyl peroxide |
| DSC | Difference scanning calorimetry |
| TEAC | Trolox equivalent antioxidant activity |
| DMPD | N,N-dimethyl-p-phenylenediamine |
| TBARs | Thiobarbituric acid reactive substances |
| HGF | Human gingival fibroblast |
| CAA | cellular antioxidant activity |
| AMLT | Acetyl-melatonin |
| BMLT | Benzoyl-melatonin |
| MLT | Melatonin |
| ET | Electron transfer |
| IAA | Indole-3-acetic acid |
| HAT | Hydrogen atom transfer |
| PAGE | Polyacrylamide gel electrophoresis |
| ELISA | Enzyme-linked immunosorbent assay |
| REM | Rapid eye movement |
| PBS | Phosphate buffer saline |
| EDTA | Ethylenediaminetetraacetic acid |

| | |
|---|---|
| MT-Se | Melatonin-selenium |
| LPS | Lipopolysaccharide |
| BCG | Bacillus Calmette-Guérin |
| BHT | Butylated hydroxytoluene |
| HIOMT | Hydroxyindole-o-methyltransferase |
| CYP1A1 | Cytochrome P1A1 |
| CYP1A2 | Cytochrome P1A2 |
| 6 HMS | 6 Sulfatoxymelatonin |
| AA-NAT | Arylakylamine-N-acetyltransferase |
| SOD | superoxide dismutase |
| ROS | Reactive oxygen species |
| RNS | Reactive nitrogen species |
| DNA | Deoxyribonucleic acid |
| FR | Free radical |
| GC | Gas chromatography |
| MS | Mass spectrometry |
| RIA | Radioimmunoassay |
| EC | Electrochemical |
| FT-IR | Fourier Transform infrared spectroscopy |
| DTGS | Deuterium tryglycine sulfate |
| MCT | Mercury cadmium telluride |
| NMR | Nuclear magnetic resonance |
| MRI | Magnetic resonance imaging |
| CT | Computed tomography |
| DMF | Dimethylformamide |
| $KH_2PO_4$ | Potassium phosphate monobasic |
| ACN | Acetonitrile |
| BHA | Butylated hydroxyanisole |
| $H_2SO_4$ | Sulfuric acid |
| DMSO | Dimethylsulfoxide |
| $NaH_2PO_4$ | Monosodium phosphate |
| NaOH | Sodium hydride |
| TCA | Trichloroacetic acid |

| | |
|---|---|
| TBA | Tertiary butyl alcohol |
| KBr | Potassium bromide |
| NaH | Sodium hydrate |
| $H_2O_2$ | Hydrogen peroxide |
| $FeCl_3$ | Iron(III) chloride |

## 1. INTRODUCTION

The human body is controlled by a group of hormones, each of which has a specific function and any defect or lack of activity of these hormones might lead to certain issues in the body's functions, which may even cause development of certain diseases. The hormone “melatonin” is one of the most important hormones that influence the bodily functions. Melatonin is a peculiar substance synthesized in pineal glands of humans and animals. The pineal body is a small gland located in the midst of the brain. This gland contains two types of cells: the pinealocytes, which secrete the melatonin, and the glial cells, which support neurons. Pineal gland secretes the serotonin in the night and this hormone is then converted to melatonin by certain enzymes. Relying on that, pineal gland and this hormone (melatonin) are considered as the key factors for the internal clock of the body. Melatonin has a numerous functions and the its regulation is amongst the most important functions of the circadian sleep-wake rhythm and antioxidant mechanisms [1].

Melatonin has other functions besides the aforementioned biological functions, like its endocrine functions which affect the pituitary, adrenal, and thyroid glands. Also, melatonin has effects on certain diseases like Alzheimer’s disease, diabetes, cancer, Bon’s disease and insomnia. Oxygen is an essential element for living, biological systems. The cells use oxygen to produce energy through adenosine triphosphate (ATP) in the mitochondria. The process of ATP production is accompanied by certain byproducts known as free radicals. These free radicals in their normal levels have no effect but when their rate increases beyond the defensive capabilities of the organism, the destruction of cells by oxidative stress occur. This is where begins the importance of antioxidants emerge, which inhibit the oxidative process [2].

There are many ways to measure the antioxidant capacity (DPPH, ABTS, FRAP, ORAC, HORAC, TRAP and CUPRAC methods). For this study the DPPH, APTS, and hydroxyl radical scavenging methods were used. New derivatives of melatonin were synthesized and the antioxidant activity and other biological activities of these derivatives were evaluated. HPLC method was used to identify the new compounds.

## 2.MELATONIN

### 2.1. A Brief History

Comparing it with BHT and melatonin, the antioxidant activity of capsaicin was measured by David E. Henderson and others. Determination process included the direct measurement of lipid hydro peroxides formed upon linoleic acid autoxidation, which in turn was started by AIBN. Via HPLC, four isometric lipid hydro peroxides were detected using a total of three types of detectors: UV absorption, glassy carbon electrode electrochemical detection, and post column chemiluminescence using luminol. As for the result of this study, in suppressing the formation of lipid hydro peroxides, capsaicin was found to be more effective than melatonin, but not as effective as BHT [3].

A study performed by Ilhami Gulcin and others consisted of using a thiocyanate method for determination of total antioxidant activity of melatonin in vitro, where melatonin exhibited potent antioxidant activity in a linoleic acid emulsion system, and its antioxidant capability was found to raise as the melatonin concentration was increased. Even though the reduction capability of the melatonin was lower than butylated hydroxytoluene's (BHT) or quercetin's, it was statistically significant compared to the control group. Melatonin was found to have a potent superoxide radical reduction capability, which was higher than that of quercetin's or BHT's, but was still lower compared to the butylated hydroxyanisole's (BHA) [4].

Russel J. Reiter and others performed a study in order to determine if walnuts contained melatonin. They tested if eating walnuts influenced the melatonin levels of the animal eating them, and if the total antioxidant status of the blood changed. The researchers extracted melatonin from walnuts and quantified them by high-performance liquid chromatography. After feeding walnuts to rats, via using a radioimmunoassay serum melatonin concentrations were measured. Using the trolox equivalent antioxidant capacity and ferric-reducing ability of serum methods helped the estimation process of the "total antioxidant power" of the serum. As for the result, it was determined that walnuts did contain melatonin and increased blood melatonin concentrations when eaten. An increase in blood melatonin levels is in direct correlation with an increased antioxidative capacity of this fluid. Which was reflected by augmentation of trolox equivalent antioxidant capacity and ferricreducing ability of serum values [5].

Carsten Pape and others used three different extraction methods based on ether, acetone or perchloric acid for the extraction of melatonin. With these methods the melatonin

in tomatoes, ginger and the marine green macroalga, Ulva lactuca were quantified. Melatonin was determined by enzyme-linked immunosorbent assay (ELISA) in high-performance liquid chromatography (HPLC)-purified extracts. After derivation of melatonin, the same HPLC method with hydrogen peroxide (HPLC-PD), was used for purifying the extracted materials by utilizing alkaline conditions. It is found that, the same outcomes were obtained by both qualification techniques, i.e. there is sufficient relationship between these plants that possess melatonin and various rate of concentration. Therefore, the method that based on HPLC purification, which is qualified by ELISA and HPLC-PD, is a high sensitive technique to specify melatonin in diverse photoautotrophic organisms [6]. Irina V. Zhdanova et al. performed a study where the induction of polysomnographically recorded sleep was investigated where similar doses of melatonin were given later in the evening, which was approximately four times of the normal endogenous melatonin release. The habitual sleep onset was then monitored for any change. The results they obtained indicate that melatonin was inefficient in suppressing REM sleep or delaying its onset. Majority of the participants in the study were able to determine if they were given melatonin and or were in placebo when the hormone was tested between 6 or 8 PM. The melatonin doses used in the study were found to be incapable of inducing 'hangover" effects, which was determined with mood and performance tests performed during the morning of the next day. These data provide new evidence that nocturnal melatonin secretion may be involved in physiologic sleep onset and that exogenous melatonin may be useful in treating insomnia [7].

Tobias W. Fischer et al. performed a study to determine the antioxidative capabilities and UV- protective effects of melatonin in an in vitro irradiation method which involved leukocytes. These leukocytes were obtained from EDTA-treated whole blood samples and were collected PBS solution. The samples were irradiated by UV light and the radicals formed were quantified through the chemiluminescence technique. The preparations were stained with trypan blue to evaluate the cell viability. The findings of this study agree that UV protective effects of melatonin observed by clinicians might be attributed to their radical scavenging capabilities [8]. Hua WANG et al, on the other hand, performed a study where they investigated the properties of a novel complex called "melatonin-selenium nanoparticle" (MT-Se). The complex was firs synthesized by casting selenium nanoparticles within a melatonin-based environment. The protective effects of MT-Se in cases of immune-related liver injuries in mice (the agent used was bacillus Calmette-Guérin

(BCG)/lipopolysaccharide (LPS)) were evaluated and the results showed that the novel complex was quite effective in protecting the liver against immunologic injury in mice [9].

Aysin Akinci et al. performed a study where they compared the potential efficacies of melatonin, ascorbic acid, and β-carotene against stress-induced gastric mucosal degredation. A group of male Wistar albino rats were separated into 7 groups including control, which were treated with stress alone, stress and saline, and stress with each of the melatonin, ascorbic acid, and stress and β-carotene. The stress factors used in the study consisted of hunger, entrapment, and chilling at +4°C. The starvation was imposed by 3-day food restriction. Following this stress period, the agents tested in the study were administered for a week for each of the corresponding groups. Gastric tissue samples were then collected and examined under microscope, besides being subjected to the biochemical tests. The results of the study clearly indicated that melatonin was more efficient compared to ascorbic acid and β-carotene in recovering from the gastric mucosal degradation due to stress factors tested. It is also possible that melatonin's secondary effect through the brain-gut pathway may also have contributed to its protective potential [10].

Seiichro Fujisawa et al. researched the antioxidant capabilities of melatonin using a biomimetic carbon-centered radical model that consisted of growing methyl methacrylate (MMA) radicals (poly-MMA radicals and PMMA•). The study focused on the kinetics of MMA polymerization taking place under anaerobic environment by thermal decomposition of isobutyronitrile or benzoyl peroxide, and in the presence of melatonin. Oxygen amount within the living cells is low, and as the anaerobic environment helped bring the this system closer to biomimetic, melatonin was found to be capable of scavenging harmful in vivo carbon-centered radicals [11].

The study of M. Isabel Rodriguez-Naranjo et al. was a systematic evaluation of the antioxidant activities of melatonin and chemically similar compounds within similar biosynthetic pathways. Ferric-reducing antioxidant power (FRAP) assay, oxygen radical absorbance capacity (ORAC) assay, and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay were used to perform the evaluations. The results show that 5-hydroxyindoles have the highest activity amongst their counterparts, and their activity is similar to those of phenolic compounds when the reaction is based on the ET mechanism. Both melatonin and IAA are antioxidants which are active at the presence of a HAT reaction (ORAC value) [12].

Chawapon Phiphatwatcharadeda et al. performed a study to investigate the anti-oxidant capacity of melatonin derivatives modified using the indole-ring method, and compared them to the activity of melatonin in primary human gingival fibroblast (HGF)

cells. Anti-oxidant activity of melatonin (MLT), acetyl-melatonin (AMLT) and benzoyl-melatonin (BMLT) was evaluated by the usual five standard methods were used to investigate the antioxidant capabilities of melatonin, acetyl-melatonin, and benzoyl-melatonin. These methods were the DPPH, FRAP, superoxide anion scavenging, nitric oxide (NO) scavenging, and TBARs methods. FRAP and superoxide scavenging assays revealed that the antioxidant activity of power acetyl-melatonin and benzoyl-melatonin were found to be slightly lower compared to that of melatonin. NO scavenging and TBARs assays, on the other hand, indicated that benzoyl-melatonin and acetyl-melatonin were more active compared to melatoning. Finally, DPPH assay indicated that melatonin was clearly more active than the other two variants. Benzoyl-melatonin and acetyl-melatonin were found to be more potent antioxidants against $H_2O_2$ in HGF cells, compared to melatonin [13].

### 2.2. Definition of Melatonin

The chemical formula of melatonin is N-acetyl-5-methoxytryptamine, and it's an amine with a molecular weight of 232. It is also a heterocyclic compound derived from indole, Figure 2.1 commonly present in nature both in plants and animals. It is well known that in mammals, melatonin is synthesized in several cells, tissues, and organs mainly for local utilization (autocrine and paracrine actions) and that circulating melatonin is largely provided by the pineal gland where it is produced and directly released to the blood and cerebrospinal fluid [14].

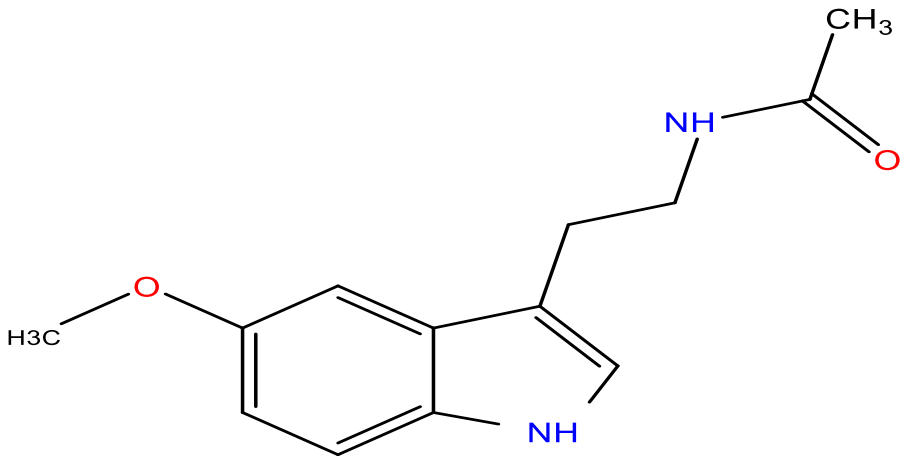


**Figure 2.1.** Melatonin molecular structure.

The pineal gland takes a small place near the central core of the brain and is a neuroendocrine organ with no pair. The gland is also called "epiphysis cerebri", and acts as

one of the primary actors of the circadian system. This system operates on the eyes and the superchiasmatic nuclei of the hypothalamus. The pineal gland shows its mediatory effects by secreting its primary hormone called melatonin in different amounts depending on the time of day. The amount of hormone secreted is also influenced by the animal's age, and for some species, the time of the year [15]. One interesting aspect of the pineal body is the fact that it's the only endocrine gland that works based on the input from the retina. In fact, the gland converts the environmental information into neuro-endocrine messages carried by physical agents within the circulation. The information is carried from the retinal photoreceptors to the superchiasmatic nuclei, which then is passed into the paraventricular nuclei. From here it reaches intermediolateral cell, the superior cervical ganglia [16].

Melatonin is mainly synthesized by the pinealocytes in the pineal gland. It can also be found in other tissues like the retina, gastrointestinal tract, skin, lymphocytes, platelets and bone marrow cells. The synthesis of the hormone is based on the ambient light and is under the mediation of the circadian clock handled by the suprachiasmaic nuclei (SCN) of the hypothalamus. Tryptophan is an amoniacid which the melatonin is synthesized of by hydroxylation first, followed by the decarboxylation, resulting in the formation of the 5-hydroxytryptamine, or serotonin [17]. Tryptophan, therefore, is the precursory substance for the biosynthesis of melatonin.

The 5-hydroxytryptophan form of the melatonin precursory substance can also transform into serotonin (5-hydroxytryptamine; 5-HT) under the influence of aromatic amino acid decarboxylase. Serotonin can also be acetylated, resulting in N-acetyl serotonin, which is performed by the arylakylamine-N-acetyltransferase (AA-NAT) enzymatic activity Figure 2.2.

N-Acetyl serotonin can further be transformed into melatonin under the enzymatic activity of hydroxyindole-O-methyl transferase. Melatonin can later be metabolized by cytochrome P-450 enzymes (CYP1A1, CYP1A2) into its main metabolites: 6-sulfatoxymelatonin (6-HMS) and 6-hydroxymelatonin glucuronide, which takes place in hepatic cells, and are later excreted with urination [18].

AA-NAT proteolysis is triggered by light received by the retina, which then leads into a rapid decrease in melatonin synthesis activity. Reduced levels of what are known as the "clock-proteins" then stimulate gene transcription, moving the cycle towards melatonin synthesis that peaks during the night time. Alternation of light and darkness act as the primary environmental factors that mediate the biological rhythm of the organism, showing

effect through the retina and retina-hypothalamic pathways and influencing the SCN directly.

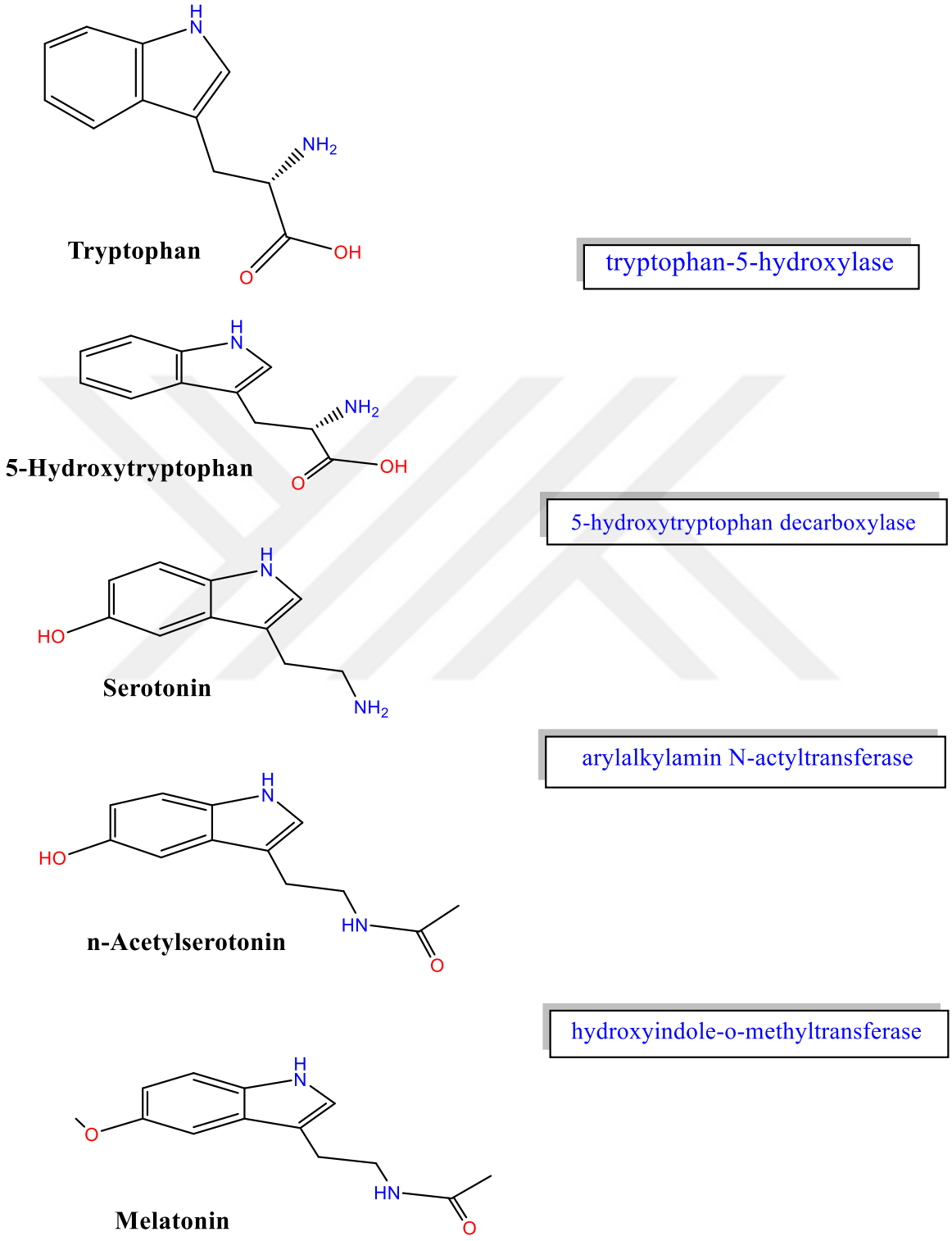


**Figure 2.2.** Schematic diagrams of melatonin synthesis from tryptophan.

Melatonin secretion is known to increase as the period of darkness gets longer. On the other side of the coin, increased light intensity causes the rate of endogenous melatonin production to drop, and shifts the pattern of release throughout the circadian clock (melatonin synchronization). Blind people completely lack this melatonin release synchronization, and instead operate on a state known as “free-running” [19].

Nocturnal and diurnal species’ metabolisms handle the melatonin production by the pineal gland during the darkness periods at night time, whereas light, particularly daylight, causes melatonin production to drop. Under the mediation of the pineal gland, melatonin circulates in a physiologically active concentration during the night period, and induces the nocturnal melatonin signal that creates the information of what the time is and how long the day may last in the central biological clock [20]. The average rate of melatonin peaks between 2:00 AM and 4:00 AM [21], and its release is reduced to almost complete halt between 7:00 and 9:00. This reduced melatonin state coincides with the peak hours of the endogenous cortisol. The average concentrations of melatonin in plasma are somewhere between 60–70 $pg/mL^{-1}$, and the rise and drop of melatonin concentration is only derived from the pineal gland [22].

Melatonin operates by means of its own receptors called the MT1 and MT2, which are amongst the G protein-linked receptor family members. Certain melatonin binding locations with lower binding potential were also suggested, like the site coined “MT3” which was recently identified as “Quinone reductase 2”. That being said, its physiological activity is yet to be determined [23].

The fetal and infancy period mammals lack the means to produce their own melatonin, but instead have to obtain the hormone either through the placental blood or through breastfeeding. A small number of studies investigating the development of the circadian functions in full-term human infants where the melatonin secretion periods, the sleeping cycles, and the body temperature regulation is researched report the lack of a circadian variation neon-tally until the ninth week. Preterm infants, on the other hand, were found to have a significantly delayed development of rhythmic melatonin synthesis. The melatonin produced was found to raise sharply during the first year of life, where the peak melatonin levels at night are observed in children between the ages of 1 to 3 years old [24].

The biological clock mediated by the melatonin and other supporting agents is essential the survival of numerous species. This mechanism regulates the sleep and wake cycle timings and prepares the organism for its daily activity periods. This is done by raising the heart rate, and glucose and cortisol levels. At the same time the 'hormone of the darkness',

melatonin, decreases. Thus, the time-of-day message penetrates into all tissues and influences the organs of the body. The suprachiasmatic nucleus (SCN) inside the brain regulates the daily rhythm through the intrinsic clock mechanism [25]. Melatonin, therefore, is capable of relaying information (signal of darkness) regarding the time of day to other organs including the SCN itself. The phase-shifting effects of melatonin in essence act in opposition to the phase-shifts of the light. SCN houses MT1 and MT2 melatonin receptors, due to which the hormone becomes capable able of phase-shifting the endogenous circadian clock. During the dark periods the hormone can also entrain the sleep-wake and neuroendocrine rhythms due to this fact. Exogenous melatonin was shown to be capable of entraining or phase-shifting the endogenous melatonin cycle in vivo [26].

A significant role of melatonin regarding the circadian cycle is the phase shifting capability, used in mediation of the sleep initiation and maintaining the sleep. These functions are mostly mediated by the membrane receptors, and due to its aforementioned influence, melatonin was suggested for the treatment of various Chrono biological disorders. These include the seasonal affective disorders, besides the more common disorders like insomnia and other sleep disorders that may arise due to blindness and jet lag. It is usually taken in a dose of 3 mg and is commonly present as a nutritional supplement substance in the USA. At a dose of 5 mg, melatonin can shift the sleep phase if taken before an imposed sleep period, which is particularly helpful for those suffering from the delayed sleep phase disorder. Furthermore, such a use of melatonin doesn't alter the sleep duration.

Melatonin use for extended periods of time was shown to entrain rest-activity regulation in blind people who suffer from the free-running disorder. Individuals who travel across multiple time zones often can greatly benefit from a daily use of melatonin in doses between 0.5 and 5 mg if they begin to suffer from jet lag symptoms.

Besides is biorhythm regulation functions, the melatonin is also known as a potent antioxidant working on two different mechanisms [1].

### 2.3. Antioxidants

Antioxidants are substances that significantly delay or inhibit the oxidation of another reduced substance present in higher concentrations. They are found as compounds or systems that inhibit auto-oxidation by preventing free radical formation or interrupting a chain of oxidation initiated beforehand with the following mechanisms: scavenging peroxidation-inducing reactive molecules, chelation of metals that would otherwise bring

about other reactive species or lipid peroxidation, scavenging the $^{\bullet}O2$ species that would form peroxides, and interrupting the chain of autoxidation reactions [27].

Oxygen is essential for the survival of living beings and sustaining an overall ecosystem. Cells utilize oxygen in order to produce adenosine triphosphate (ATP) in their mitochondria. The electron transport chain in the mitochondria uses oxygen to transport electrons, generating free radicals in the process. These are found as reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are the by-products of the cellular redox reactions. Since the main organic molecules found in an organism, namely carbohydrates, lipids, proteins and nucleic acids, contain high amounts of hydrogen, oxygen radicals have a high affinity for them. Free radicals can be beneficial in low concentrations but is potentially damaging in high concentrations.

Oxidative stress is known as the disparity between the amount of free radicals and reactive species, or oxidants and the amount of scavengers that protect the body from these metabolites, known as antioxidants. This disparity causes significant damage to key molecules and cellular structures in the body, potentially affecting the organism as a whole [28]. Oxidative stress has two sources: extrinsic and intrinsic factors. The extrinsic factors include UV light, chemicals or infectious organisms; the intrinsic factors can be classified as the electron transport chain found in the mitochondria, certain enzymatic activities and chemical outburst from the inflammatory cells [29].

Free radicals are molecules that contain unpaired electrons in the outermost atomic or molecular orbit. Since atoms need to possess paired electrons in their outermost shell to be stable, the free radical receives an electron from nearby molecule in order to become stable. This results the donor molecule to become a free radical itself [30]. The free radical levels can be detected via the spin electronic resonance, or the electronic paramagnetic method, which is based on detecting the energy exchange between atoms that have different electronic spin orientations.

Free radicals are generated from two sources: endogenously from immune cell activity, physical and mental stress, ischemia, excessive exercise, cancer, infection and aging; exogenously from environmental pollution, smoking, alcohol, some anti-cancer drugs (Tacrolimus, Cyclosporine), heavy metals, cooking, industrial solvents and radiation. These compounds become free radicals once they have decomposed in the body. The compounds that contain unpaired electrons such as superoxide, hydroxyl, peroxyl, lipid peroxyl, nitric oxide and nitrogen dioxide are free radicals, whereas the molecules that do not contain unpaired electrons such as singlet oxygen, ozone, hydrogen peroxide, hypochlorous acid,

lipid peroxide, peroxynitrite, nitrous acid and dinitrogen trioxide are considered oxidants and can produce free radicals but are not free radicals themselves [31, 32].

There are two different classes of antioxidants, known as enzymatic and non-enzymatic (Figure 2.3). Melatonin is an enzymatic antioxidant, a hormone produced at night from the pineal gland. Melatonin can be found in high concentrations in bodily fluids other than blood even if the serum levels are low, suitable to exhibit its' antioxidant properties on cells. Melatonin is also an ideal antioxidant in the sense that it can cross physiological barriers because of its' amphiphilic nature, in other words, it can reduce oxidative stress in both aqueous and lipid compartments of the body [33].

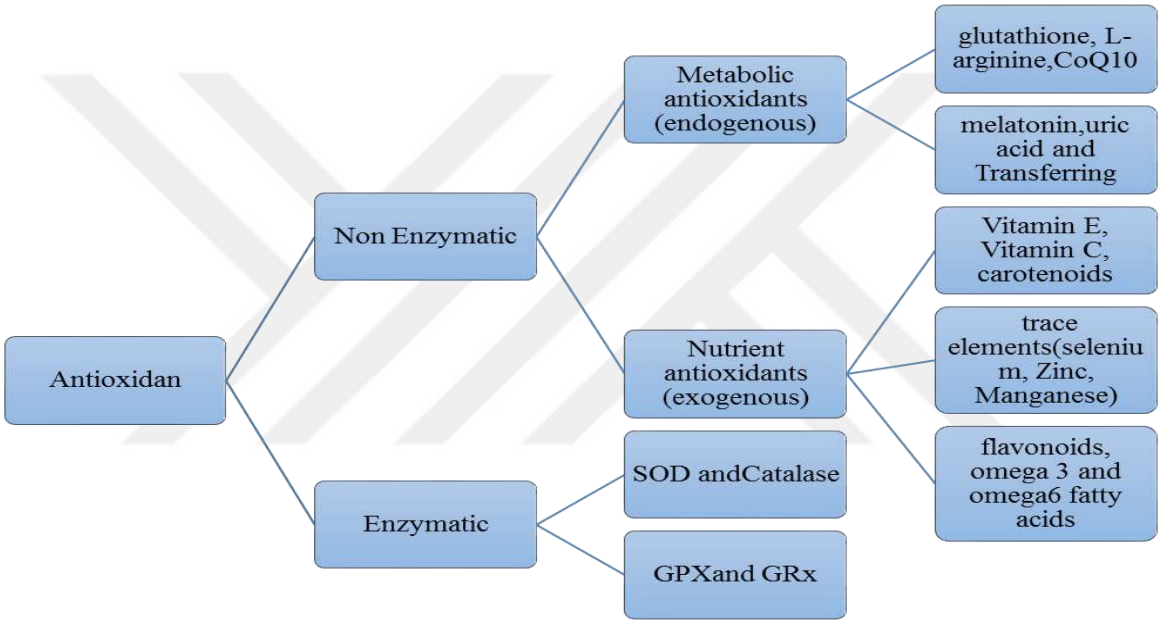


**Figure 2.3.** Antioxidant classification.

Melatonin works as an antioxidant by either by directly removing hydroxyl radicals, peroxyl radicals and the highly toxic peroxynitrite as well as inhibiting the production of isoprostanes, or by activating enzymes that remove free radicals. These enzymes include superoxide dismutase (SOD) and glutathine peroxidase, as proven by a study that recorded an increase in these enzymes in fetal rats' brain tissues when melatonin was administered to pregnant rats [34].

The fundamental workings of aging have been linked to cellular damage from free radicals such as peroxyl. Melatonin works similarly with tocopherol (Vitamin E) and glutathione in neutralizing these peroxyl radicals. An important function of melatonin is

implied to be prevention of neurodegenerative diseases by limiting oxidative stress in the brain tissue, which is significant due to cerebral tissue having a high rate of oxygen consumption and lipid peroxidation. Melatonin also inhibits dopamine oxidation, preventing neurodegenerative diseases in an even larger scale. [35].

## 2.4.Infrared (IR) spectroscopy methods

The term spectroscopy refers to the interaction of matter and light. Electromagnetic radiation present at infrared spectrum provides a very powerful tool to determine the presence of and identify molecular structures. Infrared spectroscopy also enables the researchers to quantify various biochemical materials that are otherwise challenging to count. The IR spectroscopy methods work by casting a range of infrared radiation towards the investigated sample and measuring the remaining spectrum to determine at which energy range the radiation was absorbed. Since this is determined by the frequency of the vibrations of atoms and molecules which are quite specific, the absorbed peak energy in an IR spectrum refers to the presence of that particular molecule [36].

The term infrared itself is used to described the spectrum that starts from the limit visible spectrum that ranges far into the microwave regions, usually extending from approximately 4000 $cm^{-1}$ to 600 $cm^{-1}$. The radiation passed through the sample is measured using specialized detectors to determine the absorbed portion –referred to often as the gap- to determine the specific molecular composition. The peak of the absorption at the plot corresponds to a specific wave number, which in turn is used to identify various biochemical compounds. Overall, IR spectroscopy has become an essential tool in accurate identification of various chemical compositions.

The whole infrared region of the electromagnetic spectrum consists of three regions:

- Near-infrared region (12820-4000 $cm^{-1}$): this range provides poorly specific absorptions, as it mostly is formed of overtones and combinations of the bands caused by vibrations in the mid-infrared region described below.
- Mid-infrared region (4000-400 $cm^{-1}$): this range is where most of the organic molecules correspond to due to their own specific molecular vibration frequencies.
- Far Infrared (400-33 $cm^{-1}$): this range has not been fully explored yet in terms of biochemical applications but it is being used in inorganic molecule studies [37].

IR spectrometers usually come in one of two types: dispersive spectrometers, and Fourier Transform spectrometers.

- Fourier transform infrared (FTIR) spectrometers have an internal interferometer which they use to exploit the well-known mathematical process called the Fourier transformation. Modern FTIR spectrometers have quite complex, laboratory-based instruments, as well as smaller models used routinely as portable handheld devices. The principle of FTIR spectroscopy is that the interference of radiation between two beams yielding a plot called an "interferogram". This plot is basically a signal obtained as a function path length change between two different beams. Based on the findings of this plot, the Fourier transformation method is used to convert distance and frequency properties and to obtain the molecular information (Figure 2.4) [38].

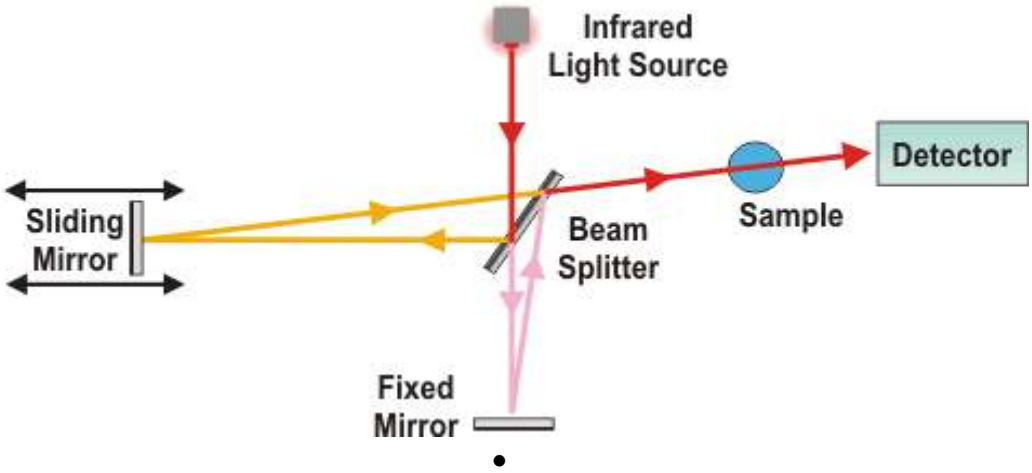


**Figure 2.4**. Schematic diagrams for principle work of IR [39].

FTIR spectrometers are slightly more complicated than standard spectrometers, as they contain an infrared radiation source, an interferometer, a sample room, a detection mechanism, and the laser. Either silicon carbide or a certain type of oxide mixture (Nernst) is used as radiation source to obtain a scanner spectrum at the mid-infrared region. To obtain radiation at near-infrared region, tungsten halogen lamps are needed as the source. Similarly, there are two primary detector types that can be utilized to detect the mid-infrared region. The routinely used detector is a pyroelectric device that utilizes deuterium tryglycine sulfate (DTGS) inside a heat-resistant alkali-halide window. Mercury cadmium telluride (MCT), on the other

hand, is used for more sensitive ranges towards the far infrared spectrum. This type of detector, however, needs to be cooled down to liquid-nitrogen level temperatures.

For the far-infrared spectrums, germanium/indium–antimony detectors can be employed. These work at liquid-helium level temperatures. Detectors used for the near-infrared region, however, simply utilize lead sulfide photoconductors [40]. FTIR spectrometers also contain an interferometer which has to mirrors: one fixed, and that can move. The moving mirror is what causes the variable optical path to create the difference between the two beams, which in turn provides a signal for the detector that carries the molecular information. The light cast from the source is split by a beam splitter, and while half of the light is cast towards the fixed mirror and back to the beam splitter, the other 50% passes through and reaches the detector.

- The other type of spectrometers called the "dispersive infrared spectrometers" have long left their place to FTIR spectrometers for routine measurements, but certain applications that involve time-resolved measurements still benefit from them greatly. This type of dispersive spectrometer uses a single beam cast from a source of IR radiation, a monochromator, and a detector. As the radiation interacts with the sample, IR radiation is then dispersed by a monochromator into the frequency components that comprise it. This information is then used to determine frequencies were absorbed, which is then measured using a photodiode array detector [41].

## 2.5. Nuclear Magnetic Resonance (NMR) spectroscopy

NMR is a spectroscopy method where the energy absorbed as the nuclear spin state changes is measured. At its core NMR spectroscopy works on the same principles as other methods of spectroscopy, where a photon causes a transition from the ground state to the excited state for a given atom. It is the absorption of a photon at a given radio frequency that promotes a nuclear spin from its ground state to its excited state. As such, it can be counter-measured to determine the change taking place, revealing the molecular structure of the sample. NMR spectroscopy is therefore a common technique for identifying organic chemicals. It relies on a phenomenon called Larmor precession, where the magnetic moment of a spin 1/2 particle in a magnetic field will rotate around the axis of the magnetic field. By measuring the frequency of this procession (the Larmor frequency), the researchers can get an extremely accurate measurement of the strength of the local magnetic field around the

particle. Because hydrogen nuclei are spin 1/2 particles, we can use proton NMR to uniquely identify many molecules containing hydrogen [42].

Many molecules the chemists are interested in as part of their studies contain hydrogen atoms. Hydrogen nucleus has a very strong resonance and as such the $^{1}H$ NMR represents the most frequently used NMR application. Carbon element, on the other hand, consists of various stable isotopes like $^{12}C$ and $^{13}C$ which are naturally present at 98.9% and 1.1% abundance, respectively. That being said, $^{13}C$ nucleus has a magnetic moment where I = 1/2, while the $^{12}C$ nucleus is not magnetic. Due to this fact, the organic chemical investigations of carbon with NMR is limited to the investigation of $^{13}C$, despite $^{12}C$ being a topic of great interest.

The magnetic moment of the $^{13}C$, on the other hand, is significantly lower compared to the proton's magnetic moment. Due to this fact $^{13}C$ is less sensitive for the NMR readings compared to the proton, and its lower natural presence makes its detection highly challenging. All things considered, it's not surprising that $^{13}C$ NMR spectroscopy is far less sensitive compared to the $^{1}H$-NMR spectroscopy [43].

Two primary versions of NMR instruments are being used in research:

- Continuous wave (CW) NMR spectrometers: These devices are similar to the optical-scan spectrometers as to how they work. While working with CW NMR devices, the sample is suspended inside a strong magnetic field, and the frequency of the source is scanned for with a slowly-varying reading scan. These devices have lost their attraction after the advent of FT-NMR devices, but are sometimes being used for wide line experiments for solid-state NMR applications.
- In NMR technology, Fourier transform operation can be used to convert time signals into the frequency signals. In FT-NMR, all frequencies in a spectral width are irradiated at the same time using a frequency pulse instead of scanning the frequency with a slowly changing scan rate. The sample can therefore be excited by a single strong radio-wave pulse, or multiple successive pulses. The scanners then look for the free induction decay (FID) detected by the receiver (Figure 2.5). This FID information can then be subjected to a Fourier transform, revealing the results. Such an operation can be completed in a matter of seconds, and as such, FT-NMR is substantially faster compared to the continuous wave spectroscopy [44].

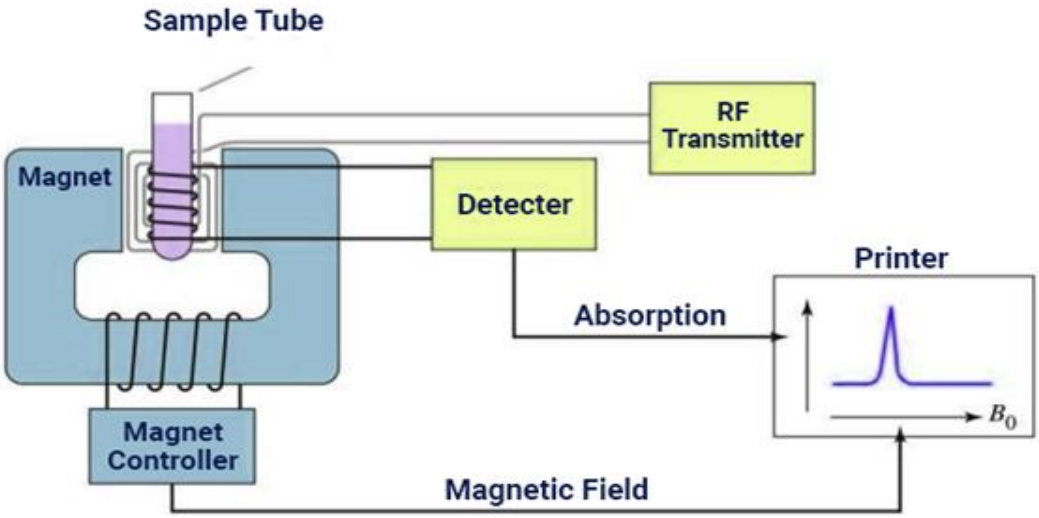


**Figure 2.5**. Schematic diagrams for principle work of NMR [45].

NMR has already become an essential tool for medicinal practices and chemistry studies. Just as MRI has become a strong and relatively safe tool for medical diagnosis, chemists are using the nuclear magnetic resonance principle to determine the molecular structures of certain biochemical compounds. Different peaks at the plot obtained by the NMR methods provide the information regarding the atoms that compose a molecule, and reveal the type of the bonds established between atoms. NMR is also being used in certain environmental studies, and as part of petroleum and other industries for process and product control [43].

## 2.6. Differential Scanning Calorimetry

Certain materials, particularly the polymers, change when they are subject to heat. These changes are called the thermal transitions, and can reveal quite significant information regarding the composition of the material. Differential Scanning Calorimetry (DSC) is the technique used in the study of such changes to determine the material compositions.

In the thermal analyses used in these techniques, certain physical parameters of the system are determined and/or recorded as a function of temperature. This function can then be analyzed further to reveal the physical and chemical properties of polymers, electronic circuit boards, and geological materials [46].

In DSC, two materials (one the sample and the other is a blank or a control) are subjected to the same temperature treatment in a controlled environment. It is the energy used to create almost the same temperature in the two materials that is measured to obtain information regarding the sample. In this technique, a calorimeter is employed to measure the heat put into (or radiated from) the sample during heating and cooling regimes of the test. At the same time, a differential calorimeter is measuring the difference of the temperatures of the sample and the control. As the sample is heated in a linearly increasing heat input and the readings from the calorimeter are used to observe how the sample reacts to the temperature regime. Since how various molecules behave under different heating and cooling regimes is quite specific, this information can then be used to deduce information regarding the composition of the sample.

One of the advantages of this method is that only a small amount of sample substance is needed, where a few mg is enough. DSC is fast and easy to apply, as all it does is to determine if a substance goes through a phase transition, which requires more heat input compared to the control, which in turn can be detected by the calorimeter. Based on the exothermic or endothermic nature of the sample, the same principle can be used in reverse and the cooling down of a sample can be monitored as well [46]

Various types of DSC instruments have been developed over the years that work with the same principle, but with some variations. These include the heat flux, power compensated, modulated, and hyper DSC's. There's also the pressure DSC, which incorporates the use of varying atmospheric pressure to either reach higher/lower temperatures or make specific measurements.

The most common types of DSC's Power compensated DSC simply use two identical furnaces to heat the sample and the reference control. The measured temperatures of both the sample and the control are then brought to the same level, but due the phase transition the sample may go through, the amount of energy used for it while bringing the temperature to the designated level will be higher compared to the control. This energy is actually a measurement of the enthalpy (or heat capacity change) in the sample, which is a relative value to the control [47].

Another type of DSC device is known as the heat flux DSC, which changes the temperatures of the sample and the control at a constant rate, while monitoring the difference in heat flow going into them. The heater disk has two elevated platforms where the sample and the reference pans are located. A chromel disk and a wire that connects them are attached under the platforms, forming the chromel-constantan thermocouples. This system

constitutes the measurement environment in order to detect the particular differential temperatures of interest. The chrome discs also present an chromel-alumel junctions due to the cables connected them, which are used to measure the sample and reference temperatures independently [48].

### 2.7. HPLC Application

HPLC, is the abbreviation to High Performance Liquid Chromatography, and is one of the most important types of chromatography techniques [49]. This method monitors separation, detection and estimation of the amount of material to be separated from a particular mixture. Figure 2.6 shows HPLC where, the work is done by injecting a small amount of the sample into a tube packed with a stationary phase. The stationary phase is typically a granular material made of solid particles such as silica or polymers. Then, the sample is pushed down the column with a mobile phase by applying a high pressure up to 400 atmospheres, whereby the pressure makes the technique become much faster compared to column chromatography and, after that, the components will detect and quantified as they exit the column [50]. Next, a detector that shows the retention times of the molecules sample retention times, which will vary depending on the interaction between the stationary phase, analyzes the molecules and the solvent (or solvents) used [51].

In general, there are five types of HPLC which are classified depending on the phase system used in the process. The first one is Normal phase chromatography that depends on the polarity in the work where it is stationary phase polar and mobile phase non-polar. The second is reversed phase chromatography with the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar analyst, and the nonpolar stationary phase [52]. The third one is based on size exclusion chromatography which is a technique that is widely used for the molecular weight determination of polysaccharides. The fourth is ion exchange chromatography. This type of chromatography is widely used in purifying water, ligand exchange chromatography, ion exchange chromatography of proteins, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides. The fifth is bio-affinity chromatography. This type separation is based on specific reversible interaction of proteins with ligands. The last technique has numerous of applications such as chemical separations, purification, Identification and also used for Pharmaceuticals like aspirin, ibuprofen, or acetaminophen (Tylenol) [53]. Melatonin measurement in body fluids such as serum and cerebrospinal fluid

has always been desired by researchers. Several different methods have been reported in the literature for determination of melatonin such as radioimmunoassay and gas chromatography with mass spectrometry (GC-MS) [54-56]. However, equipment for GC-MS is scarce and costly, sample preparation is time-consuming and the assay is labor intensive [57]. The RIA is sensitive but requires expensive radioactive substrate and complicated post incubation sample preparation by organic extraction [58].

High-performance liquid chromatography (HPLC) with electrochemical detection (EC) has widely been used to measure indolamines including melatonin [59, 60]. Furthermore, the determination of melatonin at very low levels in biological samples was carried out by HPLC with fluorescence detection after derivatization in the pineal gland or fish plasma [61, 62] or HPLC-UV detection only in tablets [63]. However, HPLC-EC systems have a high initial cost and are also sensitive to the contaminant, an issue that can be troublesome at high sensitivity [54, 64].

The aim of the present study was to develop a fast, cheap and reproducible method for determination of melatonin in human serum samples by HPLC with UV detection.

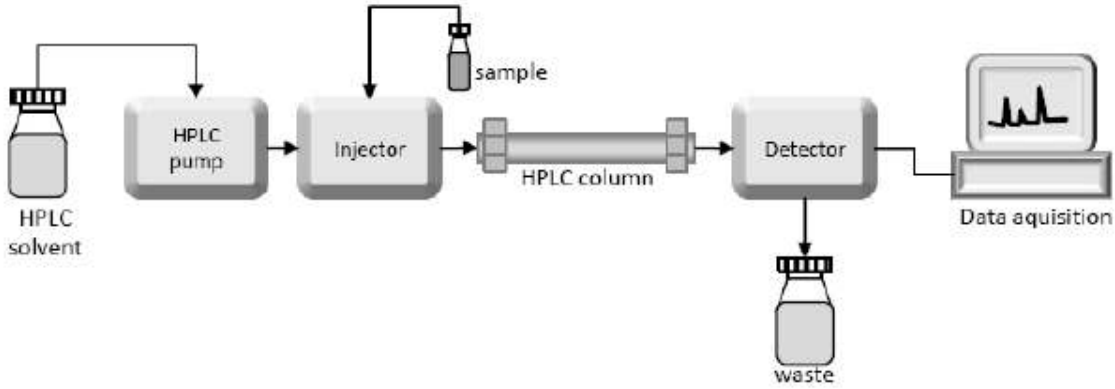


**Figure 2.6.** Schematic diagram of (HPLC) system [65].

## 3.EXPERIMENTAL PROCEDURE

### 3.1.Synthesis of N-[2-(1-benzyl-5-methoxy-1H-indol-3-yl) ethyl] acetamide (L)

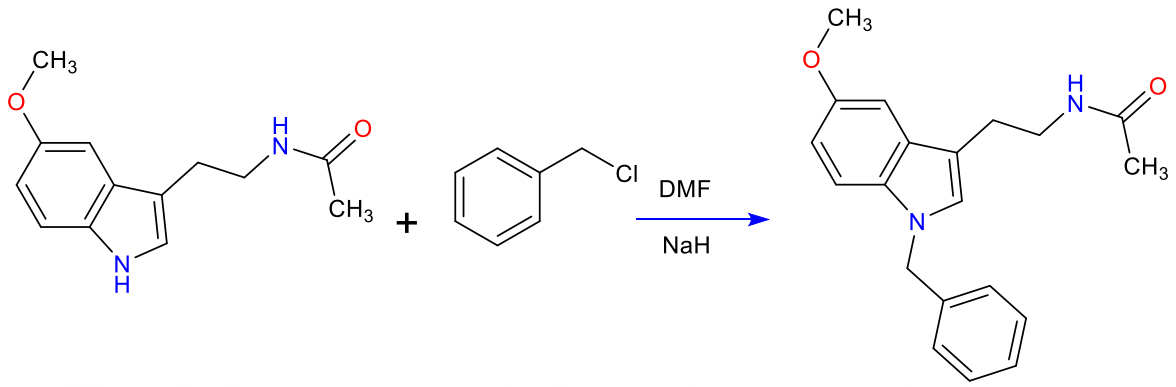


**Figure 3.1.** The obtained reaction of N-[2-(1-benzyl-5-methoxy-1H-indol-3-yl) ethyl] acetamide (L).

Melatonin (2.42 g, 0.01 moL) was dissolved in 15 mL of DMF under $N_2$ atmosphere. 1.35g (0.056moL) of dry NaH was added in portions at $0^0$C. After stirring at room temperature for 1 hour, 6.7 mL (0.056 moL) of benzyl chloride were added dropwise. The reaction continued to be stirred at room temperature for 24 hours, poured into ice at the end of the period and a few drops of acetic acid added to the oily substance. When this material was taken up in methanol with the aid of a baguette, the precipitate was crystallized from the ethanol-water mixture to give a white colored product. The structure of the product obtained was clarified by FT-IR, $^1$H-NMR, $^{13}$C-NMR and DSC techniques. The general reaction of the product is given in Figure 3.1. Yield % 63.

### 3.2. HPLC quantification of new melatonin derivative

The new method is developed for the new melatonin derivative in serum. Firstly, chromatographic conditions were determined for HPLC analysis of the newly synthesized compound. For this purpose, primarily mobile phase composition, flow rate, wavelength, and column temperature were optimized. It was then studied and prepared for the preparation of serum samples.

The mobile phase was 10 mM $KH_2PO_4$ -ACN (35+ 65, v/v %, pH 4) and the flow rate was 1.0 mL min-1. Chromatograms were monitored at 250 nm and injection volume was 50 µL. The retention times of the new derivative was 3.52 min.

### 3.2.1.Preparation of serum sample

100 µL of BHT and 500 µL of methanol were added to 1000 µl of the serum sample and vortexed for 2 min. and the medium was removed. Then 50 µL (50 mM) benzyl chloride in DMF, ACN and NaH were added in medium, vortexed and incubated for 24 h. in refrigerator. After incubation 100 µL ethanol (contain $H_2SO_4$) was added for the precipitation of protein residue and centrifuged for 15 mint 4500 rpm. Addition of ACN was necessary to dissolve and release the new derivative other groups and compounds. Then, supernatant (50 µL) was used for HPLC analysis.

### 3.2.2.Instrumentation

Liquid chromatographic system [30]. (Shimadzu, Kyoto, Japan) which consists of the following:

- 2x LC-20AD pumps
- 1x DGU-20A5 degasser
- 1x SIL 20A auto sampler
- 1x CTO-10AS VP column oven
- 1x SPD-M20A DAD system
- 1x RF-10AXL FLD system
- 2x detectors connected in series

These devices were connected via a communication module (Model CBM-20A) and controlled by a Shimadzu LC Solution workstation. A Shimadzu Shim-pack VP-ODS column (150L×4.6) was also used.

### 3.2.3.Preparation of standards

Standards from a new derivative stock solution (300 ppm) in the range of 0.25 to 60 ppm were prepared by dilution with ACN.

The intra- and inter-assay coefficients for new derivative were 2.7 % and 5.6 %; 1.0 % and 2.3 %, respectively.

### 3.2.4. Sample collection

Blood samples were given from by us, Firat University Medical School, Biochemistry Laboratory, Elazig. Serums were obtained by centrifugation then were stored at –80 $^0$C until analyzes.

## 3.3.Antioxidant activity

Determination of Antiradical Activities: The standard antioxidant and synthesized compounds were dissolved in DMSO (for HPLC Grade) at 5000 µM concentration.

### 3.3.1.$ABTS^{\bullet+}$ Radical Scavenging Activity

The spectrophotometric analysis of $ABTS^{\bullet+}$ radical scavenging capacity was determined according to the method of Re et al. (1999). $ABTS^{\bullet+}$ was produced by reacting 2 mM ABTS (in 0.1 M sodium phosphate buffer, pH=7.4) with 2.45 mM $K_2S_2O_8$, and it was stored for 16 h at room temperature in the dark. The $ABTS^{\bullet+}$ solution was diluted to give an absorbance of 1.650 ± 0.015 at 734 nm. Then, 3 mL of $ABTS^{\bullet+}$ solution was added to 500 µL L compound from stock solution (5000 µM concentration). After 0.5 h, absorbance was recorded at 734 nm [66].

### 3.3.2.Hydroxyl (OH•) Radical Scavenging Activity

The extract's inhibitor capabilities over n hydroxyl radical-mediated peroxidation were measured as suggested by Halliwell et al. (1987). The reaction mixture contained the following:

- 500 µL synthesized compounds
- 500 µL 3.6 mM 2-deoxy-D-ribose (inside a $NaH_2PO_4$–NaOH buffer material 20 mM with pH=7.4)
- 200 µL 100 mM $FeCl_3$
- 200 µL 104 mM EDTA
- 100 µL 1.0 mM $H_2O_2$
- 100 µL 1.0 mM aqueous ascorbic acid

The sample tubes were vortexed and incubated at 37 °C for 1 hour. 1 mL of 2.8% TCA and 1 mL of 1.0% TBA were then added to the sample tubes which were then vortexed again

and heated in a water bath at 50 °C for ½ hour. The level of oxidation 2-deoxyribose underwent was determined by the absorbance of the solution at 532 nm wavelength [67].

### 3.3.3.DPPH Radical Scavenging Activity

The DPPH radical scavenging capacity of compounds was measured by 2,2-diphenyl-1-picryl-hydrazil using the method of Brand-Williams et al. (1995). A solution of 0.1 mM DPPH in methanol was prepared and 4.0 mL of this solution was mixed with 500 µL of synthesized compounds (Figure 3.2). The reaction mixture was stored in darkness at room temperature for 30 min (Figure 3.3). The absorbance of the mixture was measured at 517 nm in a spectrophotometer [68].

**Figure 3.2.** DPPH Radical test for compound (L).

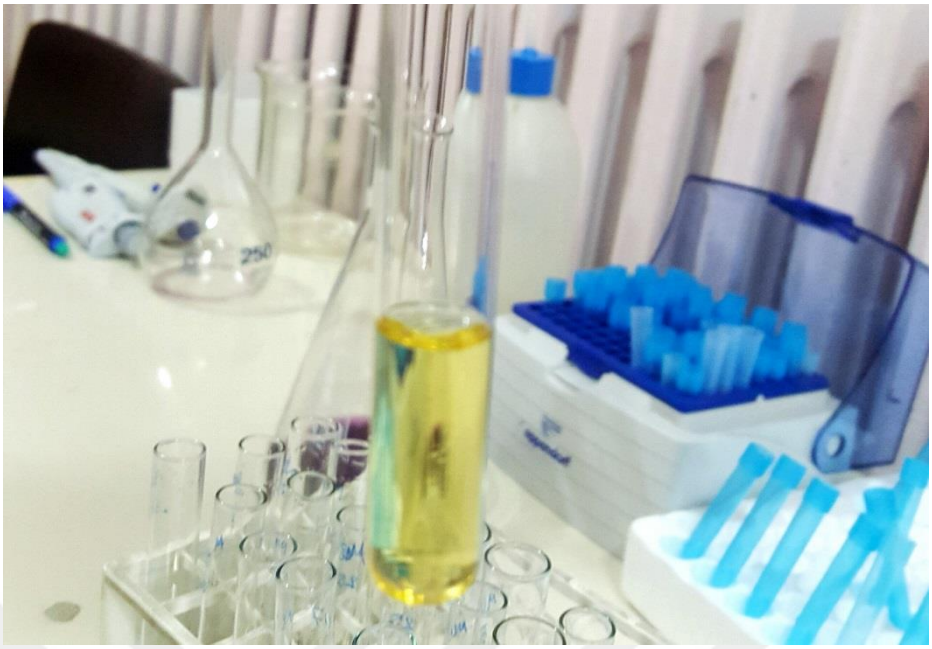

**Figure 3.3.** DPPH Radical test for compound (L) after 30 min.

The antiradical activities of samples were estimated by the following equation:

$$\% \text{ Scavenging Activity} = [(A_0 - A_1)/A_0] \times 100$$

where $A_0$ is the absorbance of control, and $A_1$ is the absorbance of the sample in the presence of extracts or standard.

### 3.4. Antimicrobial activity

The *Staphylococcus aureus* ATCC25923, *Klebsiella pneumoniae* ATCC700603, *Escherichia coli* ATCC25322, *Bacillus megaterium* DSM32 and *Candida albicans* FMC17 strains were used in the study. The microorganisms were taken from the culture collection of the Microbiology Laboratory of Fırat University.

Prepared bacteria, yeast and fungi in the broth culture respectively; After inoculation 1% of Müeller Hinton Agar, Sabouraud Dextrose Agar and Potato Dextrose Agar ($10^6$ bacteria/mL, $10^4$ fungus/mL), 25 mL of sterile Petri dishes were placed in 9 cm diameter and homogeneously distributed. Figure 3.4 shows the antimicrobial discs (Oxoid) 6 mm in diameter, which was aseptically impregnated into the solidified agar medium, each containing 100 µL of compound L, were placed slightly. Petri dishes prepared in this way were kept at 4 °C for 1.5-2 hours and the inoculated plates were incubated at 37 ± 0.1 ° C

for 24 hours and the yeasts and dermophytes plates were incubated at 25 ± 0.1 °C for 72 hours. Standard discs were used for control. (Streptomycin sulfate (10 mg / disk, Nystatin 30 mg / disk). the end of the incubation time, the inhibition zones were evaluated formed on the medium as mm. In addition Figure 3.5 illustrates the result of *s.aureus* after 24 hours of all process.

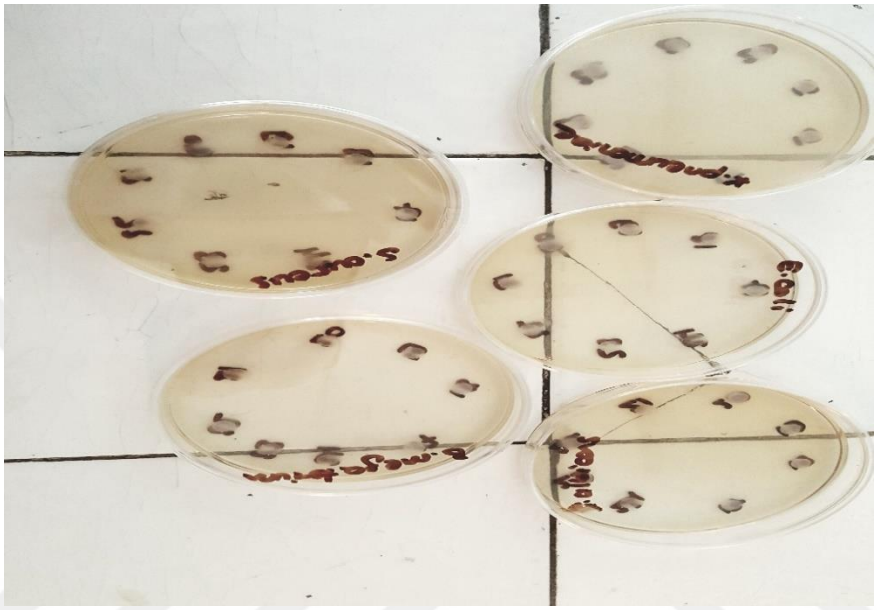

**Figure 3.4.** Antimicrobial test for compound (L).

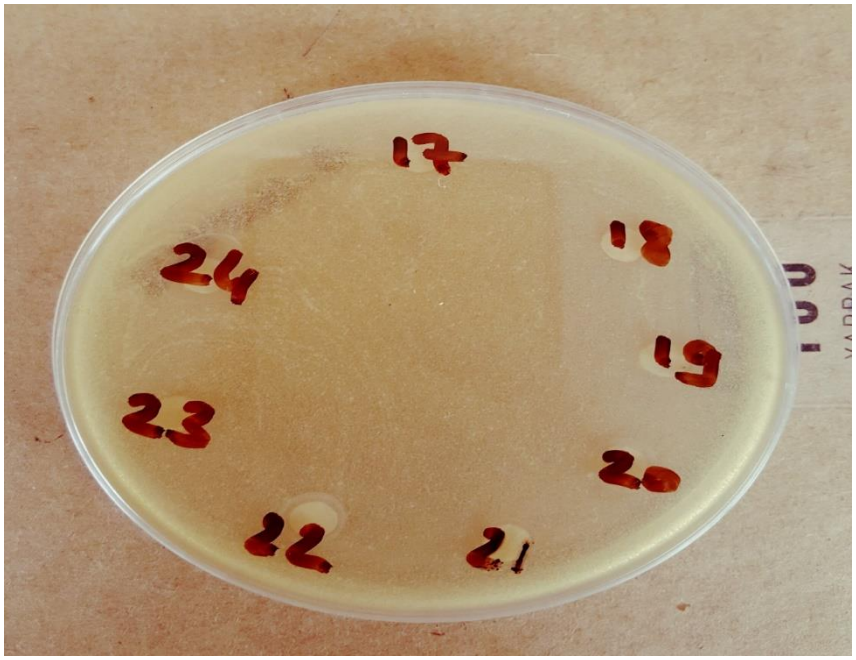


**Figure 3.5**. *S. aureus* after 24 h for compound (L).

## 4. RESULTS AND DISCCUSIONS

In this study, it is aimed to synthesize a new derivative of melatonin which has antioxidant activity as well as other known biological activities and to develop a method for analyzing the biological properties of this new derivative as well as HPLC. For this purpose, the indole -NH group in melatonin was taken as a target.

### 4.1.The characterization of N-[2-(1-benzyl-5-methoxy-1H-indol-3-yl) ethyl] acetamide (L)

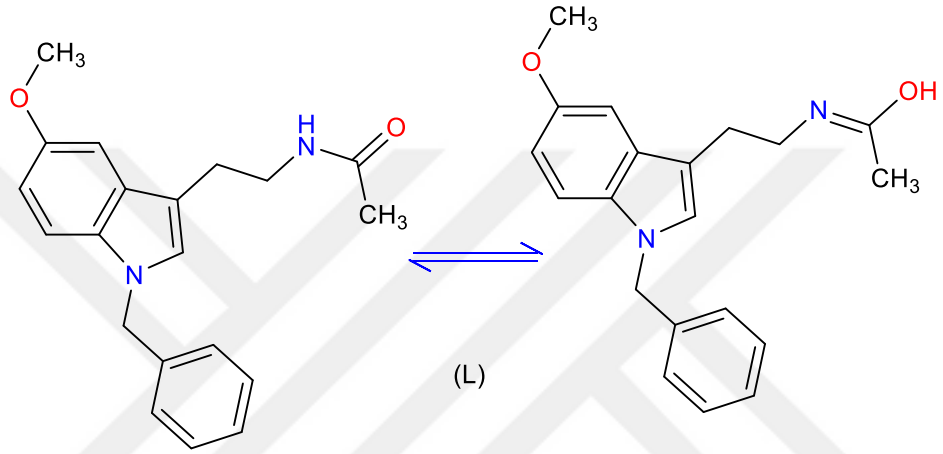


**Figure 4.1.** The tautomerization of compound (L).

The tautomerization of compound (L) (Figure 4.1.) The FT-IR, $^{1}$H-NMR, $^{13}$C-NMR and DSC spectra of molecule (L) are shown in Figure 4.2, Figure 4.3, Figure 4.4, Figure 4.5. and Figure 4.6, respectively.

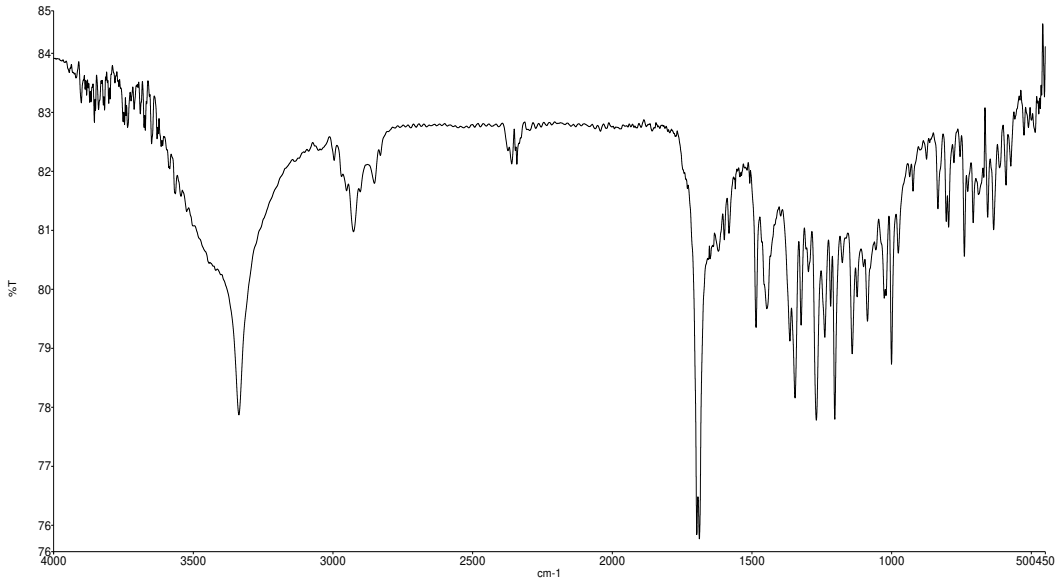

**Figure 4.2.** FT-IR spectrum of molecule (L).

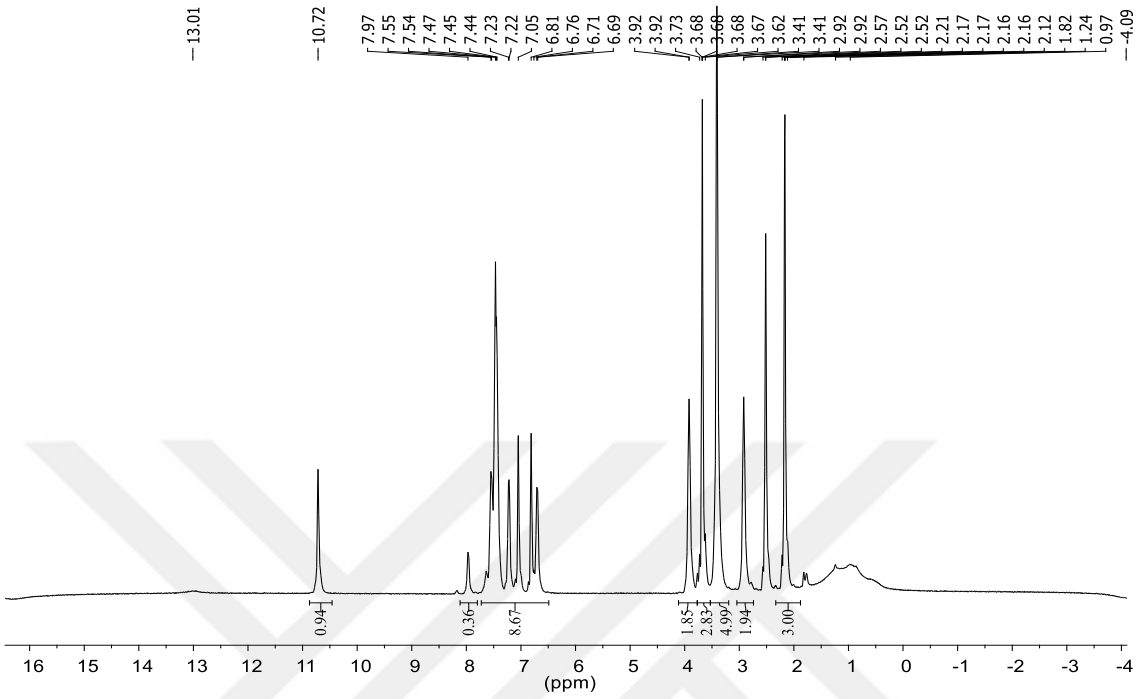


**Figure 4.3.** [1]H-NMR spectrum of molecule (L- iminol form).

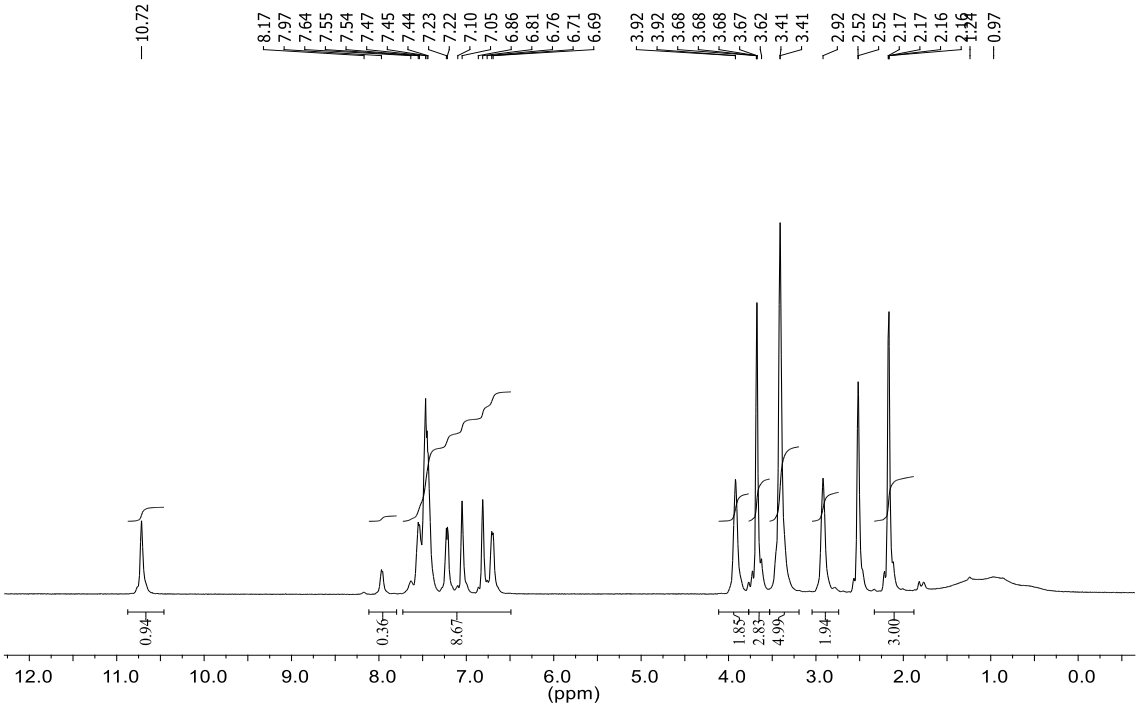


**Figure 4.4.** [1]H-NMR spectrum of molecule (L-amid form ).

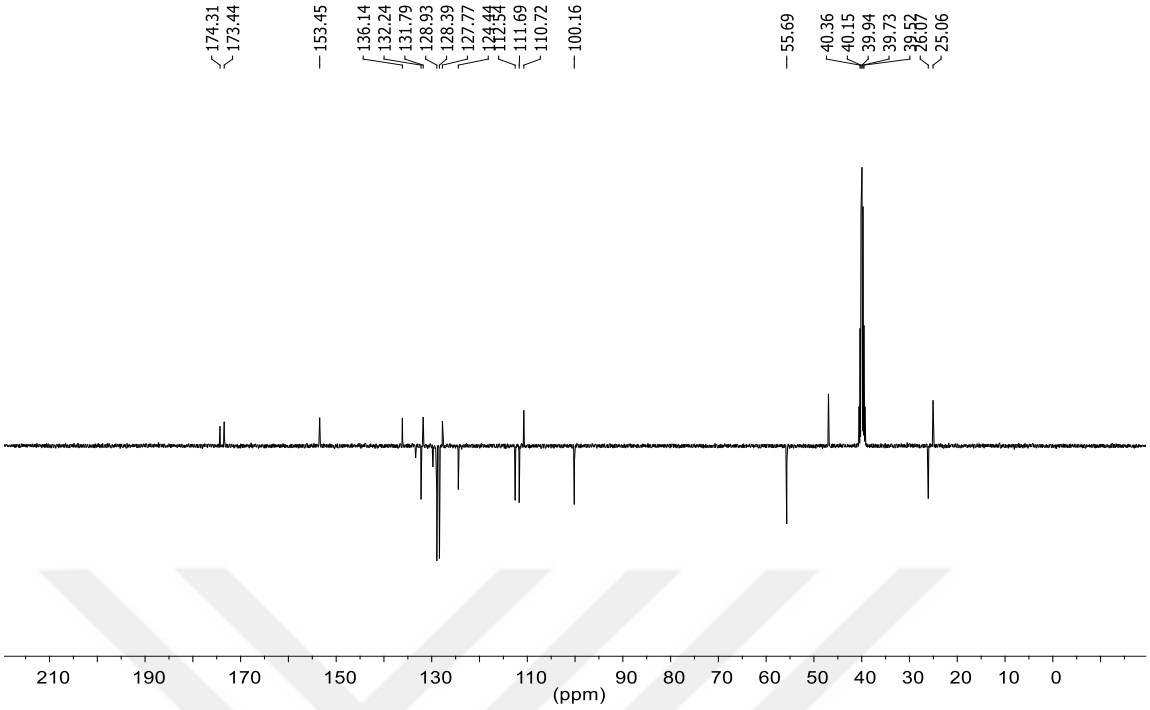


**Figure 4.5.** $^{13}$C-NMR spectrum of molecule (L).

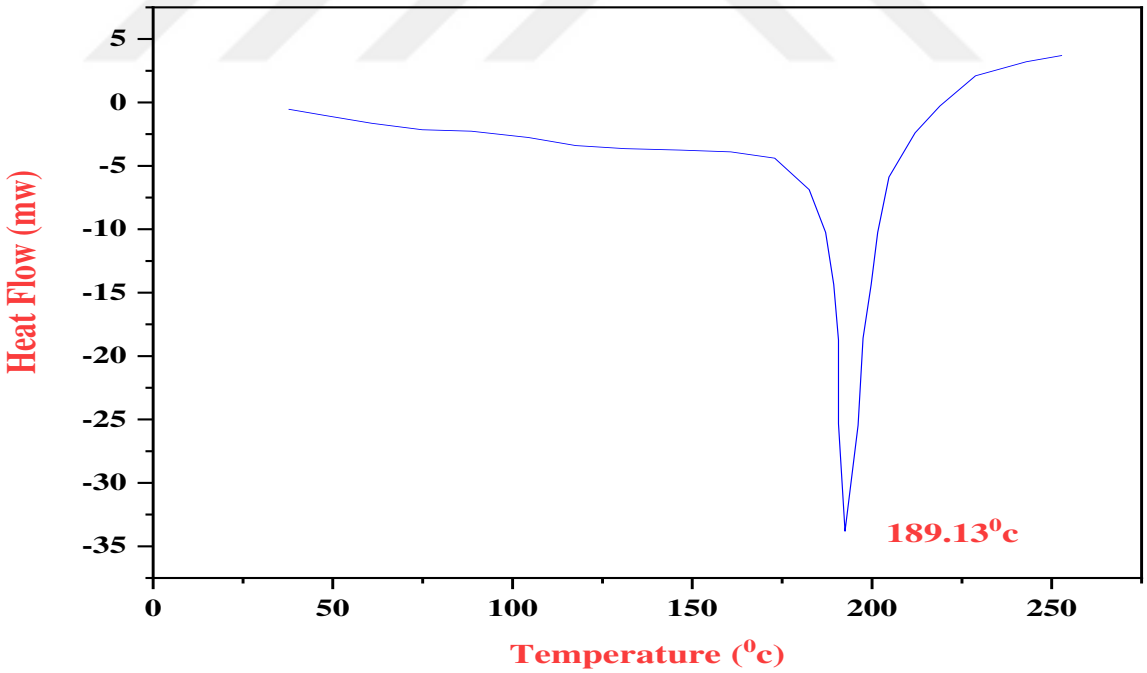


**Figure 4.6.** DSC spectrum of molecule (L).

Melting point: 189.13 ºC; FT-IR (KBr, $cm^{-1}$, υ): 3400-3390 ( OH, iminol form), 3339 (NH), 3072-3107 (Ar-H), 2890-2968 (C-H), 1697 (C=O), 1581 (C=N), 1269 (C-O); $^{1}H$ NMR (400 MHz, DMSO-d6, δ, ppm): 2.12 (s, 3H, $CH_3$-C=O), 2.91 (t, -$CH_2$-$CH_2$-NH), 3.41 (t, 2H, $CH_2$-NH), 3.68 (s, 3H, -O-$CH_3$), 3.92 (s, 2H N-$CH_2$-Ar), 7.55-6.70 (m, 9H, Ar-H), 10.71 (s, 1H, -NH); $^{13}C$ NMR (100 MHz, DMSO-d6, δ, ppm): 25.06, 40.15, 40.36, 55.69, 100.16, 110.72, 111.69, 112.54, 124.44, 127.77, 128.39, 128.93, 131.79, 132.24, 136.14, 153.45, 173.44, 174.31. Molecular weight: 322 ($C_{20}H_{22}N_2O_2$). Elemental analysis (theoretical): C, 74.51; H, 6.88; N, 8.69; experimental, C, 74.11; H, 6.78; N, 8.52; S, 9.17.

### 4.2.Antiradical Activity

The results of antiradical activity for synthesized compound is shown in (Table 4.1). the test compound (99.29%) was showed higher scavenging activity than standard antioxidant BHT (95.87%) in the OH radical scavenging assay. L compound (99.03%) was exhibited higher scavenging activity than standard antioxidant BHT (94.22%) for the ABTS radical scavenging and moderately DPPH tests.

**Table 4.1.** ABTS+•, OH•, DPPH• radicals scavenging activities of compound (L).

| **Samples** | **ABTS+• Scavenging** (%) (5000 µM) | **OH• Scavenging (%)** (5000 µM) | **DPPH• Scavenging** (%) (5000 µM) |
|---|---|---|---|
| L | 99.03 | 99.29 | 90.17 |
| BHT | 94.22 | 95.87 | 94.64 |

### 4.3.Antimicrobial activity

The result showed in antimicrobial activity of L only inhibited the growth of L *S. aureus* (7 mm) and could not prevent the development of other microorganisms (Table4.2).

**Table 4.2.** Antimicrobial activity for compound (L).

| | *S.aureus* | *B.megaterium* | *Klebsiella* | *C.albicans* | *E.coli* |
|---|---|---|---|---|---|
| L | 7 | - | - | - | - |
| control | 10 | 17 | 13 | 8 | 38 |

Characterization of compound L, and the derivative of this compound was prepared with benzyl chloride. The benzyl substitution to indole nitrogen was carried out using DMF and NaH. The reaction proceeded through the nucleophilic substitution mechanism.

The purity control of the obtained derivative was made by means of melting point and the chemical structure was confirmed by IR, $^{1}H$ NMR and $^{13}C$-NMR spectral analyses and results with elemental analysis were supported. When the IR spectrum of the compound is examined; Bending and stretching band of amide functional group were observed (Figure4.7).

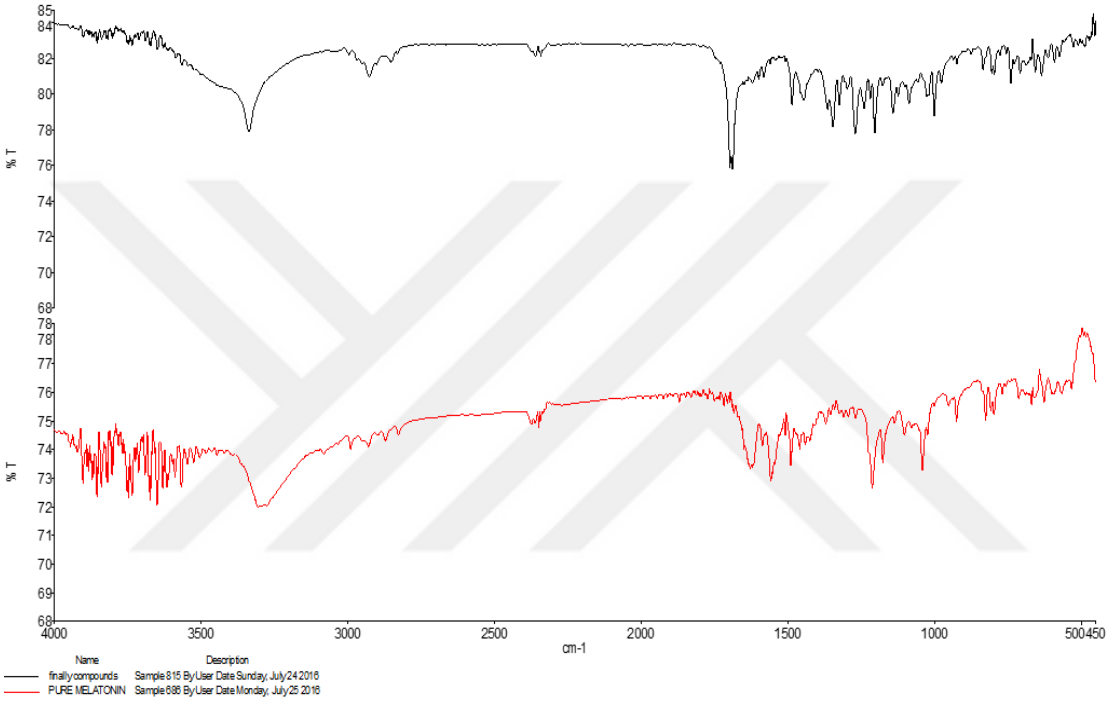


**Figure 4.7.** Compared between melatonin and new compound (L) synthesis.

In the IR spectra, the disappearance of the acidic -NH in the indole ring indicates that the targeted molecule is formed.

Amide I band specific for amide functional group (CONH, C = O Stretch) 1697 $cm^{-1}$, amide II band (CONH, NH bending) 1536-1550 $cm^{-1}$, amide III band (CONH, CN Stretch) 1450-1467 $cm^{-1}$. The Stretch of $cm^{-1}$ and NH was observed in the range of 3267-3372 $cm^{-1}$. These values are consistent with literature data Breteche et.al.

When $^{1}H$-NMR was taken into account, the disappearance of NH proton in the indole ring, the formation of singlet -$CH_2$ at 3.92 ppm, and the presence of 9 hydrogen equivalent protons at 7.55-6.70 ppm.

In addition, the $^{1}H$-NMR spectrum of the obtained derivative was determined; $CH_2$ protons in the benzyl group attached to the nitrogen atom of the indole ring were observed

as a singlet in the range 3.92 ppm. $CH_2$ protons in the carboxamide group attached to the indole ring were observed as a triplet at 3.41 ppm. NH protons in the carboxamide group attached to the indole ring were observed as a singlet in the range of 10.71 ppm. The NMR interpretations of the synthesized compounds are detailed in (Figure4.3).

When $^{13}$C-NMR is considered, it is thought that amide carbonyl at 174.31 and 173.44 ppm gives two peaks due to amide iminol tautomerization of carboxylic acid amides [69]. In the $^{13}$C-NMR spectrum drawn by the ATP method, one of the 3 $CH_2$ carbons in our structure appears in the solvent and is present on the upper side in the order of 25.06, 40.15, 40.36 ppm. The two $CH_3$ peaks in the structure signaled at 26.07 and 55.69 ppm.

9 protonated carbons in the aromatic ring on the lower side, 6 unprotonated carbons on the upper side of the signal is fully compatible with the structure.

The results of the elemental analysis of the synthesized derivative were found within the limits determined in comparison with the calculated values and proved the chemical structure of the molecule.

Characterization of compound L, the identification, and quantification of melatonin have been described using a variety of techniques such as GS-MS, radioimmunoassay, and HPLC. In the analysis of melatonin by using electrochemical or fluorescence detectors, some researchers applied $KH_2PO_4$-methanol [54, 70]. water-acetonitrile phosphate buffers containing $Na_2EDTA$ at various pH value [71, 72]. and acetate buffers with organic solvents [58, 73] as mobile phases. To the best of our knowledge, no HPLC method has been reported with UV detection for biological samples, except drug tablets (at 223 nm). In view of the relevant literature, we have attempted to prepare a new derivative of melatonin and determine it by using HPLC/UV. For this purpose, different mobile phases (mentioned above with modifications) and columns (C18 and ODS) were applied in the new derivative and then human serum sample. By using both columns, the best separation of (L) was observed with 100 % ACN for standards. However, the retention period was quite long. In order to optimize the HPLC procedure, different percentages of ACN in 25 mM $KH_2PO_4$ (20 %, 40 %, 60 %, 80 %) were tested.

In order to obtain a suitable mobile phase for the new derivative, we have made many attempts Mobile phase1 contains 60 mL acetonitrile, 10 mL methanol, 15 mL dichloromethane, 10 mL chloroform with 5 mL hexane. The result was a peak resulting from overlapping a set of peaks with each other Figure 4.8.

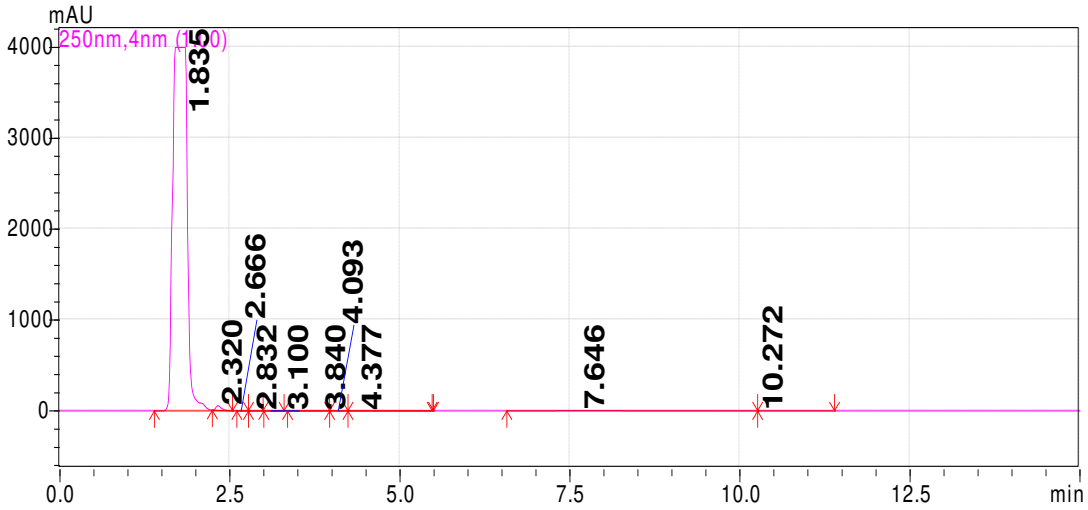


**Figure 4.8.** Chromatography detection of Mobile phase1 for compound (L).

Mobile phase2 contain 200 mL acetonitrile, 33 mL methanol, 50 mL dichloromethane, 33 mL chloroform and 17 mL Hg. Then take 100 mL from this solution with 25 mL $KH_2PO_4$ but this mobile gives the same result. We continued to work on the same mobile phase with the change of focus, noting that there was no change in the results so we have been working on a new mobile phase taking into consideration the results from past mobile phase.

Mobile phase3 contains 150 mL acetonitrile, 150 mL methanol and 50 mL $KH_2PO_4$ this new mobile phase gives the first peak for the new derivative Figure 4.9.

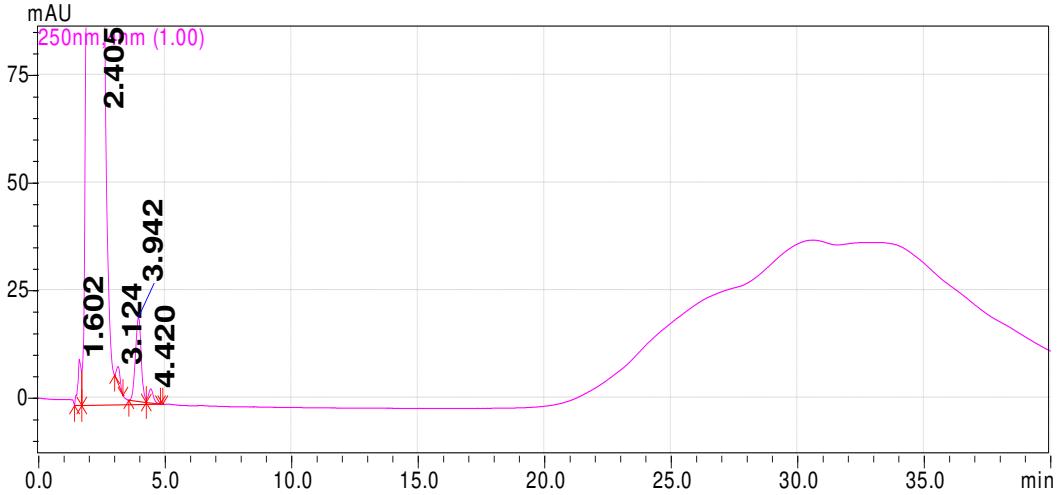


**Figure 4.9.** Chromatography detection of Mobile phase3 for compound (L).

This new mobile phase gives the first peak for compound L but this not enough result retention time and the intensity for this peak. We have worked to develop this mobile phase to get the best results in Mobile phase 4 used 150 mL acetonitrile, 150 mL methanol with 50 mL $KH_2PO_4$ and take 100 mL from this solution with 100 mL D.W.

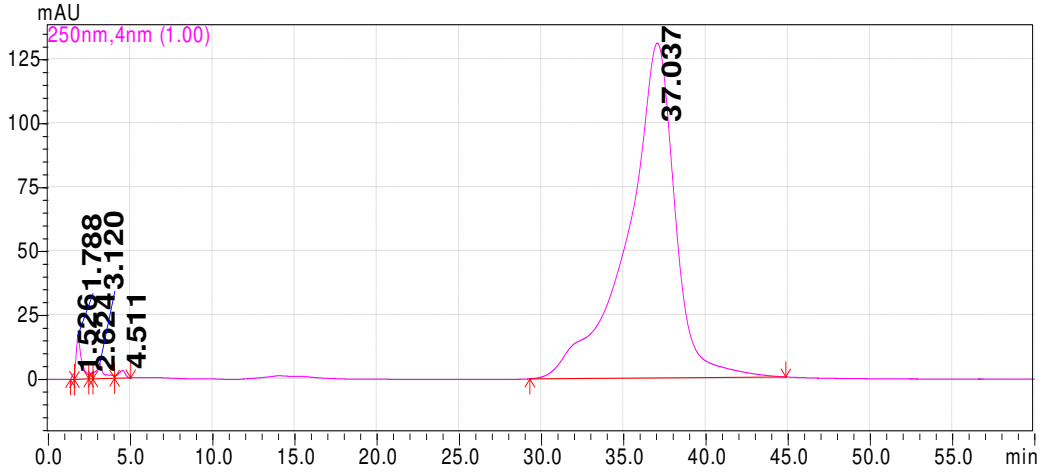


**Figure 4.10.** Chromatography detection of Mobile phase4 for compound (L).

This mobile phase gives a peak at 37.03 mint but the intensity for this peak very low, for this reason, we tried work on the same mobile phase by taking 200 mL from mobile phase with 200 mL D.W the results have not changed Figure 4.10.

Mobile phase 5 contains 150 mL acetonitrile, 150 mL methanol with 50 mL $KH_2PO_4$ take 200 mL from this solution with 100 mL D.W.

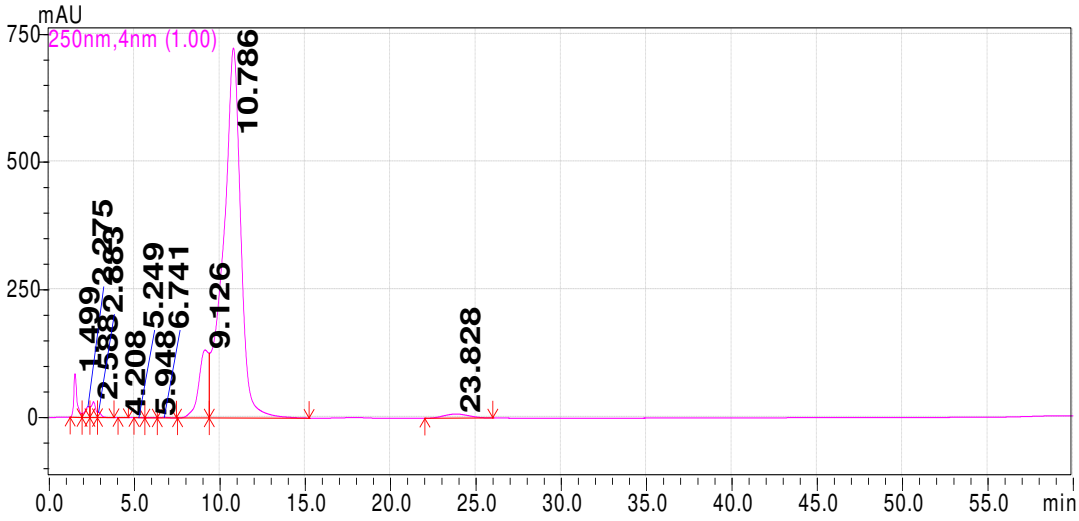


**Figure 4.11.** Chromatography detection of Mobile phase6 for compound (L).

The intensity of the peak did not change the change occurred only in retention time peak at 23.82 mint Figure 4.11.

Mobile phase7 150 mL acetonitrile, 150 mL methanol and 50 ml $KH_2PO_4$ take 200 mL from this solution with 100 mL D.W and add phosphoric acid to change PH.

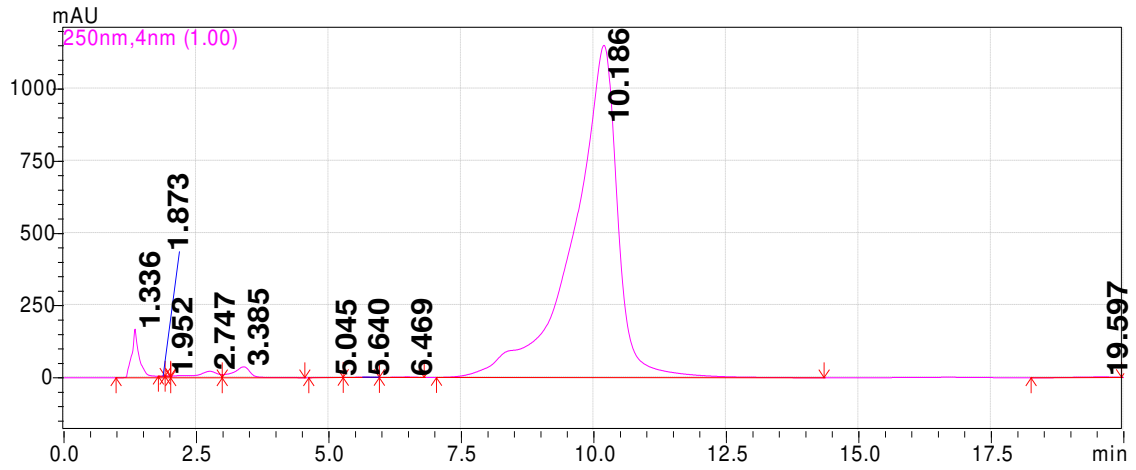


**Figure 4.12**. Chromatography detection of Mobile phase7 for compound (L).

In others mobile phase the PH solution in range (3,5,6) but in mobile phase7 the PH solution 4 and this change in PH give a different result for intensity and retention time this solution gives high peak and decrease retention time (peak at 10.18 mint). From this, we chose PH 4 from this mobile phase Figure 4.12.

Mobile phase8 contains 150 mL acetonitrile, 150 mL methanol and $KH_2PO_4$ take 200 mL from this solution with 100 mL D.W and added 20 µL phosphoric acid and add 10 mL DMSO for the total solution. In this mobile phase, we work to develop the result Figure4.13.

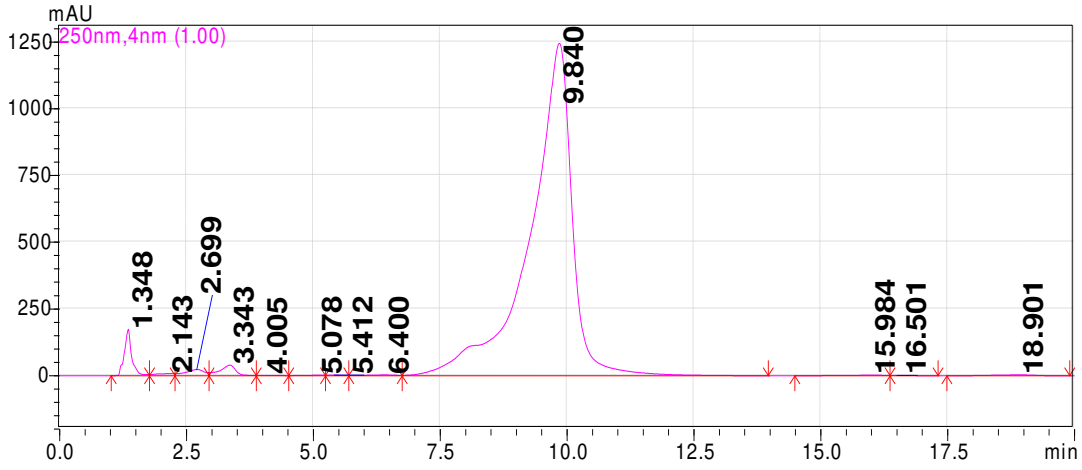


**Figure 4.13.** Chromatography detection of Mobile phase8 for compound (L).

In this mobile phase, the intensity for Peak not changed but the retention time changes Peak at 9.84 mint.

Mobile phase9 contains 300 mL acetonitrile, 50 mL $KH_2PO_4$, 175 mL D.W, 10 mL DMSO and PH 4 in this solution we chose acetonitrile solvent for this new derivative and don't use methanol for this mobile phase.

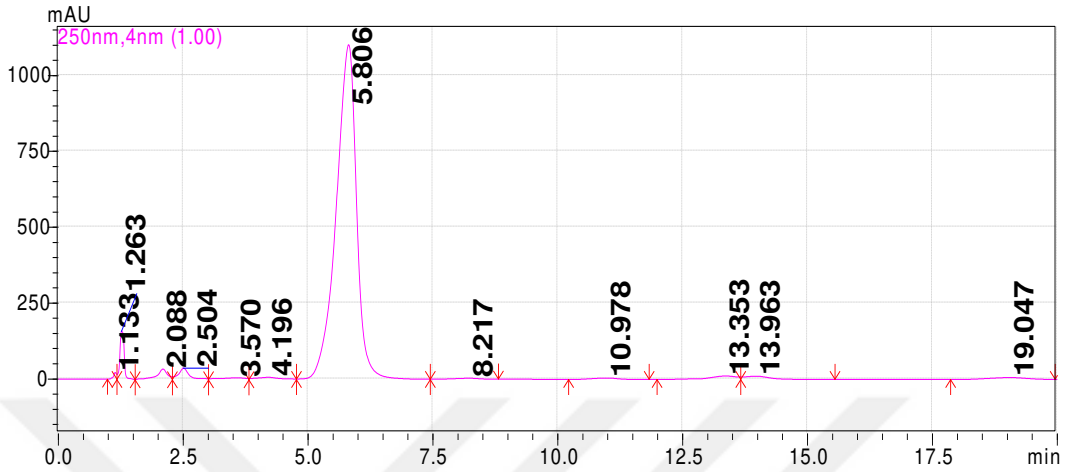


**Figure 4.14.** Chromatography detection of Mobile phase9 for compound (L).

From this result, we can see the change in the result when the change of concentration for ACN. The intensity for peak not change but the retention time decreased to give a peak at 5.80 mint Figure 4.14.

Mobile phase10 contain 300 mL acetonitrile, 50 mL $KH_2PO_4$, 175 mL D.W, 15 mL DMSO, with PH 4 and we mixed 150 mL from this solution with 25 mL acetonitrile.

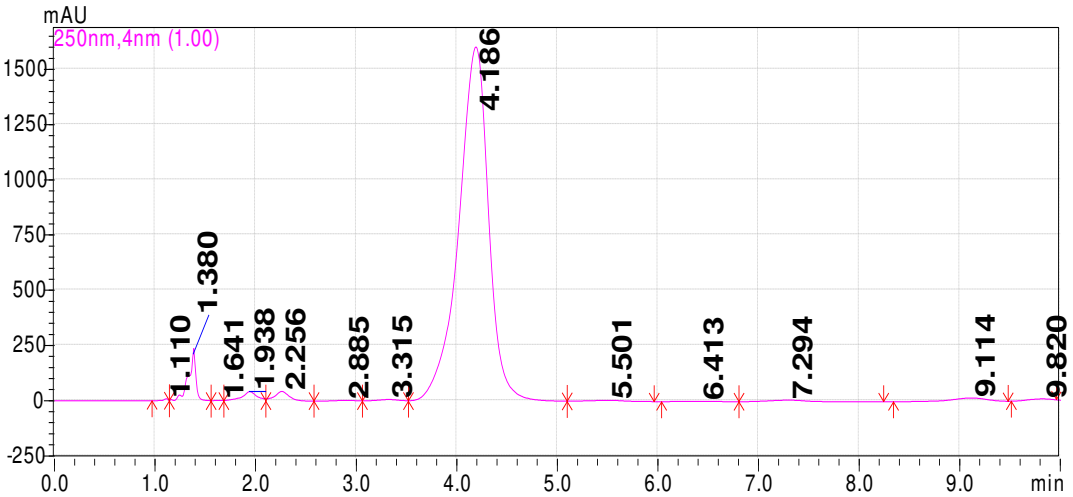


**Figure 4.15.** Chromatography detection of Mobile phase 10 for compound (L).

In this mobile phase observed with increased acetonitrile concentration, there was a significant change in peak intensity with a slight decrease in retention time. In order to obtain better results, we increased acetonitrile concentration Figure 4.15.

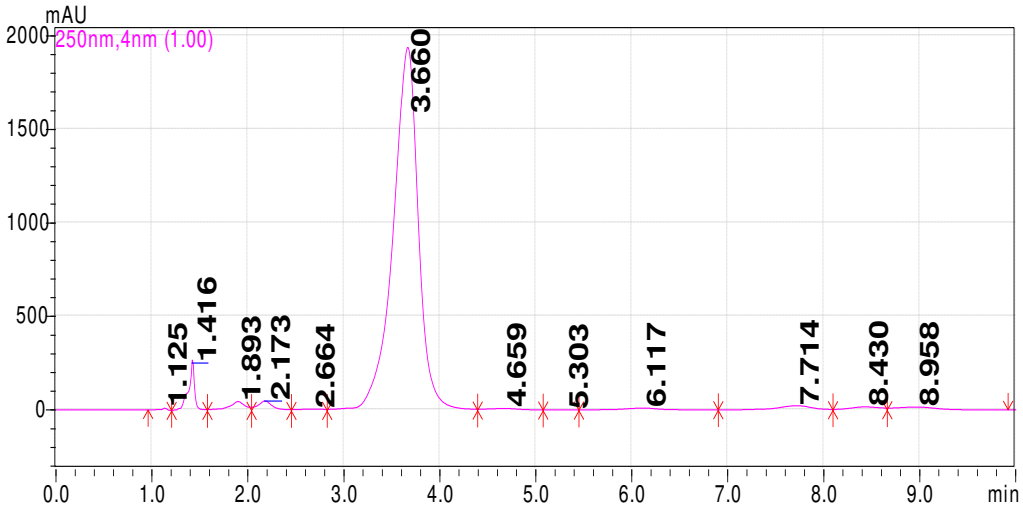


**Figure 4.16.** Chromatography detection of Mobile phase10 for compound (L) with increase acetonitrile concentration.

For this result, we used the same concentration for mobile phase10 and took 150 mL from this solution with add 375 mL acetonitrile. Observed the intensity for Peak increase for weakness and give the retention time at 3.66 mint Figure 4.16.

From the previous attempts to obtain the best mobile phase, we were able to obtain the required results. and Based on these results, we chose the mobile phase10 the best mobile phase to compound L. and we applied this mobile phase on standards from a new derivative stock solution (300 ppm) in the range of 0.25 to 60 ppm Figure 4.17.

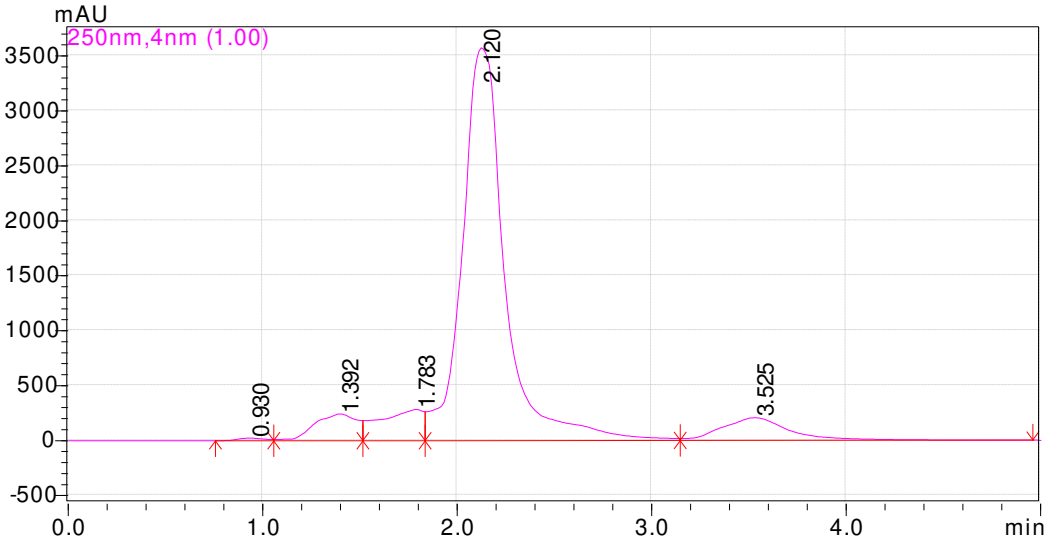


**Figure 4.17.** Chromatography detection for compound (L) at range 0.25 ppm.

The peak heights of (L) were low at a low ratio of ACN; however, they increased with the increasing percentage of ACN. When ≥ 50% of ACN was used, the peak of (L) began suit and have high detection limits. Optimum analysis conditions were supplied by % 64 ACN percentage Figure 4.18.

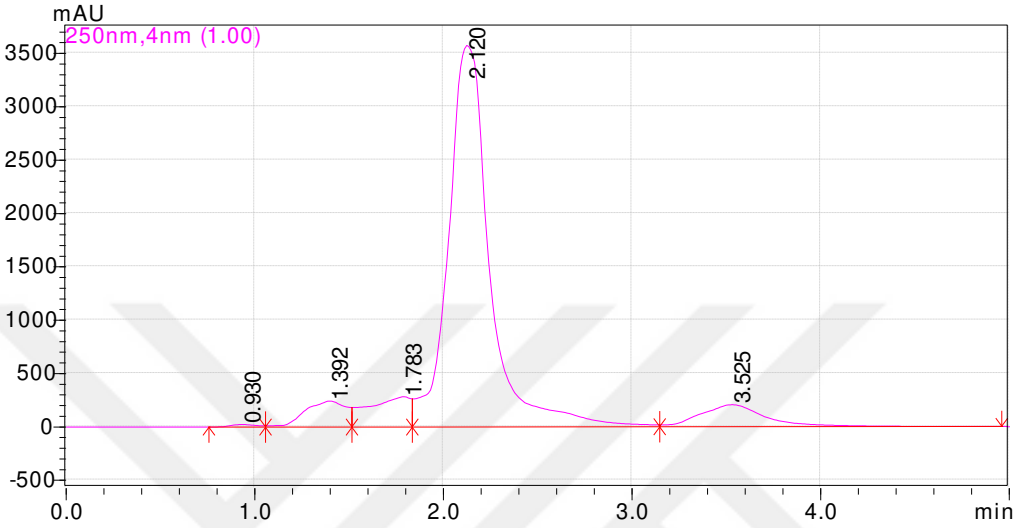


**Figure 4.18.** Chromatography detection of Serum samples were analyzed at optimized conditions.

Liquid extraction method was found to be a satisfactory method for sample preparation. Because, melatonin peak was overlaid by an unknown peak in some serum samples processed by liquid extraction method. Moreover, liquid extraction method was labor extensive with several preparation procedures and time demanding.

In this study, the antioxidant properties of synthesized melatonin derivative were investigated using ABTS, hydroxyl radical and DPPH methods. The synthesized derivative can be evaluated as a promising antioxidant due to the inhibition of ABTS and OH radicals (Table 4.1).

This indicates that the aromatic structure on the side chain containing the amide functional group can be effective.

When the situation in terms of antimicrobial activity is evaluated, as it is understood in the table, the compound has been found to be effective on a few microorganisms. (Table 4.2). Therefore, it is concluded that the synthesized derivative has no significant effect on microorganisms, moderately.

## 5. CONCLUSION

In conclusion, we suggest a new melatonin derivatives have high antioxidant activity and low antimicrobial activity and for the first time used HPLC/UV that characteristics of the proposed method are rapid, simple and easy sample preparation. The important results of this study has been summarized as following bullet points:

- Synthesis new melatonin derivative by using benzyl chloride, DMF and NaH
- DSC for measuring the melting point for new melatonin derivative and in result the melting point for new compound 189.13$^0$C.
- IR, $^1$H NMR and $^{13}$C-NMR to prove chemical structure for new melatonin derivative. In IR bending and stretching band of amide functional group were observed.$^1$H NMR give iminol and amide form mean that the new derivative its tautomer compound.
- HPLC/UV to detect the new derivative and gives absorbance 250 nm and the retention time 3.52 min.
- For antioxidant result give high inhibition of ABTS and OH radicals
- Antimicrobial test inhibited the growth of *S. aureus* (7mm)

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