**TURKEY**
**FIRAT UNIVERSITY**
**GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES**

# THE ISOLATION OF AMYGDALIN MOLECULE FROM BITTER ALMOND KERNEL

**Pshtiwan Raheem SABIR**

Master's Thesis

DEPARTMENT OF CHEMISTRY

Organic Chemistry

DECEMBER 2019

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# THESIS APPROVAL

The 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 accepted unanimously as a result of the defense exam made open to the academic audience

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This thesis was approved at the meeting of the Administrative Board of the Graduate School on ...../...../20.....

Signature

Prof. Dr. Soner ÖZGEN
Director of the Graduate School

# DECLARATION

I hereby declare that I wrote this **Master's thesis**, titled **The Isolation of Amygdalin Molecule from Bitter Almonds Kernel** 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, cite 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.

30/12/2019

**Pshtiwan Raheem SABIR**

# PREFACE

Amygdalin is one of the most commons members of the cyanogenic glycosides group. Naturally found in the seeds of the *Rosaceae* family included as (almonds, apples, apricots, cherries, peaches, pears, plums, and quince). It plays an important role in Traditional and Modern Medicine. We isolated the amygdalin molecule from the bitter almond kernels without using standard amygdalin. The diastereomeric *R*-amygdaline was purified by chromatographic methods and Structural characterization of Amygdalin was performed using spectroscopic techniques.

First of all, I would like to thank Allah, Above all, who helped me to write this thesis. Also, I would like to express my deepest gratitude to my research supervisor Prof. Dr. Süleyman SERVI. I profoundly valued him for his precious advice, encouragement, guidance that helped me to comprehend the technical elements of this work effectively, and his capacity to explain even the most complex ideas so that it was entirely logical and clear.

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**Pshtiwan Raheem SABIR**

ELAZIĞ, 2019

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## Acı Badem Çekirdeğinden Amigladin Molekülünün İzolasyonu

**Pshtiwan Raheem SABIR**

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Kimya Anabilim Dalı

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Acı badem çekirdeğinde bulunan bir siyanoglikosit olan amigdalin ve onun epimeri olan neoamigdalin geleneksel tıpta önemli bir rol oynamaktadır. Bu tezde, amigdalin molekülü diastereomerik saflıkta acı badem çekirdeğinden elde edilen ekstraktların katı-sıvı, sıvı-sıvı ekstraksiyonu ve kromatografik metotlarla saflaştırılarak izole edildi. Amigdalin'in yapısal karakterizasyonu, FT-IR, UV-VIS, $^{1}H$ ve $^{13}C$ NMR gibi spektroskopik teknikler kullanılarak yapıldı.

**Anahtar Kelimeler:** Epimer, Siyanoglikozit, Neoamigdalin, Amigdalin, Acı badem

# ABSTRACT


The Isolation of Amygdalin Molecule from Bitter Almond Kernel

**Pshtiwan Raheem SABIR**

Master's Thesis

FIRAT UNIVERSITY
Graduate School of Natural and Applied Sciences

Department of Chemistry

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Amygdalin and its epimer neoamygdalin, a cyanoglycosides found in the bitter almond kernel, play an important role in traditional medicine. In this thesis, the amygdalin molecule was isolated in diastereomeric purity by purification of extracts obtained from bitter almond kernels by solid-liquid, liquid-liquid extraction and by chromatographic methods. The structural characterization of amygdalin was performed using spectroscopic techniques such as FT-IR, UV-VIS, $^{1}H$, and $^{13}C$ NMR.



**Keywords:** Cyanoglycoside, Neoamygdalin, Amygdalin, Bitter almond

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# SYMBOLS AND ABBREVIATION

## Abbreviation

TCM : Traditional Chinese Medicine

HCN : Hydrogen Cyanide

ppm : Parts Per Million

NCI : National Cancer Institute

FDA : Food and Drug Administration

UGT : Uridine 5'-diphospho-glucuronosyltransferase

CYP71 and CYP71: Both of them enzymes

CNG : Cyanogenic glycosides

AST : The enzyme aspartate aminotransferase

ALT : The enzyme alanine aminotransferase

TNF-α : Tumor Necrosis Factor-Alpha

Interleukin-1β (IL-1β): Leukocytic Pyrogen or Leukocytic endogenous mediator

C-Fos : Proto-Oncogene

(COX)-2 : Cyclooxygenase-2

IL-2 : Interleukin-2

TGF-β : Transforming Growth Factor-Beta

CMM : Chinese Materia Medica

HPLC : High-Performance Liquid Chromatography

TLC : Thin Layer Chromatography

$^{1}H$ –NMR : Proton Nuclear Magnetic Resonance

$^{13}C$-NMR : Carbon-13 Nuclear Magnetic Resonance

TMS : Tetramethylsilane

$CDCl_3$ : Deuterated chloroform

LLE : Liquid-Liquid Extraction

FT-IR : Fourier-Transform Infrared Spectroscopy

DSC : Differential Scanning Calorimetric

$D_2O$ : Deuterium oxide

DMSO-$d_6$ : Dimethyl sulfoxide-$d_6$

CC : Column Chromatography

IUPAC : International Union of Pure and Applied Chemistry

# 1. INTRODUCTION

Plants have been used worldwide to cure many illnesses. In different parts of the global native persons use different parts of herbs to inhibit and cure illnesses [1, 2]. Based on the traditional use of these plants, researchers examine the effects on many diseases [3, 4]. Because plants are natural, are compatible with the body's immune system and have few side effects, they have a curative effect in modern medicine and these plants are used for the development of pharmaceutical products [4].

Almond kernels (*Prunus amygdalus*) are a product that is useful for human health. It is a naturally edible kernel and it has high commercial value for the food and cosmetics industries. Almond kernels are classified as sweet and bitter depending on the composition of the chemicals their contain. The bitter almond core contains 40 times more hydrogen cyanide (HCN) than the sweet almond core. While sweet almonds are widely used as the major ingredient in food products, bitter almonds provide the primary source of almond oil, which is used both as a sweetener and as a component in cosmetics [4, 5].

First, before use as a drug, bitter almonds must be processed under the supervision of Traditional Chinese Medicine (TCM) theory. According to TCM theory protective amygdalin in the bitter almonds and enzymatic preventing. Amygdaline concentration from the bitter almonds rises after handling and good conserved. In TCM scalded and stir-fried bitter almond specimens of popular medical medicines [1, 9]. The effectiveness of asthma and cough relief in processed yields is improved. Bitter almond toxicity is decreased in scalded products, while bitter almond form as stir-fried products is better appropriate for cure asthma and cough of long-term from almonds. Two kind isomers of amygdalin are exist were found in recent research. *D*-amygdalin is noted to be racemized to neoamygdalin in hot water or weak basic medium [6, 7].

Amygdalin's chemical name is *D*-mandelonitrile-*β*-*D*-glucoside-6-*β*-glucoside and chemical formula $C_{20}H_{27}NO_{11}$ (Figure 1.1). An outdated drug, amygdalin has several benefits such as antipyretic, antitussive, anticancer impacted. It is used in ancient times to treat many diseases such as asthma, nausea, leprosy, bronchitis, and leukoderma [8, 9].

Amygdalin is one of the most traditional participants of the natural cyanogenic glycoside group. Cyanogenic glycosides are described as the glycosides of α-hydroxy nitrile. The role of amygdalin in nature is thought to be a toxic substance that has the potential to produce hydrogen cyanide and thus defends an insect and herbivorous plant [10, 11]. Amygdalin was first separated in 1830 by French biochemists Robiquet and Boutron-Chalard and investigated in clarity by the German chemist Emil Fisher [12]. Naturally found in the seeds of the *Rosaceae* family containing almonds, apples, apricots, cherries, peaches, pears, plums and quince [13]. Between all organic cyanogenic glycosides, amygdalin was the attention of nutrition chemists' concern as a cause of

bitterness in the regulatory organs and fruits of the plant. Amygdalin also is found at ppm level in several product apple juices [11, 14].

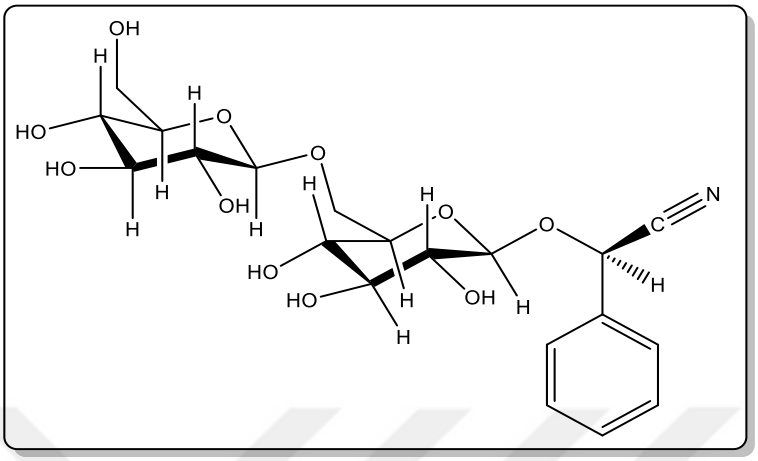


**Figure 1.1.** Chemical structure of amygdalin

## 1.1. Discovery of Amygdalin

First, amygdalin was discovered and purified in bitter almonds (*Prunus dulcis*) via a couple of chemist Robiquet and Boutron-Charlard in 1830 [15]. Amygdalin in 1845 has been used in Russia as an anticancer compound. Amygdalin in the United States used for cancer treats from 1920. Oral and intravenous forms of amygdalin produced by Mexican companies didn't fulfill USA pharmaceutical product standards when evaluated [16]. Moreover, many Americans still use Mexico-produced amygdalin. Effectiveness of the amygdaline cure investigated by the National Cancer Institute (NCI). Of the 22 patient treatment cases, only 6 had good cancer an effect, this report does not support amygdalin effectively to the antitumor effects [17]. Amygdalin was banned in the United States. Nevertheless, in 23 USA states use of amygdalin in the advanced cancer patient's treatment [18]. (NCI) the USA Food and Drug Administration (FDA) decided to assess the effectiveness of the amygdalin cure. An amygdalin clinical study proves to fail to show anti-cancer activity. Later in the USA and Europe, the FDA banned amygdalin. Amygdalin is as it produces cyanide in the UK labeled as a recommended medicine and it can be used under the guidance of a physician. So, like an antitumor medicine manufactured in big quantities in Mexico and used also a medicine. Results much experimental indicates that it has anti-cancerous activity [9, 19, 20].

## 1.2. Stereochemistry of Amygdalin

The amygdalin compound has only the *R*-configuration in the natural and mandelonitrile carbon is asymmetric centers (Figure 1.2). Amygdalin has a total of 11 stereogenic centers, 10 of which are located in two parts of the glycopyranose and are not easily isomerized. The epimerization of this carbon stereocenter occurs in the existence of hot water or a weak basic medium.

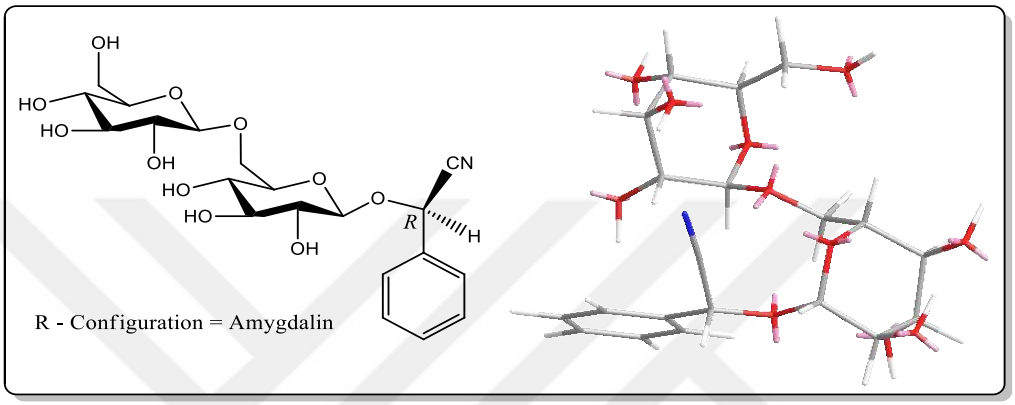


**Figure 1.2.** Chemical structures of amygdalin, *D*-mandelonitrile-*β*-*D*-gentiobioside with the *R*-configuration of the labelled carbon

The *S*-amygdaline (neoamygdalin) has an *S*-configuration on mandelonitrile carbon (Figure 1.3). Since the stereogenic center of the gentiobiose fragments 10 has the same configuration, *R*-amygdaline and *S*-amygdalin are the epimers of each other and not the enantiomer. Therefore, *R*-amygdaline and *S*-amygdalin are diastereomers and could be isolated by using chromatographic and different separation methods. The physical properties of *R*- and *S*- amygdalin can have different [11, 21].

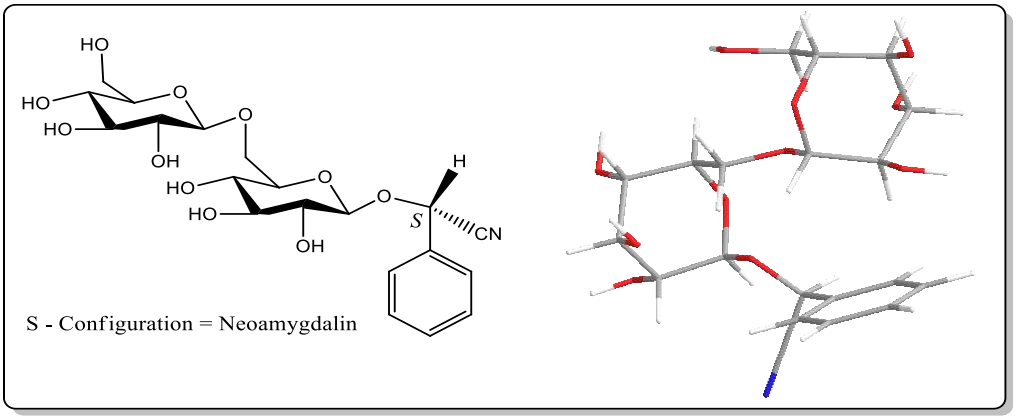


**Figure 1.3.** Chemical structures of *S*-amygdalin (neoamygdalin) epimer or *L*-mandelonitrile-*β*-*D*-gentiobioside

## 1.3. The Physical Property of Amygdalin

Although amygdalin called *D* (-)-mandelonitrile-*β*-*D*-gentiobioside, the chemical IUPAC name is (*R*)-alpha-(6-*O*-beta-*D*-glucopyranosyl-beta-*D*-glucopyranosyloxy)phenylacetonitrile or *R*-amygdalin (Figure 1.1) the molecular formula $C_{20}H_{27}NO_{11}$ and a molecular mass of 457.4 g/mole [22]. It is white fine crystalline powder with a melting point temperature between 213-214 ℃ [23]. But in this study founding melting point of pure amygdalin is 218 ℃ according to the DSC curve. The density of amygdalin is found to be 1.59 $g/cm^3$ [23]. In ethanol-soluble in a certain ratio (83 g/L at 25 ℃) [24]. Insoluble from non-polar solvents such as benzene and chloroform and soluble from polar solvents. Optical activity and molar absorptivity of amygdalin [α] 20/D = 38.3° and 287.4 at lambda max = 261 nm respectively [22, 23].

## 1.4. Amygdalin Biosynthesis

Amygdaline biosynthesis from trees generally occurs in two steps. In the first step, the amino acid phenylalanine convert to phenylacetaldoxime by cytochrome P450s (CYPs) enzymes belong to the CYP79 family in two sequence reactions [25-27]. First decarboxylation and hydroxylated of phenylalanine second dehydration of phenylacetaldoxime it is produced phenyl acetonitrile. The second step phenyl acetonitrile is hydroxylated to mandelonitrile by the CYP71 enzyme added one molecule of glucose to the α-hydroxyl group of mandelonitrile by catalyzed a uridine diphosphate glucose-glucosyl transferase (UGT) prunasin is produced. This is lastly changed to amygdalin by adding another glucose molecule to the 6'-hydroxyl group thus diglucoside gentiobiose makes at the end (Figure 1.4) [22, 28, 29].

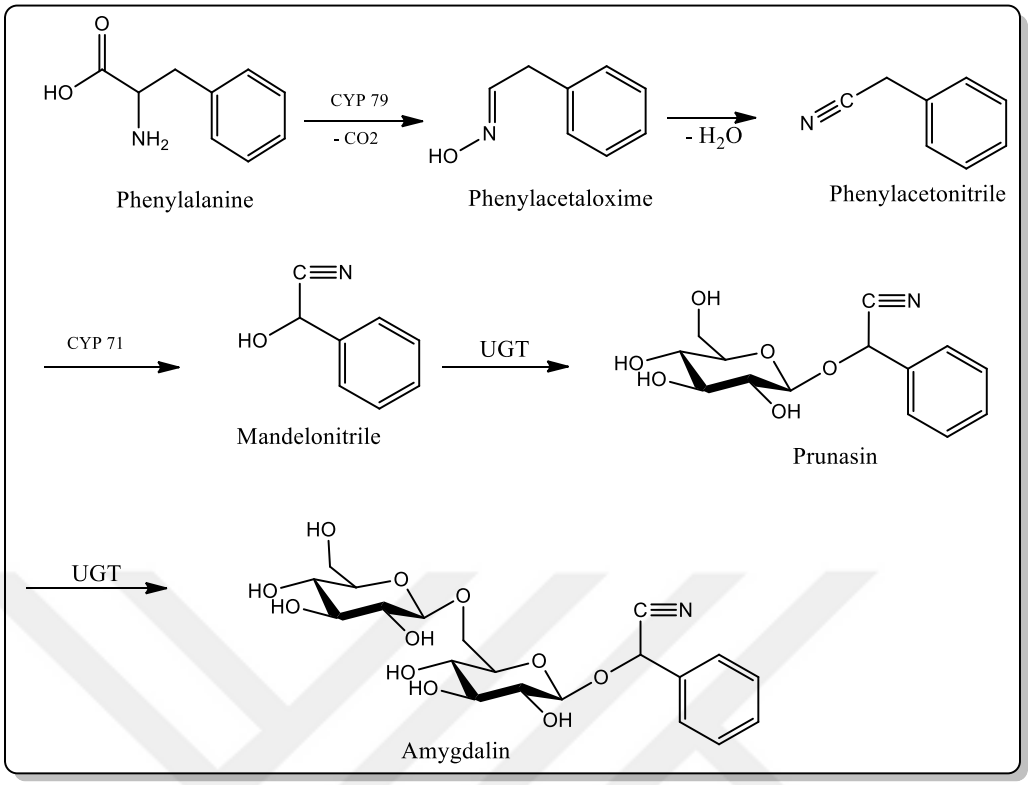


**Figure 1.4.** Biosynthesis of amygdalin

## 1.5. Pharmacokinetics of Amygdalin

Recognized two various metabolic processes for amygdalin orally administered [30]. The first path has been defined as metabolism by enzyme *β*-glucosidase, amygdalin hydrolase in which prunasin (*D*-(-)-mandelonitrile-*β*-*D*-glucoside) is formed and one molecule of glucose released in the proximal small intestine. Prunasin is being hydrolyzed to create mandelonitrile and glucose via another *β*-glucosidase. In neutral or alkaline pH, mandelonitrile non-enzymatically decomposes to benzaldehyde and HCN but the enzymatic existence of mandelonitrile *lyase* is a much quicker change to benzaldehyde and HCN. The second path in the colon by microflora was the *β*-glucosidase driven completely amygdalin hydrolysis to benzaldehyde glucose and cyanide (Figure 1.5) [30, 31].

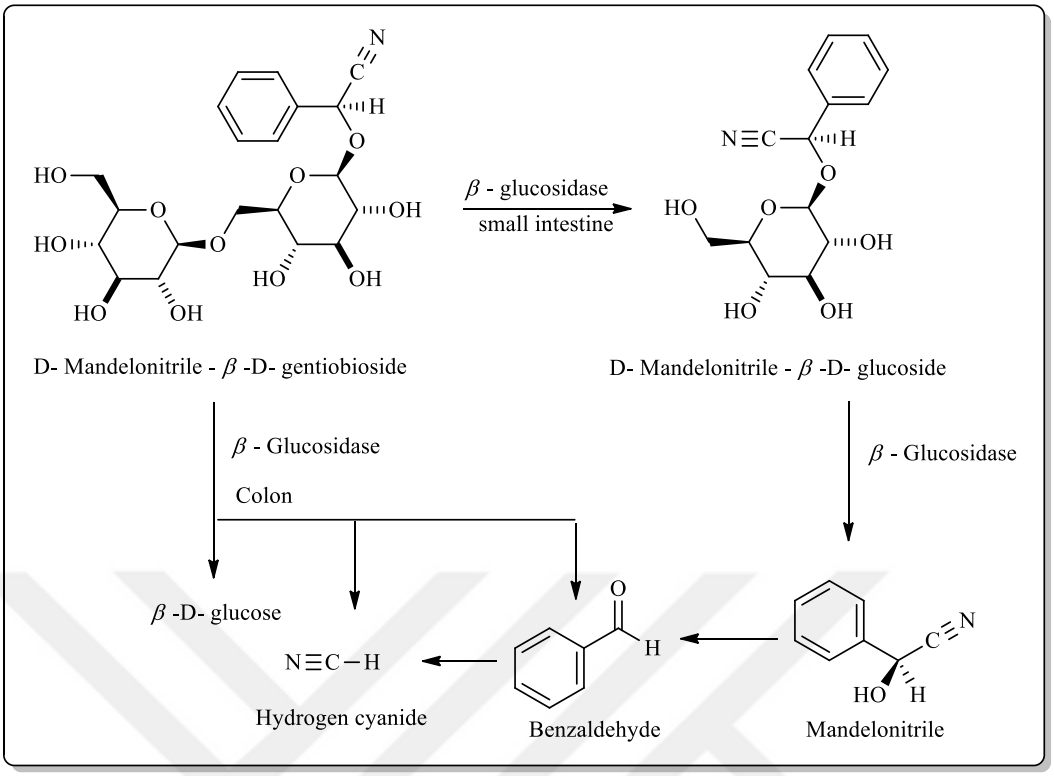


**Figure 1.5.** The route of the amygdalin Metabolization

## 1.6. The Pharmacological Effect of Amygdalin

Amygdalin in bitter almond is an effective component of Traditional Chinese Medicine (TCM). That has always been used as a cough expectorant and cancer treatment auxiliary medicine. The pharmacological effects of amygdalin were classified as follows [19].

### 1.6.1. Antitussive, Antiasthmatic Effect

Amygdalin is decomposed into benzaldehyde and hydrogen cyanide after oral administration that prevents the respiratory center from reaching a certain level and slows the breathing motion and produces antitussive and antiasthmatic effects. Amygdalin used in Traditional Korean Medicine to prevent asthma. Amygdalin can promote pulmonary surfactant synthesis and improve the disease [9, 19, 32-34].

### 1.6.2. Effect on the Digestive System

When the amygdalin decomposed by the enzyme, it produced benzaldehyde and hydrogen cyanide. It can prevent pepsin function and cause damage to the digestive system. 500 mg/kg dose of pepsin hydrolysate achieved from almond water on $CCl_4$ is administered to the rats inhibiting extension of euglobulin lysis time by inhibiting the rate of AST, ALT and increasing

hydroxyproline content. The almond water could prevent the proliferation of rat liver connective tissue and lead to an increase in the ALT, AST level of rat [9, 19, 35, 36].

### 1.6.3. Improve the Apoptosis of Human Renal Fibroblast

Collagenases are enzymes that break the peptide bonds in collagen. The collagenase type I is a crude collagenase preparation that can be used for the isolation of primary cells or for tissue dissociation by enzymatic means [37]. Amygdalin increased kind I collagenase activity released by human renal fibroblast within a time of action and concentration which preventing the certain expression of type I collagenase and renal fibroblasts cell proliferation, enhancing renal fibroblasts cell apoptosis [9, 19, 35, 38].

### 1.6.4. Painkillers Affect

During the examination of the mouse on the hot plate and induced by acetic acid writhing the mouse. It is confirmed that amygdalin has pain-relieving effects. Amygdalin found from (*Prunus armeniaca* L.*)* can lessen formalin and induced alleviate pain in rats. The mechanism has included inflammatory cytokines for example tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and interleukin-1$\beta$ (IL-1$\beta$), as well as c-Fos. In addition, amygdaline produces an anti-inflammatory analgesic effect by preventing the production of prostaglandin and nitric oxide by breaking lipopolysaccharides promoted by cyclooxygenase (COX)-2. From some research, the amygdalin constituent benzaldehyde part plays an important portion in antinociceptive action. Amygdalin has recently been documented to be anti-inflammatory and antinociceptive in microglial cells of the mouse [9, 19].

### 1.6.5. Enhance the Immune Function.

Amygdalin could produce polyhydroxyalkanoates that caused T lymphocytes prevalence and increase blood lymphocytes stimulated by polyhydroxyalkanoates secrete IL-2 and interferon and then inhibit the secretion of TGF-$\beta$1, as a result, enhance immune function. Because regulatory T cells are stimulated. The functions of amygdaline depend on the TGF-$\beta$ process and promote cell death in the plaque region mediated by apoptosis of caspase. Amygdalin has the ability to cure and prevent atherosclerosis [9, 19, 27, 39].

### 1.6.6. Prevent Hyperglycaemia

Amygdalin inhibits the alloxan made hyperglycemia that relies on the effective concentration of medicine in the blood. Some research has shown that amygdalin has a

therapeutic effect on gastric ulcers. Research has shown us that the amygdalin will have a therapeutic effect on gastric ulcers [9, 19, 40].

### 1.6.7. The Toxicity of Amygdalin

Research has shown that amygdalin through the intravenous route is less toxicity and good than the oral administration toxicity. Because of after oral administration amygdalin was hydrolyzed by intestinal microbial and more hydrocyanic acid produced. For avoiding the toxicity of amygdalin we need reduced oral doses and prevent the growth of intestinal microbial [9, 19, 40-42].

# 2. LITERATURE REVIEW

Amygdalin one of the most important compounds of bitter almond. Almond seed extract also has antioxidant and antibacterial properties. It has been shown that the use of bitter almond seed or essential oil helps prevent stress and prohibit tumor growth during pregnancy [43]. According to some studies, the oral use of bitter almonds and the products derived, therefore cannot be said to have anticancer properties [44, 45]. Bitter almond kernels contain various nutrients such as minerals, vitamins, and fatty acids. The main bioactive components of bitter almonds are prunasin, amygdalin, flavonoids, and phenolic acids, which show anticancer and antioxidant effects [4, 46].

In 1923/1924 first published various synthetic approaches of amygdalin and the structural elucidation. Approximately ten years later Viehoever and Mack discussed the biochemistry of amygdalin and so, chemical and physical property with the physiological characterization of the compound in particular from the hydrolysis hydrogen cyanide is produced [21, 47].

Amygdalin was one of the first cyanoglycosides to be isolated. Containing sugar from any plants and animals compound is a glycoside. Cyanogenic glycoside is a substance that has the capacity of producing cyanogen or hydrocyanic acid and containing sugar. Cyanogenic glucoside sources usually consumed by humans. These include almond kernels, apricot seed, plum seed, cherry seed, and apple seed, in addition to around 150 other ingredients. In 1837 isolated crystalline amygdalin by two French chemists Robiquet and Boutron-Charlard. The scientific name of amygdalin is (*Amygdalin Communis Amara*) the term comes from the bitter almond and could derive much of the substance. Identifies amygdalin as *D*-mandelonitrile-*β*-*D*-glucosido-6-*β*-*D*-glucoside (Figure 1.1). Later demonstrated hydrolysis of amygdalin was possible in two ways. One way, amygdalin enzymatic decomposition into *D*-glucose, benzaldehyde and hydrocyanic acid. The other way, achieving the same end yields as the first ways by hydrolysis of amygdalin in hot dilute mineral acids [48].

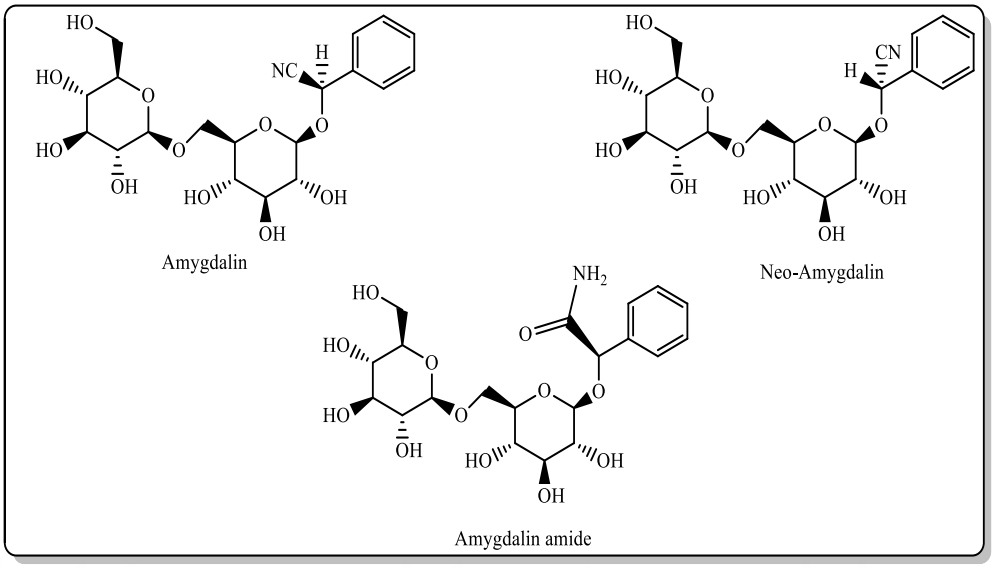


**Figure 2.1.** Chemical structures of the Cyanogenic Glycosides (CNG) present in the almond kernel

On the one side, this compound is described greatly therapeutic effects such as anti-inflammatory and analgesic drugs [49] and as an alternative cure for asthma, bronchitis, emphysema, leprosy, and diabetes [49, 50]. On the other side, amygdalin is recorded as a hazardous substance able to produce highly toxic effects in humans, even including death [50, 51].

Naturally in bitter almonds might exist amygdalin, neoamygdalin, amygdalin amide (Figure 2.1). The amygdalin isomers different the concentration ratio in raw, scalded and the stir-fried seed of bitter almonds [6].

## 2.1. Traditional and Modern Medicine Used Amygdalin

Under advice to Traditional Chinese Medicine (TCM), the theory processing of Chinese Materia Medica (CMM) is a pharmaceutical process used to accomplish the various necessities of treatment. The purposes of processing are to decrease the toxicity of crude medicines [52]. From medicinal materials, almonds are edible kernels that have two kinds in its natural and high commercial value for the food and cosmetic productions. There have been many studies conducted to check the effects of bitter almonds in inhibiting and curing illnesses. Whereas bitter almonds have different health advantages. It will have toxic and harmful effects on the body because of containing hydrogen cyanide which is the source of bitter almond tasting [4, 53, 54]. The use of almonds in China as an antitussive drug has a long history [4, 6]. It was shown that bitter almond seed causes antioxidant, antibacterial and anticancer effects because certain compounds such as amygdalin are contained [4]. Amygdalin was used for treatment aplastic anemia, asthma, high blood pressure, atherosclerosis, diabetes antitussives, migraine [55].

Amygdalin is not toxic by itself but it is decomposed by enzyme produces toxic material HCN [33]. According to several modern studies the small quantity of hydrocyanic acid can help to alleviate asthma and cough. Moreover, hydrocyanic acid and benzaldehyde are useful for cancer patients [49]. Amygdalin has recently been found to be able to selectively kill cancer cells at the tumor site without systemic toxicity [56]. Amygdalin is an ancient drug that was used for treating asthma, bronchitis, antipyretic, leukoderma, antitussive and anticancer [55]. Amygdalin's antitumor effect is one of the newest issues in recent times. Amygdalin has been used in many countries for treatment [9].

## 2.2. Quantification of Amygdalin

The analytical procedure is one crucial aspect of the extraction step from food samples. It was reached the conclusion that the efficiency of the extraction of cyanogenic glycosides can vary significantly depending on the grinder and the methods of extraction. In hot water, amygdalin cans epimerize to neoamygdalin in addition to enzymatic decomposition [57-60]. Approximately 35% of the amygdalin level in methanol/water was stated to be transformed into neoamygdalin during the 24-hour room temperature extraction process using methanol. In several situations, this could lead to a significant underestimation of the sum of cyanogenic glycosides. Water containing 0.1% citric acid, methanol and ethanol are often used for the extraction of amygdalin [61, 62]. High-Performance Liquid Chromatography (HPLC) with ultraviolet detection and HPLC with diode-array detection were commonly used to measure amygdaline after extraction of food samples [59]. Suggested use of cyclodextrin chiral columns and porous graphite carbon columns to selectively isolate amygdalin and neoamygdalin [11]. The researchers found that the transition of amygdalin to neoamygdalin relies on glassware and therefore proposed inert plastic containers for the storage of aqueous solutions amygdalin. Throughout their analysis, commercial amygdalin preparations contained higher quantities of neoamygdalin and about 5 percent of other products for degradation [22, 62]. Thin Layer Chromatography (TLC) is mostly an external process that demonstrates more qualitative than quantitative concepts. It can be used as a method of evaluation [63].

## 2.3. Isolation of Amygdalin

Conventionally the process of extraction of chemical compounds of pharmaceutical materials is decoction in boiling hot water. But from now the popular amygdaline extraction techniques are soxhlet extraction, reflux extraction, and ultrasound extraction. By emulsion enzyme, amygdalin is degraded into *D*- glucose, benzaldehyde, and hydrocyanic acid. In aqueous solvents, hot water or basic medium amygdalin converted to neoamygdalin its epimerization

occurs [61]. By convert the pH of the basic medium or before boiling of the aqueous solution added 0.05% citric acid for preventing the change of amygdalin to neoamygdalin [57]. They have some method directly or indirectly for recognition of amygdalin direct method by used gas chromatography, mass spectrometry, HPLC and TLC indirect by measurement of enzymatically derived benzaldehyde [64]. HPLC was performed mainly for the determination of amygdalin. Generally, the stationary phase with a C18 column and mobile phase as a mixture of (methanol, acetonitrile, water). A solid-phase extraction is a standard form of pretreatment of samples. The benefits include the retention of solvents, high selectivity, and simple procedure. It has been commonly used to isolate and concentrate goal compounds in complex specimens, for example, herbal medicines (Figure 2.2) [61].

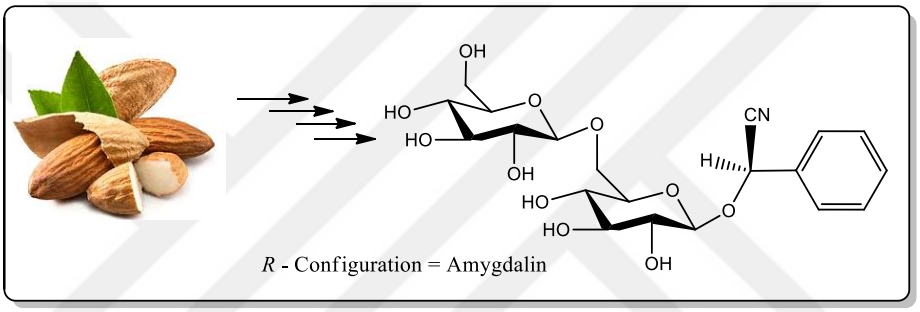


**Figure 2.2.** Isolation of amygdalin molecule

## 2.4. The aim of this Thesis

The aim of this thesis is to isolate the amygdalin molecule from bitter almond kernels. The diastereomeric *R*-amygdaline was purified by chromatographic methods such as thin-layer chromatography and column chromatography. The structural characterization of amygdalin was performed using spectroscopic techniques such as FT-IR, UV-VIS, $^{1}H$, and $^{13}C$ NMR.

# 3. MATERIALS AND METHODS

In this work, instruments, laboratory equipment and apparatus available in the chemistry department were used.

## 3.1. Materials

We used the soxhlet and reflux apparatus for extracting compounds from the solid sample also digital scale (Denver APX-200) for weighing. Rotary evaporator under reduced pressure used for evaporating solvents (Heidolp 4001). Silica gel 60 (0.063-0.200 mm) for column chromatography. TLC aluminium sheets 20 X 20 cm silica gel-60 (Merck). HPLC was performed with the Shimadzu Prominence-I LC-2030C 3D Model with a PDA detector. $^{1}H$ and $^{13}C$-NMR spectrums were recorded with BRUKER 400 MHz-NMR and 100 MHz-NMR spectrometer respectively with TMS as internal standard, all the spectrum stated were carried out in DMSO-$d_6$, $D_2O$ and were reported in ppm. FT-IR spectra of the samples were recorded on Perkin Elmer spectrum. UV-VIS spectra were recorded on the HR 2000 CG-UV-NIR High-Resolution spectrometer. The melting points were determined by differential scanning calorimetric with a Perkin Elmer instrument operated at a scanning rate of 10 ℃/min on heating. Vacuum oven for drying sample and we use solvents methanol (Sigma-Aldrich), ethanol (Sigma Aldrich), chloroform (Sigma-Aldrich), *n*-hexane (Sigma-Aldrich), petroleum ether (Fisher Scientific), ethyl acetate (Sigma- Aldrich). Some of the apparatus include beakers, separation funnel, dropper, measuring cylinder, funnels, glass column for preparative chromatography, hairdryer, and pipettes, round bottom flask, mortar and pestle, oven for drying apparatus and use bitter almond for sampling.

## 3.2. Methods

In this study, we used two different solid-liquid extraction methods to obtain the extracts. One of these methods is Soxhlet and the other is reflux extraction. The extracts were subjected to liquid-liquid extraction with different solvents.

### 3.2.1. Sample Preparation

Braked the bitter almond and isolated the seed removed the foreign matters. The seed transferred to the hot water for 5 minutes after the seed was put out to eliminate the coatings by hand to give the scalded seeds. Stay at the room temperature until drying after dehydrated at 75℃ for 24-hours, seeds crushed by mortar and pestle. Some of the crashed seeds heating with stirring when scalded almond seeds became yellow we obtained the stir-fried samples [6].

### 3.2.2. Extraction of Amygdalin

Extraction and purification of natural products is the most important and critical process to isolation compound from plants. This is to ensure that most, if not all, of the target molecule, is within the extraction process. There are also several very significant reasons to this end, in order to achieve maximum productivity, also to know the exact rate in w/v, w/w or v/v of the main product of a whole sample. Also, this would make it possible to interact with other work/research. Different extraction methods mentioned for extracting amygdalin compound ultrasonic extraction, soxhlet extraction, and reflux extraction for any methods have some beneficial and damage. We choice the Soxhlet extraction and reflux extraction method for this thesis [65].

## 3.3. Soxhlet Extraction Method for Amygdalin

For the extraction and isolation of the amygdalin compound from the bitter almonds seed, we used the soxhlet extraction method. According to [50] we gained higher extraction quantities of amygdalin from the seeds by used Soxhlet at the same time extracted three or twice time excess quantities of amygdalin from apple seeds gained compare to the reflux and microwave method.

**Figure 3.1.** Soxhlet extraction setup used for solid-Liquid extraction of amygdalin from powdered bitter almond seed

The soxhlet procedure is as follows. 50 gram dried powder of the scalded and stir-fried bitter almonds seed is added into the thimble. The thimble containing the sample is placed in the

Soxhlet extractor chamber. 750 mL of ethanol is added into the flat-bottomed flask. Continuous extraction for 29.5 hours with heating until poling point of the ethanol and approximately 59 cycles. By attaching a condenser, the setup is completed (Figure 3.1).

Then all of the solvent (ethanol) extracted collecting and evaporated by using a rotary evaporator until concentration ethanol, after used for liquid-liquid extraction.

## 3.4. Liquid-Liquid Extraction (LLE)

Liquid-liquid extraction (LLE) also known as solvent extraction and partitioning is a technique of separating compounds in two separate immiscible fluids, depending on their relative solubility. Immiscible fluids are those that, when shaken together, cannot be blended together and divided into layers. LLE is an extraction into another liquid phase of material from one liquid [66]. After the extraction stage, a clean-up of the residue by solvent partition is required in all methods developed for liquid-liquid extraction. Many solvents (petroleum ether, chloroform, and hexane) were used to remove the saturated or unsaturated fatty acid [67].

### 3.4.1. Liquid-Liquid Extraction with Hexane

All of the solvent (ethanol) collecting from the soxhlet extraction, after concentrated and poured into the separation funnel, added 100 mL of hexane shaking together 1-2 minute stay until separated hexane layer from the ethanol layer. The hexane layer is upper phase ethanol layer is a bottom phase because of the density of hexane is less than ethanol (Figure 3.2). Amygdalin doesn't soluble in hexane. Hexane phase removed any hydrophobic contaminants from ethanol such as oils and stay at room temperature until evaporating.

**Figure 3.2.** Liquid-liquid extractions, separation of hexane and ethanol

### 3.4.2. Liquid-Liquid Extraction with Petroleum ether

After the hexane phase was removed, the ethanol phase was washed by 100 mL of petroleum ether. Petroleum ether and ethanol phase poured into the separation funnel shaking together 1-2 min. stay until separated petroleum ether layer from the ethanol layer. The petroleum ether layer is upper phase ethanol layer is a bottom phase because of the density of petroleum ether is less than ethanol (Figure 3.3). Petroleum ether phase removed contaminants from ethanol and stay at room temperature until evaporating.

**Figure 3.3**.Liquid-liquid extractions, separation of petroleum ether and ethanol

### 3.4.3. Liquid-Liquid Extraction with Chloroform

The ethanol phase was transferred into a separation funnel and washed with 100 ml of chloroform. When chloroform added into separation funnel, shaking together 1-2 min. directly acquire precipitate (Figure 3.4). We removed precipitate and re-washed by chloroform.

**Figure 3.4.** Liquid-liquid extractions, separation of chloroform and ethanol

### 3.4.4. Liquid-Liquid Extraction with Ethyl Acetate

The remaining ethanol phase was washed with 100 mL ethyl acetate. When ethyl acetate added into separation funnel, shaking together 1-2 min. directly acquire precipitate (Figure 3.5). We removed precipitate collecting extract and concentrated 30 mL by using a rotary evaporator. Concentrating extracted stay at room temperature until dry it and used for the analysis of TLC and Column Chromatography.

**Figure 3.5.** Liquid-liquid extractions, separation of ethyl acetate and ethanol

## 3.5. Reflux Extraction Method for Amygdalin

The suitable method for extraction from the bitter almonds of the amygdalin is the reflux extraction method [53]. Reflux extraction is required less solvent and extraction time [69]. The reflux procedure will be as followed. 50 gram dried powder of the scalded and stir-fried bitter almond seed is added into the flat round bottom flask. 750 mL of ethanol added into the flat-bottomed flask. Continuous extraction for 3 hours with heating until the boiling point of the ethanol with stirring (Figure 3.6).

**Figure 3.6.** Reflux extraction setup used for solid-liquid extraction of amygdalin from powdered bitter almond seed

After extraction, the extract was separated from the solid material by the filtering process. The extracted was evaporated by using a rotary evaporator until it remains 60 mL (Figure 3.7). For cleaning and removed unwanted organic compounds, we used hexane, petroleum ether and chloroform as solvents for the decantation method.

**Figure 3.7.** Extracted after filtration

## 3.6. Decantation Methods

Decantation is the method of separating the liquid from solid and other non-mixable fluids by removing the liquid layer at the top of the solid or liquid layer below. Many solvents (petroleum ether, chloroform, and hexane) were used to remove the saturated and unsaturated fatty acid [67].

### 3.6.1. Washing Extracted with Hexane

After reflux extraction, the extract was concentrated until around 60 mL by using a rotary evaporator. Added 20 mL of hexane to concentration extracted and stirring one minute after stay until the acquire upper layer (Figure 3.8). The bottom layer contains the target compound. The upper layer which is the hexane phase contains the unwanted compound, for example, a fatty compound. We removed the hexane phase by the decantation method.

**Figure 3.8.** Washing extracted with hexane

### 3.6.2. Washing Extracted with Petroleum Ether

The ethanol phase was washed with petroleum ether. 20 mL of petroleum ether was added to the ethanol phase and shaken 30 seconds after directly precipitate acquire (Figure 3.9). We separated the upper phase and remaining precipitate with decantation methods.

**Figure 3.9.** Washing extracted with petroleum ether

### 3.6.3. Washing Precipitate with Chloroform

The dry precipitate washing by chloroform (Figure 3.10). The precipitate obtained here was used for the analysis of TLC and Column chromatography.


**Figure 3.10.** Washing precipitate with chloroform

## 3.7. TLC for Choosing the Best Solvents (Mobile Phase) for Extracts

For the determination of the best solvents system we used different solvent mixture ratios and run the TLC plate. That can be used to separate amygdalin by column chromatography. Some

TLC solvents system for amygdalin has been reported [63]. We use TLC aluminium sheets 20 X 20 cm Silica gel 60 (Merck) and different solvent mixtures. The best solvent systems gave us the highest number of visible spots and better separation.

**Table 3.1** Different solvent mixture ratios used in this thesis for TLC as eluents

| TLC Plate Number | The mixture of Solvent System | Solvent Ratios |
|---|---|---|
| 1 | Ethyl acetate / Hexane | 1:1 |
| 2 | Ethyl acetate / Hexane | 1:3 |
| 3 | Ethyl acetate / Hexane | 2:1 |
| 4 | Ethyl acetate / Hexane | 1:4 |
| 5 | Ethyl acetate / Hexane | 1:4 |
| 6 | Ethyl acetate / Hexane | 3:1 |
| 7 | Ethyl acetate / Hexane | 4;1 |
| 8 | Ethanol / Chloroform | 1:1 |
| 9 | Ethanol / Chloroform | 1:2 |
| 10 | Ethanol / Chloroform | 1:3 |
| 11 | Ethanol / Chloroform | 1:4 |
| 12 | Ethanol / Chloroform | 2:1 |
| 13 | Ethanol / Chloroform | 3:1 |
| 14 | Ethanol / Chloroform | 4;1 |
| 15 | Methanol / Chloroform | 1:1 |
| 16 | Methanol / Chloroform | 1:2 |
| 17 | Methanol / Chloroform | 1:3 |
| 18 | Methanol / Chloroform | 1:4 |
| 19 | Methanol / Chloroform | 2:1 |
| 20 | Methanol / Chloroform | 3:1 |
| 21 | Methanol / Chloroform | 4:1 |
| 22 | Methanol / Chloroform | 1:1 |
| 23 | *n*-Butanol / Acetic acid | 1:2 |
| 24 | *n*-Butanol / Acetic acid | 1:3 |
| 25 | *n*-Butanol / Acetic acid | 1:4 |
| 26 | *n*-Butanol / Acetic acid | 2:1 |
| 27 | *n*-Butanol / Acetic acid | 3:1 |
| 28 | *n*-Butanol / Acetic acid | 4:1 |
| 29 | *n*-Butanol / Formic acid / Water | 3:2:1 |
| 30 | *n*-Butanol / Formic acid / Water | 1:1:1 |
| 31 | *n*-Butanol / Acetic acid / Water | 3:2:1 |
| 32 | *n*-Butanol / Acetic acid / Water | 1:1:1 |
| 33 | *n*-Butanol / Acetic acid / Water | 2:1:1 |
| 34 | Methanol / Chloroform / Hexane | 1:1:1 |
| 35 | Methanol / Chloroform / Acetone | 1:1:1 |
| 36 | Methanol / Chloroform / Ethyl acetate | 1:1:1 |
| 37 | Methanol / Chloroform / Hexane | 1:2:1 |
| 38 | Methanol / Chloroform / Hexane | 1:1:2 |
| 40 | Methanol / Chloroform / Ethyl acetate | 1:2:1 |
| 41 | Methanol / Water | 1:1 |
| 42 | Ethanol / Water | 1:1 |

TLC chromatograms obtained from these tests were shown in the following (Figure 3.11), (Figure 3.12) and (Figure 3.13).

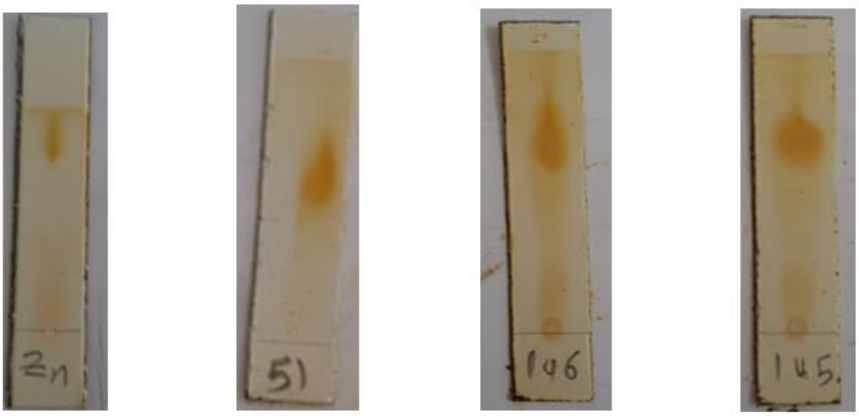


**Figure 3.11.** (A) Pure amygdalin

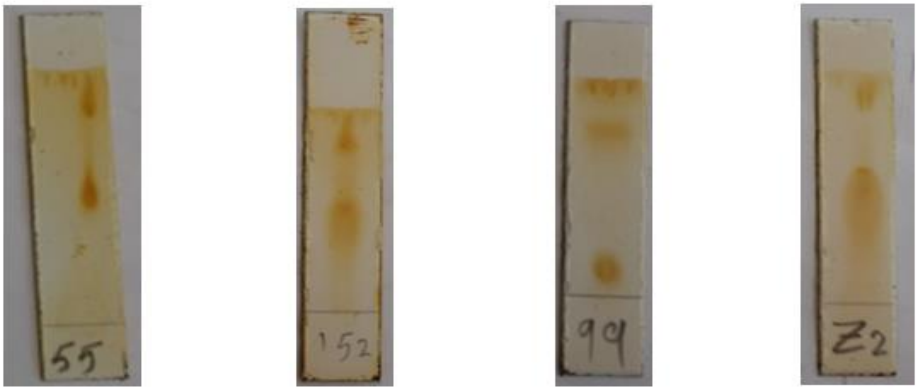


**Figure 3.12.** (B) Semi pure amygdalin

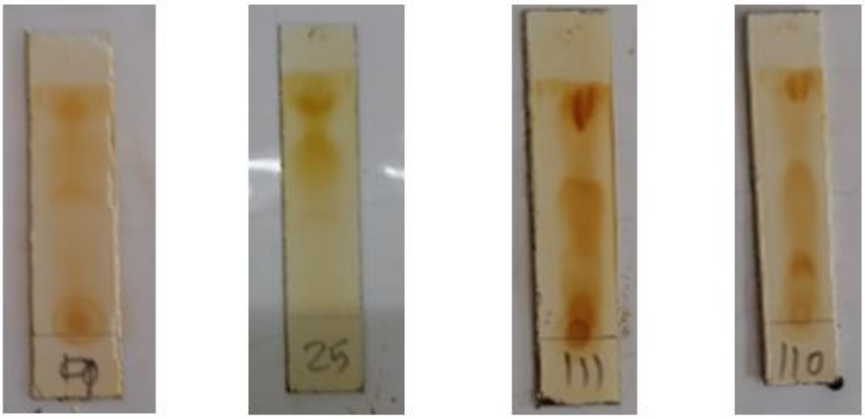


**Figure 3.13.** (C) Is not pure amygdalin

## 3.8. Column Chromatography by Using Silica Gel

40.0 gram of silica-gel was dissolved in the solvent mixtures. Dissolves 1.0-2.0 grams of the sample added to the middle of the sand loading column. The solvent mixture ratio of the eluent is chloroform: methanol (2:1). Collecting 5.0 mL of eluate from each beaker and stay until evaporated eluate and ready for analysis (Figure 3.14).

**Figure 3.14.** Silica gel column chromatographic set-up

## 3.9. Differential Scanning Calorimetric (DSC) of Amygdalin Isolated from the Bitter Almond Kernel

For each sample, the amount of 3.7 mg was placed in a special container. The sample was heated under an inert atmosphere (nitrogen) at a temperature of 50 ℃. The instrument was then injected with oxygen gas until the temperature was adjusted to 310 ℃. For control, the empty container was used. The oxygen flow rate through the instrument was 50 mL/Minute during this dynamic process, while the heat flow rate was 10 ℃ / Minute.

# 4. RESULT AND DISCUSSION

There are serious deficiencies in the current analytical methods used to determine the amount of amygdalin in food samples. When literature studies are investigated, amygdalin and neoamygdalin epimers are analyzed together and this results in misleading results. Amygdalin is epimerized to neo-amygdalin in basic medium, high temperature, and aqueous solution. This situation arises to be an invisible danger in determining the amount of pure amygdalin. Their use as a diastereomeric mixture in biological activity studies leads to some controversy. This causes serious problems over the medicine and pharmacological properties of amygdalin in the scientific world.

The bitter almond cores were divided into three different parts (Figure 4.1).

- the dried almond kernels that are not treated,
- the only peeled almonds,
- The peeled and roasted almond kernels.

The bitter almond kernels were subjected to three different treatments and the ratio of cyanogenic glycosides in their contents changed. In order to obtain extracts of almond kernels, Soxhlet and reflux extraction methods were used.

Each of these almond kernels was obtained total cyanogenic glycosides and other organic compounds by the Soxhlet and reflux extraction method using polar protic solvents such as methanol-water or ethanol.

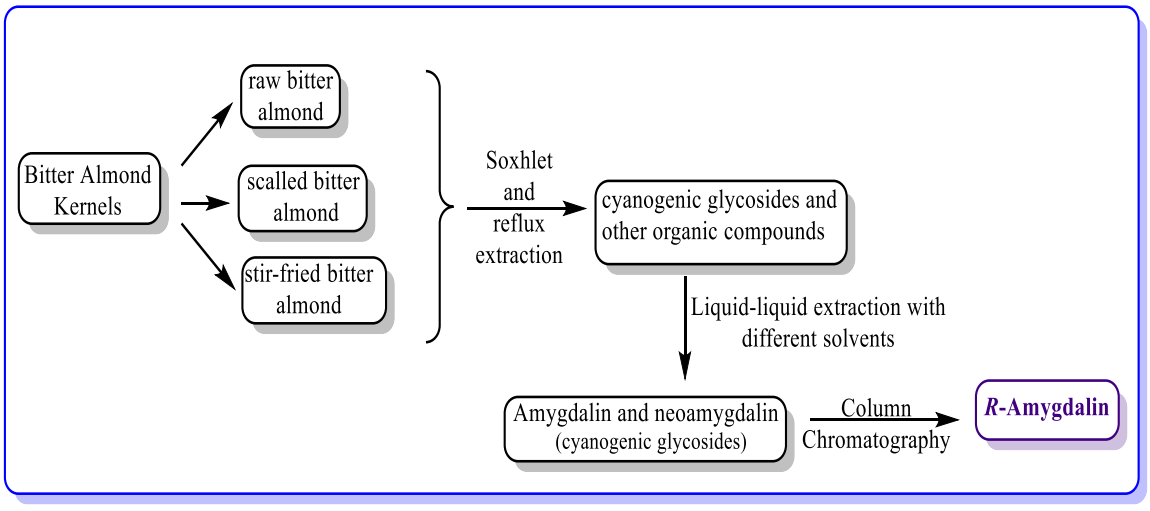


**Figure 4.1.** The stages of the method used in the isolation of amygdalin

In order to obtain the mixture containing total cyanogenic glycosides, such as amygdalin, neo-amygdalin, and amygdalin amide, the extracts were done liquid-liquid extractions by using different solvents, such as chloroform, hexane and petroleum ether.

## 4.1. Observations on the Best Mobile Phase for Separation of Amygdalin by TLC

We tried some solvent system running more than 150 TLC plates and alternating solvent composition and ratios to give better resolution and amygdaline separation. Methanol/chloroform separates our target molecule (amygdalin).

The separation and purification of amygdalin were carried out by using CC.

## 4.2. Determination of Extraction and Pure Amygdalin Yield

The extraction yield (%) was calculated as follows:

$$Extractionyield(\%) = \frac{Weight\ of\ dry\ extract}{Dry\ weight\ of\ sample} \cdot 100 \quad (4.1)$$

$$Extractionyield(\%) = \frac{1.3417g}{100g} \cdot 100 = 1.3417\ \% \quad (4.2)$$

The weight of extracted is 1.3417g using for column chromatography by solvent mixture rate 2:1 (chloroform/methanol). We achieved 0.7481g of the stereoisomers of the amygdalin after column chromatography process.

After purification, we obtained 0.4575 g of stereoisomers amygdalin with 0.2906 g of *R*-amygdalin. This sample contains 97.1% amygdalin and 2.9% neoamygdalin (Figure 4.8).

We isolated amygdalin from the bitter almond kernels without using standard amygdalin in this thesis. The pure amygdalin obtained from the bitter almond kernel is white powder without odor. For the verification of the purity of the extracted amygdalin was used to spectroscopic methods, such as FT-IR, UV-VIS, $^{1}H$ $^{13}C$- NMR, and DSC techniques.

## 4.3. Structural Characterization of the Isolated Amygdalin by FT-IR Analysis

In the first stage of structure characterization, the FT-IR spectrum and melting point of the standard amygdalin obtained from internet sources were used as a reference [71]. Figure 4.2 shows the FT-IR spectrum of the standard amygdalin with KBr disc. Spectra taken with FT-IR device with ATR unit are given in (Figure 4.2) and (Figure 4.3).

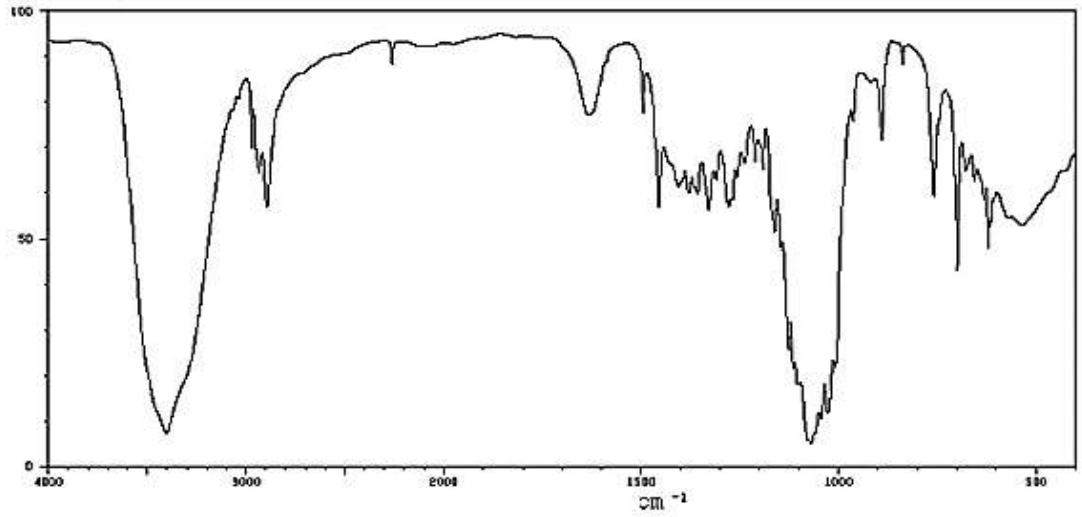

**Figure 4.2.** The FT-IR Spectrum (KBr) of pure amygdalin (Standard amygdalin)

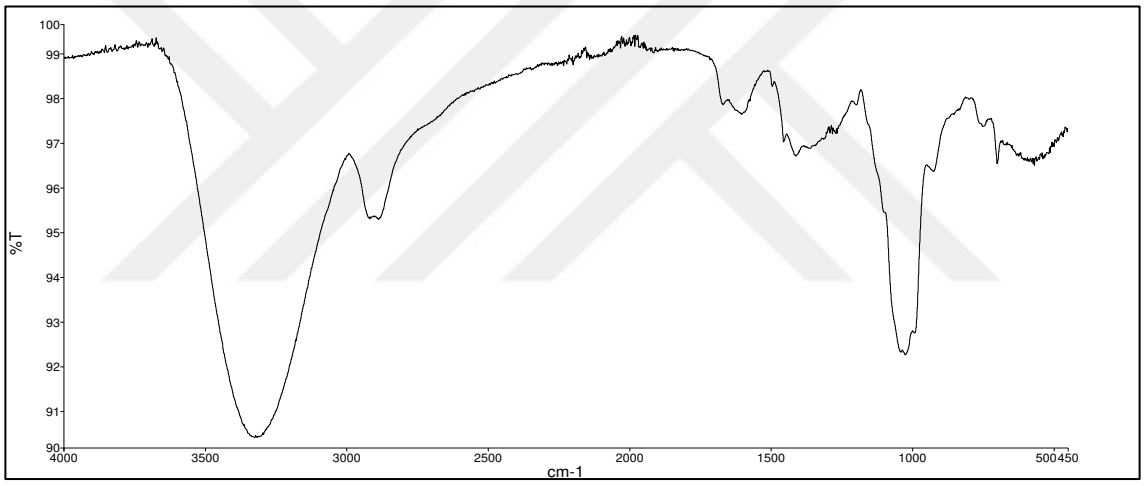


**Figure 4.3.** The FT-IR Spectrum (ATR) of the obtained solid material after solid-liquid extraction from the bitter almond kernel without purification

The A and B rings of the amygdaline molecule have seven different hydroxyl groups (Figure 4.6). One of them is a primary alcohol and the other six are the secondary alcohol functional groups. These have dense and broad bands in the range of 3597-3300 $cm^{-1}$. When the 3600-3300 $cm^{-1}$ region of the FT-IR spectrum of amygdaline was carefully examined, O-H stretching vibrations of five different hydroxyl groups were observed. Of these, the stretching vibrations at 3399 $cm^{-1}$ showed the intramolecular hydrogen bond, while the other bands (3598, 3513, 3461 and 3301 $cm^{-1}$) belong to the O-H stretching vibrations resulting from the intramolecular hydrogen bond (Figure 4.4).

The aromatic and aliphatic C-H stretching vibrations were attributed to lower intensity absorptions at 2971.23 and 2892.67 $cm^{-1}$, respectively. Due to the presence of the CN functional

group in the amygdalin structure, the band at 2263.62 $cm^{-1}$ can be noticed. The two 1632.90 $cm^{-1}$ and 1382.92 $cm^{-1}$ bands are the products of stretching vibration from the benzene ring of C=C bonds. The deformation of the aromatic ring C-H vibrations caused the band to appear at 1455.14 $cm^{-1}$. The benzene ring is monosubstituted by the out-of-plane C-H bending vibrations at 699.36 $cm^{-1}$.

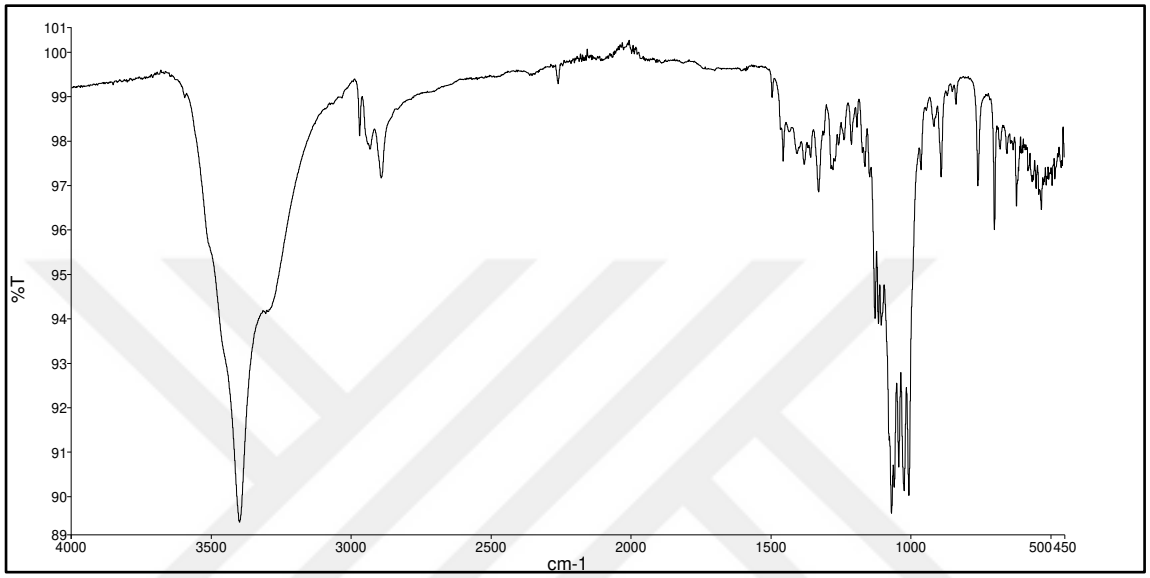


**Figure 4.4.** FT-IR Spectrum (ATR) of the pure amygdalin obtained from the bitter almond kernel in this thesis

The amygdaline molecule contains eleven different C-O bonds. Four of these are C-O-C bonds, while the remaining seven are C-O-H bonds. When the region of 1211-1022 $cm^{-1}$ was analyzed, eleven C-O-C and C-O bond stretching vibration bands were observed. Four of these are C-O-C bonds (two cyclic ethers and two acyclic ethers) and the remaining seven are C-O-H bonds. All these bands that refer to the amygdalin molecule groups without any new vibrations in the IR spectrum indicate the isolated amygdalin's satisfactory purity [68]. In this study, all information concerning the FT-IR spectrum of amygdalin extracted from bitter almond kernel is consistent with its chemical structure (Figure 4.8).

## 4.4. Structural Characterization of the Isolated Amygdalin by UV-VIS Analysis

In this study, the absorption maximums occurred at 215 nm, as well as between 250–254, 255–259, 261– 265, and 267–271 nm. The absorption maximum of the CN group did not notice data due to its low intensity. In simple nitriles, $\pi$ -$\pi$* absorption is in the distant ultraviolet region. Conjugated nitriles are observed at 213 nm and are strong. The UV-Vis spectrum of the isolated

amygdalin was taken in ethanol, mainly 218 nm σ - σ* transitions and π -π* transitions of 256 nm aromatic rings are observed (Figure 4.8).

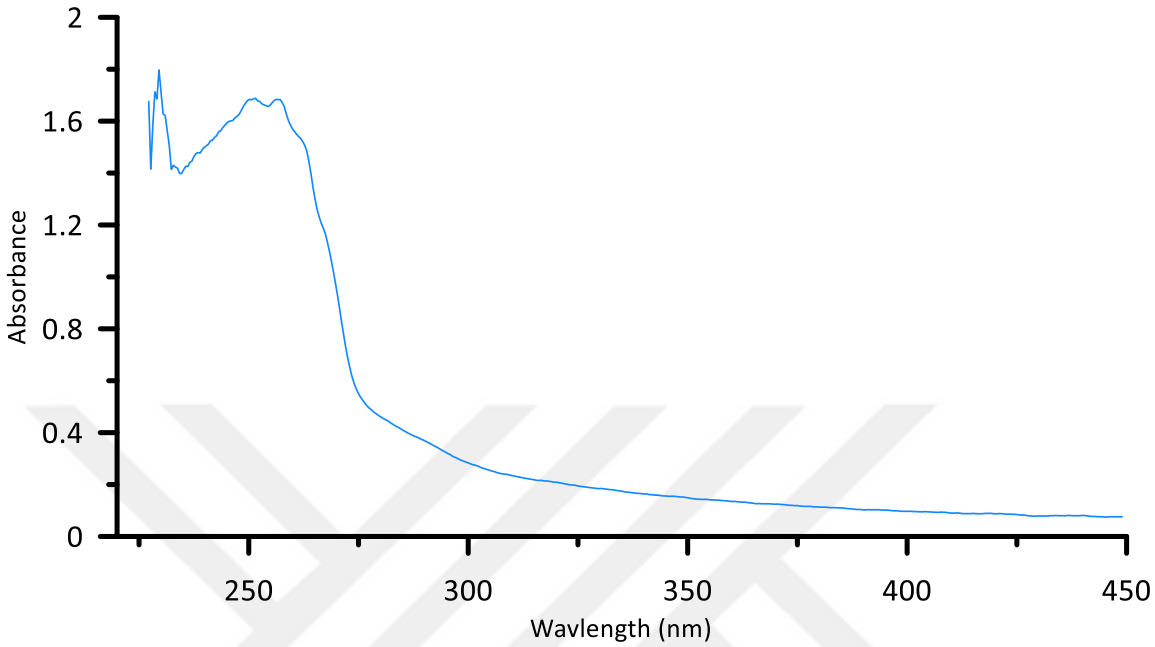


**Figure 4.5.** UV-VIS spectra of amygdalin isolated from the bitter almond kernel's

## 4.5. Analyses of the $^{1}$H-NMR Spectrums of Amygdalin and its Stereoisomers

The $^{1}$H NMR spectrum of the amygdaline and related cyanogenic glycosides are generally taken in the DMSO-$d_6$ solvent [69]. In this thesis, we analysed the detailed $^{1}$H and $^{13}$C-NMR spectra of amygdalin in both $D_2O$ and DMSO-$d_6$ solvents. The $^{1}$H NMR spectrum taken in DMSO-$d_6$ is equivalent to 27 protons, whereas the spectrum is equivalent to 22 protons when taken with $D_2O$.

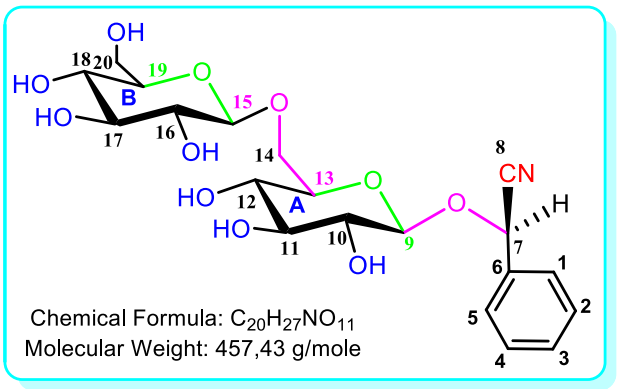


**Figure 4.6.** The numbering system used in the structural characterization of the amygdaline molecule.

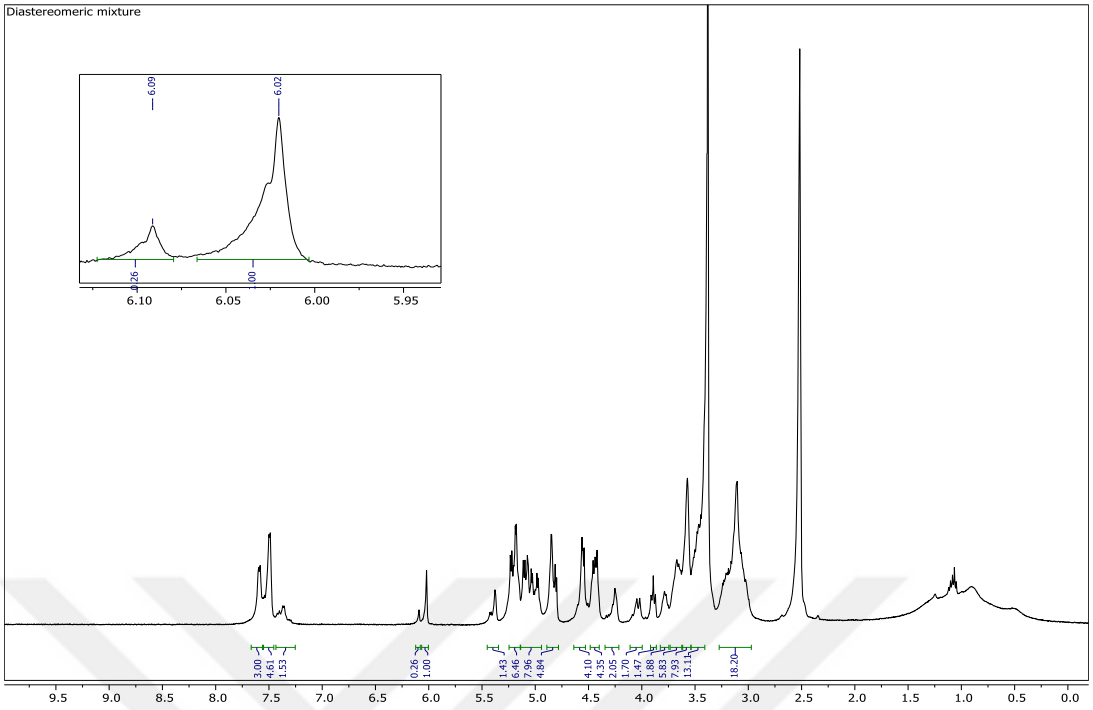


**Figure 4.7.** $^{1}$H-NMR (400 MHz) Spectra amygdalin isolated from the bitter almond kernel's and its stereoisomers (DMSO-$d_6$)

In (Figure 4.7) the spectrum taken in the DMSO-$d_6$ solvent belong to the mixture of amygdaline and neoamygdalin. In this spectrum, the aglycone proton-7, which leads to epimer formation, shifted to a lower area of 0.35 ppm for neoamygdalin compared to the spectrum taken with $D_2O$ in (Figure 4.8). The neoamygdalin proton-7 having an integration rate of 0.26 at 6.09 ppm and the amygdaline proton-7 having an integration rate of 1.00 at 6.02 ppm belong. The amount of amygdaline in this sample has 79.4%, while neoamygdalin has 20.6%. The $^{1}$H-NMR spectrum indicates that it does not belong to a pure compound, but it is a mixture of amygdaline and neoamygdalin epimers. In this study, this diastereomeric mixture was purified using column chromatography and the compound containing 97.1% amygdaline was isolated.

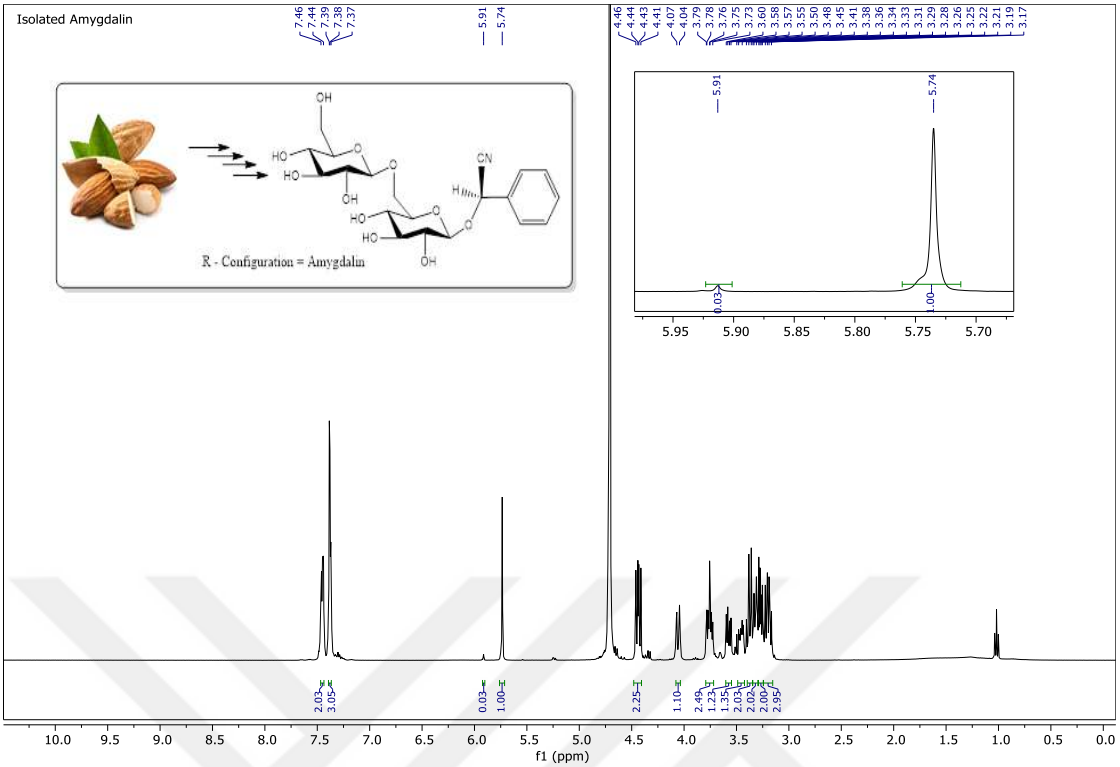


**Figure 4.8.** $^{1}$H-NMR (400 MHz) Spectra pure amygdalin which was isolated from the bitter almond kernels in the thesis ($D_2O$)

In the $^{1}$H-NMR spectrum (Figure 4.8) taken in $D_2O$ solvent of amygdaline, H-1 and H-5 aromatic protons (*ortho*) equivalent to 2 protons at 7.46 ppm and H-2, H-3, H-4 (*meta* and *para*) equivalent to 3 protons at 7.39 ppm aromatic ring protons were observed as a multiplet.

The sharp singlet at 5.74 ppm integrates for one proton in intensity and can be assigned to the CHCN moiety. This aryl-CHCN moiety is an aglycone group with a chiral center. *R* and *S* forms can exist, with epimerization aided by basic or extreme temperature conditions. The CH proton signal of the *S* epimer appears at the lower field than the CH proton signal of the *R* epimer [69].

Two different signals of H-7 (benzylic hydrogen) were observed in the $^{1}$H-NMR spectrum (Figure 4.8) taken in the $D_2O$ solvent. The signal with an integration rate of 0.03 at 5.92 ppm belongs to neoamygdalin whereas the signal with an integration rate of 1.00 at 5.74 ppm belongs to the amygdaline. The amount of amygdaline in the sample was found as 97.1% and neoamygdalin was found as 2.9%. This result is consistent with the results given in Section 4.2.

The remaining 14 proton signals derive from the two glucopyranose rings and yield multiplet signals in a 2: 1: 2: 1: 8 proton resonance pattern between 4.07 and 3.15 ppm. The eight-proton is a highly congested region spanning only 0.3 ppm from 3.40 to 3.34 ppm [69].

$^{1}$H-NMR ($D_2O$) $\delta$ = 7.46-7.44 (H-1 and H-5, m, 2H); 7.39-7.37 (H-2, H-3 and H-4, m, 3H); 5.91 (H-7, s, for neoamygdalin); 5.74 (H-7, s, 1H, for amygdalin); 4.07-4.04 (d, 1H); 3.79-3.73

(m, 2H); 3.60-3.55 (m, 2H); 3.50-3.46 (m, 2H); 3.40- 3.35 (m, 2H); 3.34-3.30 (m, 2H); 3.29-3.24 (m, 2H); 3.24-3.15 (m, 2H).

The signal at 4.70 ppm belongs to the proton residue in the $D_2O$ solvent. In the $^1$H-NMR spectra taken in both $D_2O$ and DMSO-$d_6$ solvents, the signal of impurity around1.00 ppm was observed.

When the $^1$H-NMR spectrum taken in DMSO-$d_6$ was compared with $D_2O$, all signals shifted to the low fields. The solvent change caused a little change in the epimer ratios. The amygdalin amount was 96.2%, while the neoamygdalin amount was calculated at 3.8%. These data were obtained from the integration rates which provided this sample from the $^1$H-NMR spectrum (DMSO-$d_6$).

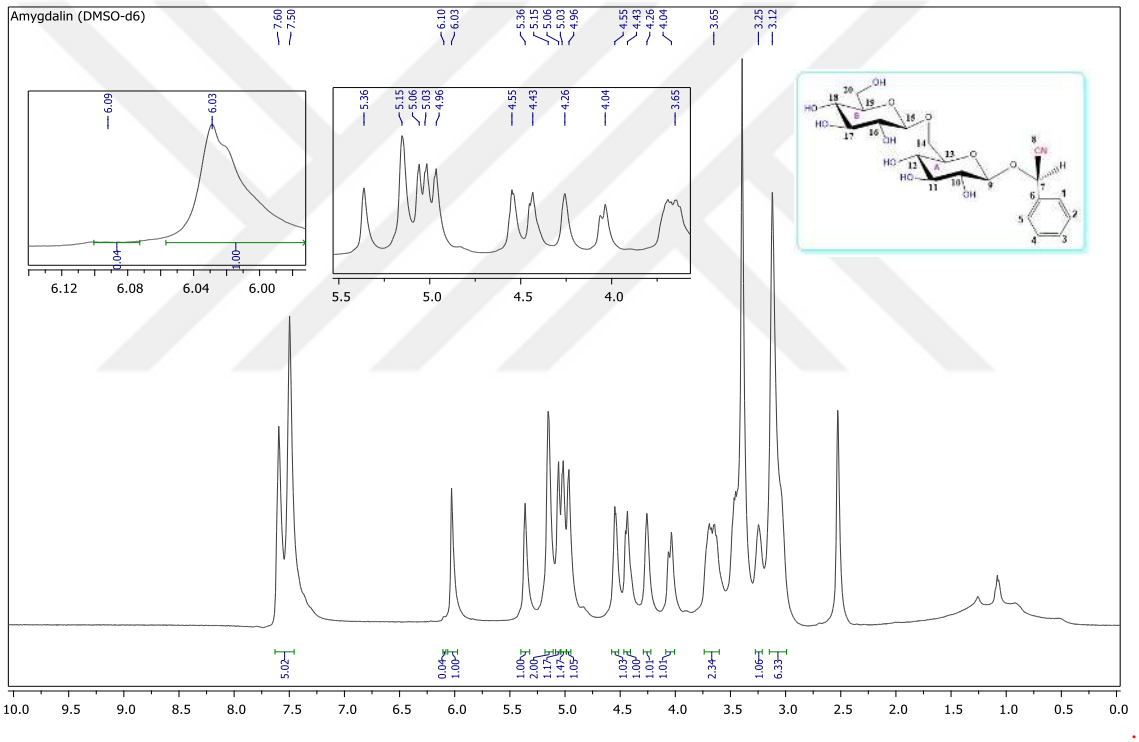


**Figure 4.9.** $^1$H-NMR (400 MHz) Spectra pure amygdalin which was isolated from the bitter almond kernel's in this thesis (DMSO-$d_6$)

## 4.6. $^{13}$C-NMR Spectrum of Amygdalin and its Stereoisomers

17 different carbon signals were observed in two separate $^{13}$C-NMR spectra taken in both $D_2O$ and DMSO-$d_6$ solvents. Two *ortho* and two *meta* protons of the aromatic ring were observed as two different carbon signals. Both C-13, C-17 carbons were observed as the same signal. All other carbons, except C-3, C-7 C-14, C-16 and C-20, resonated in the low fields, as in the $^1$H-NMR spectrum.

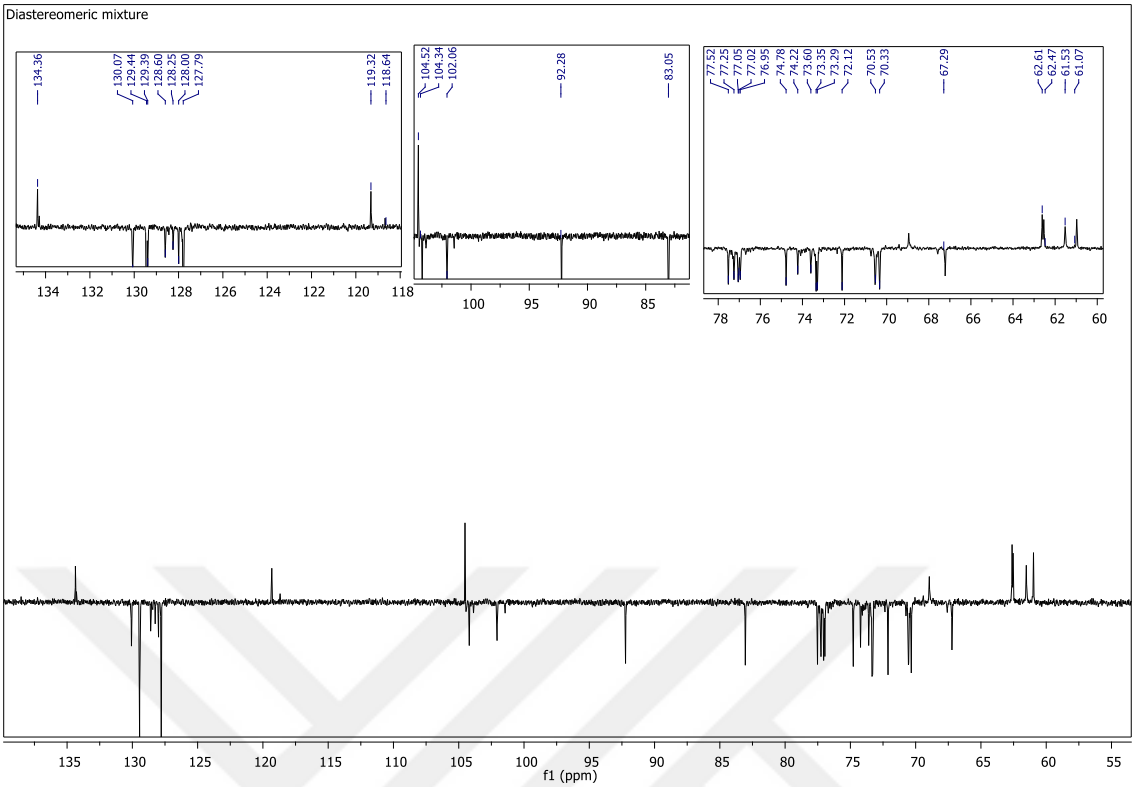


**Figure 4.10.** $^{13}$C-NMR (100 MHz) Spectra of amygdalin isolated from the bitter almond kernel's and its stereoisomers (DMSO-$d_6$)

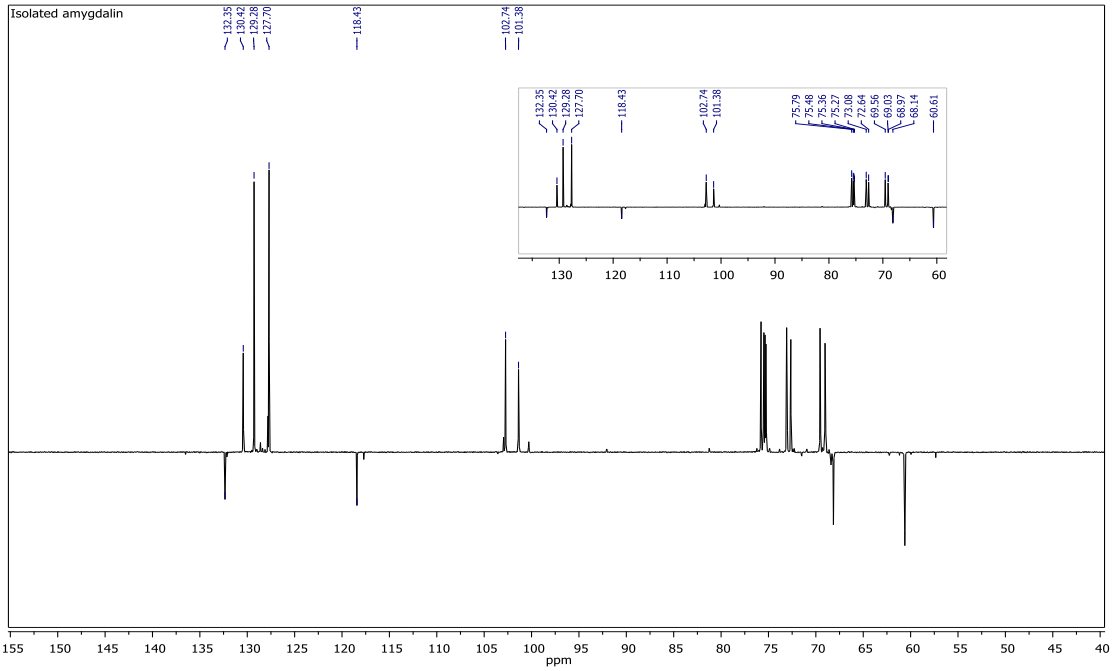


**Figure 4.11.**$^{13}$C-NMR (100 MHz) Spectra of amygdalin which was isolated from the bitter almond kernel's in this thesis ($D_2O$)

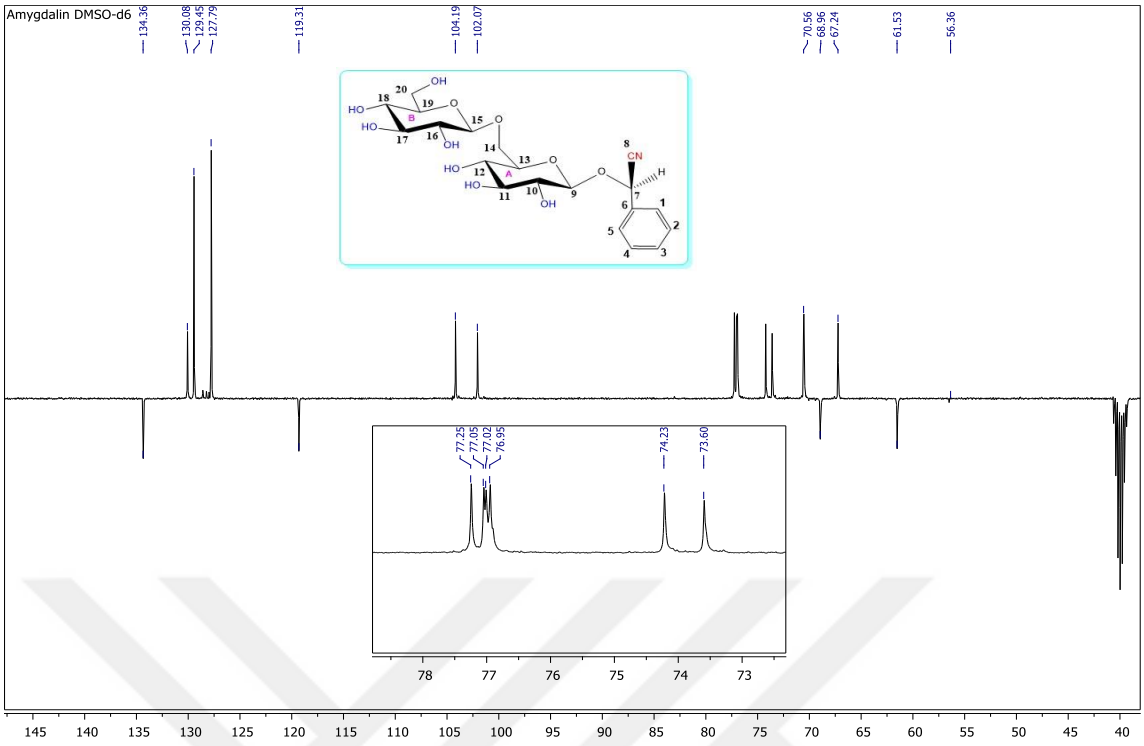


**Figure 4.12.** $^{13}$C-NMR (100 MHz) Spectra of amygdalin which was isolated from the bitter almond kernels in this thesis (DMSO-$d_6$)

While the initial values belong to the carbon signals in $D_2O$, the values in parentheses are the chemical shift values of the carbon signals in the spectrum taken in DMSO-$d_6$.

$^{13}$C-NMR, $D_2O$ (DMSO-$d_6$) ppm, $\delta$: 132.35 (134.36) C-6, aromatic quaternary carbon; 130.42 (130.08) C-3, *para*; 129.28 (129.45) C-2 and C-4, *meta*; 127.70 (127.79) C-1 and C-5, *ortho*;118.43 (119.31) C-8, CN; 102.74 (104.19) C-15; 101.38 (102.07) C-9; 75.79 (77.25) C-19; 75.48 (77.05) C-17; 75.36 (77.02) C-11 and C-17; 73.08 (76.95) C-16; 72.64 (75.23) C-10; 69.56 (70.56) C-12 ; 69.03 (68.96) C-18; 68.97 (67.24) C-7 benzylic carbon; 68.14 (61.53) C-14; 60.61 (56.36) C-20.

The carbon resonances at 132.5 and 118.7 ppm, while showing the negative phase in the APT spectrum and are less intense than the other carbon signals. Hence, these signals were assigned to the two quaternary carbons, respectively C-6 and C-8. Similarly, the carbon resonances at 68.14 and 60.61 ppm were assigned to the two methylene carbons, respectively C-14 and C-20. The remaining carbon signals show the positive phase in the APT spectrum, confirming them as odd multiplet carbon resonances. Since *D*-amygdalin possesses no methyl groups, these remaining carbon signals must arise from methane carbons. The aromatic CH carbon signal at 130.42 ppm (with about the same intensity as the glucose CH signals) derives from a single carbon and can be assigned to the *para*-CH carbon. The remaining CH carbon

signals at 127.70 and 129.28 ppm each represent two carbon signals and arises from the *ortho* and *meta* carbons.

## 4.7. Differential Scanning Calorimetric (DSC) of Amygdalin Isolated from the Bitter Almond Kernels

The DSC curve showed a large, sharp endothermal peak at 207.9–224.9 °C temperature range for *R*-amygdalin 97.1% (curve A, Figure 4.13). The temperature started to melt of the *R*-amygdalin compound is 207.9 °C. The DSC curve thus confirms the purity of amygdaline by a sharp endothermic peak at 220.7 °C, corresponding to the maximum melting rate. After melting, thermal decomposition begins immediately. Stereoisomers of the amygdalin (curve B) showed a large, broad endothermal peak at 203.8–236.0 °C temperature range. The temperature started to melt of stereoisomers of the amygdalin is 203.8 °C. The DSC curves broad endothermic peak at 223.1°C, corresponding to the maximum melting rate. After melting thermal decomposition begins immediately. *R*- amygdalin (curve C) 100% according to the DSC curve melting point is 218 ℃ [70].

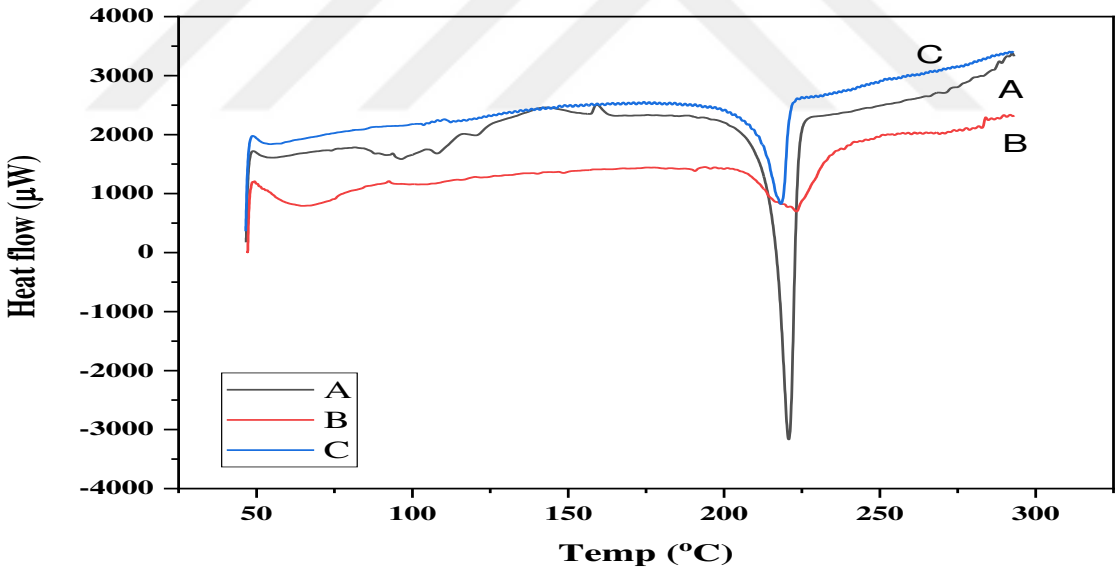


**Figure 4.13.** Differential Scanning Calorimetric (DSC) obtained for the amygdalin isolated from the bitter almond kernel

(A) *R*-amygdalin, (B) Stereoisomers of the Amygdalin and (C) Pure Amygdalin.

# 5. CONCLUSIONS

- In this study, we isolated amygdalin from the bitter almond kernel without using standard amygdalin.
- The structural characterization of the isolated amygdalin was confirmed by FT-IR, UV-VIS, $^{1}H$ $^{13}C$- NMR, and DSC techniques.
- As a result, *R*-amygdaline was isolated from bitter almond kernel with 97.1 % purity.
- Amygdalin extractions were carried out using two different solid -to-liquid methods (reflux and Soxhlet extractions).
- Higher amounts of amygdalin obtained from the bitter almond kernels with the Soxhlet extraction method,.
- Ethanol is the best solvent for the extraction of amygdalin from the bitter almond kernel.
- The suitable solvent to remove the unwanted component from the extract are *n*-hexane, petroleum ether, and chloroform.
- The reflux extraction is more efficient more than the Soxhlet extraction for the extraction of amygdalin from the bitter almond kernel.

# RECOMMENDATIONS

The isolation method of amygdalin applied in this thesis can be adapted to other seeds to isolate amygdalin and other components like flavonoid.

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