HPLC-DAD ISOLATION OF ANTIOXIDANT COMPOUNDS IN AESCULUS HIPPOCASTANUM BARK EXTRACTS AND CYTOTOXIC EFFECTS ON HL-60 CELLS

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HPLC-DAD ISOLATION OF ANTIOXIDANT COMPOUNDS IN *AESCULUS HIPPOCASTANUM* BARK EXTRACTS AND CYTOTOXIC EFFECTS ON HL-60 CELLS

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ABSTRACT

HPLC-DAD ISOLATION OF ANTIOXIDANT COMPOUNDS IN AESCULUS *HIPPOCASTANUM* BARK EXTRACTS AND CYTOTOXIC EFFECTS ON HL-60 CELLS

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This study was designed to investigate the cytotoxic and antioxidative properties of *Aesculus hippocastanum* L. (*A. hippocastanum*) bark extracts. Dried and powdered barks were extracted in ethanol, methanol, water and ethylacetate at a ratio of 1:6 (w/v). Antioxidative capacity of each extract (ethanol, methanol, water and ethylacetate) were determined by their ability to scavenge 1, 1 - diphenyl-2-picryl-hydrazyl radical (DPPH). Effective concentration (EC_{50}) values were calculated as 0.010 mg/mL 0.011 mg/mL, 0.009 and 0.019 mg/mL, respectively for ethanol, methanol, ethylacetate and aqueous extracts. The highest DPPH radical scavenging activity was demonstrated by ethyl acetate among the four bark extracts of *A. hippocastanum*. Nevertheless, methanol extract was preferred for the separation, identification and further quantification of ıts

phenolic compounds using HPLC method. Analytical and semi–preparative HPLC methods were applied to qualify and quantify the isolates. Human Myeloid Leukemia (HL - 60) cell line was used as a model system for the proliferation studies. HL - 60 cells were cultured in the presence of various concentrations (0 to 100 µg/mL) of methanol bark extract and, also, with the various concentrations of standard esculetin. HL-60 cell viability was examined by tryphan blue and the metabolism of tetrazolium salt XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) -carbonyl]-2H-tetrazolium hydroxide). XTT effective dose (ED_{50}) values for the proliferation studies of methanol extract and standard esculetin were calculated as 56.18 µg/mL and 21.23 µg/mL, respectively. These results suggested that *A. hippocastanum* methanol bark extract and esculetin could be considered as a potent antioxidant and cytotoxic agent.

Keywords: Aesculus *hippocastanum* L., Antioxidant, Free radical, RP-HPLC, Isolatated compounds, Charectrization, Cytotoxicity,

ÖZ

AESCULUS HIPPOCATSANUM KABUK ÖZÜTLERİNDEKİ ANTİOKSİDAN BİLEŞİKLERİN HPLC-DAD İZOLASYONU VE HL-60 HÜCRELERİ ÜZERİNDEKİ SİTOTOKSİK ETKİLERİ

ÖZDOĞAN, Nizamettin

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Bu çalışma, *Aesculus hippocastanum* kabuk özütlerinin sitotoksik ve antioksidan özelliklerini araştırmak için tasarlanmıştır. Kurutulmus ve toz haline getirilmiş kabuklar, metanol, etanol, su, ve etil asetat çözeltileri içerisinde 1:6 (v/w) oranında özütlenmişlerdir. Her özütün antioksidan kapasitesi, DPPH radikalini yakalama yeteneğiyle tespit edilmiştir. Metanol, etanol, su ve etilasetatlı özütler için etkili konsantrasyon (EC_{50}) değerleri sırasıyla 0.010 mg/mL, 0.011 mg/mL, 0.019 mg/mL ve 0.009 mg/mL olarak hesaplanmıştır. At kestanesinin dört farklı kabuk özütü arasında, en yüksek DPPH radikali yakalama yeteneğini etilasetat göstermiştir. Bununla birlikte, yüksek basıçlı sıvı kromotografisi metodunu kullanarak fenolik bileşiklerin ayırma, tanımlama ve ileriki miktar belirlenmesinde methanol kullanılmıştır. Analitik ve yarı-preperatif yüksek basınçlı sıvı kromotografisi, izolatların nitelik ve niceliğini ölçmede uygulandı.

Çoğalma çalışmaları için model olarak insan promiyelositik lösemi hücre dizisi (HL-60) kullanıldı. HL-60 hücreleri, methanol kabuk özütü ve esculetin standardının farklı konsantrasyonları varlığında büyütüldü. HL-60 hücrelerinin yaşayabilmesi, tripan mavi boyası ve tetrazolyum tuzunun metabolizması (XTT) ile incelendi. Çoğalma çalışmaların da, metanol özütü ve esculetin standardının XTT etkili doz değerleri, sırasıyla 56.18 µg/mL ve 21.23 µg/mL olarak hesaplandı. Bu sonuçlar, at kestanesi metanol kabuk özütü ve esculetin'in etkili bir antioksidan ve sitotoksik ajan olarak değerlendirilebileceğini göstermektedir.

Anahtar sözcükler: *Aesculus hippocastanum* L., At kestanesi, Antioksidanlar Serbest radikal, Bileşiklerin izolasyonu, Karekterizasyon, Ters fazlı- yüksek basıçlı sıvı kromotografisi, Sitotoksisite,

To my mother...

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HPLC-FU	Fluoresence dedector:
FT-IR	Fourier transform infrared spectroscopy
HPLC	High pressure liquid chromatography:
HPLC DAD	Photo diode array dedector:
LOD	Limits of detection
LOQ	Limits of quantification:
NMR	Nuclear magnetic resonance spectroscopy:
RSA	Radical scavenging activity:
RSD	Relative standard deviation
SD	Standard deviation:
UV	Ultra violet visible spectroscopy

CHAPTER I

INTRODUCTION

Since the begining of civilizations, in every culture, plants have been used for medical treatments. *Aesculus hippocastanum* L.(horse chestnut) is a well known plant, native to Asia and northern Greece, and it has been cultivated in many areas of Europe and Northern America now**.** *A. hippocastanum* is a large majestic tree used for ornamental and medicinal purposes for ages, it blooms, with white or pink flowers, from May through June, and its pricky green seedpots split open to release the shiny brown seeds in September (Dobelis, 1986; Hickman, 1993). This plant has found its place in medicine since the ancient times, especially, Native American Indians have utilized *A. hippocastanum* seeds in many medicinal remedies for, bronchial, rheumatical diseases.

1.1 *Aesculus hippocastanum L.*

The genus aesculus comprises 20–25 species of decidueous trees and shrubs which can be found widely in northern hemisphere. 7–10 species of this genus is native to north america and 13–15 species to eurasia, several natural hibrids can also be found scientists traditionally treat these species in their own monogeneric family hippocastacea but there are also genetic evidence that this family along with the maples (formerly *Aceraceae*) are better included in the soapberry family (Sapindaceae). The North American species are named as Buckeyes and the eurian species as ‘‘horse – chestnut’’. Some of these horse – chestnut are also known as ‘‘white chesnut’’ or ‘‘red chestnut’’ (as in some of the bach flower remedies). To avoid confusion with the true chestnuts (Castanea,

Fagaceae) the name horse chestnut is hyphenated here, but ‘‘Horse Chestnut’’ or ‘‘Horsechestnut’’ are also common in scientifical texts. The common Horse – chestnut, which is very widely cultivated, is also known as just ‘‘Horse – chestnut’’. The name Aesculus was given after an edible acorn. Name in Latin by linneceus. The use of the term ‘‘horse’’ is for the strength or inedibility of this specieces. The word horse originally meant strong or powerful, and the usage here does not refer to their fitness as foddex for horses, expect in folk etymology.

1.2 Botany

Medicinal species : *Aesculus hippocastanum*

Common names : Horse chestnut, buckeye, seven leaves tree

Botanical family : Hippocastanacea

Plant description : Aesculus are woody plants from 4 to 35 m tall(depending on species) and shout shoots with resinous, often sticky, buds ; opposite palmetaly divided eaves often very large and attractive insect – pollinated flowers, with a single four or five = lobed pedals joining at the base. Flowering starts after to 110 growing degree days. The fruit of this plant is a rich glossy brown to blackish – brown nut 2 – 5 cm diameter, ıts shape is usually globose.It has usually one nut in a green or brown husk, but sometimes two nuts together in one husk in which case the nuts are flat on one side.

Where it’s grown : The widely known member of the genus is the common horse – chestnut *Aesculus hippocastanum.* It is originally a plant native to a small area of the Balkan peninsula, but now widely cultivated throughout the temperate regions of the world.

1.3 Employment of *A. hippocastanum* in field of medicinal

Various crude extracts from *A. hippocastanum* have been used in the treatment of mammary indurations, some types of topical ulcer and cancer (Konishima and Lee, 1986; Chandler, 1993). The leaves and seeds of this plant

have also been used as a cough remedy and for reducing fever, varicous pain and inflamation of arthritis and rheumatism. Both the barks and the leaves have been used to prepare an expectorant and an asringent in reducing mucous congestion (Chandler, 1993).

Some important pharmacological constiuents have been found in A. *hippocastanum* including aescin, quercetin, esculetin, rutin, kaempherol, isquercetin, asragalin fraxin and scopolin.

1.4 Free radicals

Electrons occupy regions called orbitals. These orbitals can hold two electrons which spin in directions opposite to each other. A free radical is any species which can act indepently with one or more unpaired electrons in their make – up (Halliwell et al., 1995). An unpaired electron is in an orbital and this causes the species to be attracted slightly to a magnetic field. This situation can be defined as paramagneticity and it sometimes gives a considerable degree of reactivity as the free radical (Gutterridge and Halliwell, 1990).

Some examples of free radicals can be considered as hydrogen atom trichloromethyl, superoxide, peroxyl, hydroxyl, alkoxyl, and oxides of nitrogen. Hydrogen peroxide, lipid peroxide, singlet oxygen and hypochlorous acid which are also strong oxidizing agents (Punchard and Kelly, 1996).

As to radical generating reaction, we can name a Fenton type reaction, which involves an iron (II) salt with hydrogen peroxide which produces hydroxyl radicals as shown below.

$$Fe^{2+} + H_2O_2 \longrightarrow \text{intemediate complex} \longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$$

Traces of Fe^{+3} can also react with H_2O_2 as shown below, but this is a very slow reaction at physlogical pH.

$$Fe^{+3} + H_2O_2 \longrightarrow \text{intermediate complex} \longrightarrow Fe^{+2} + HO_2^{\bullet} + H^{+}$$

$$Fe^{+2} + O^2 \rightleftharpoons Fe^{+2}\_O^2 \rightleftharpoons Fe^{+3}\_O^{-2} \rightleftharpoons Fe^{+3} + O^{-2}$$

The oxygen molecule as it occurs naturally, has two unpaired electrons each located in degenerate π-antibonding orbital, if a single electron is added to the ground state O_2 molecule, it must enter to one of the π- antiboonding orbitals, forming a superoxide radical $\cdot O^-_2$

1.4.1 Free radicals in biological systems

Biological system have factors that causes free radical formation in themselves, but they face other factors which come from the surrondings in which they are exposed to, such as chemicals and irradation. Factors that causes to form reactive species constantly within a biological system can have strong effects on the system.

Most of the oxygen radicals are produced within the electron transport system. Save the basal antioxidantion cytchrome P450 and cyclooxygenases as

well as certain lipoxygenases and dehydrogenases and peroxidases generate free radical intermediates (Punchard and Kelly, 1996).

Oxygen is the terminal electron receptor during the production of ATP in the electron transport chain. And nearly 1-5% of all oxygen used in meatbolism escapes as free radical intermediates. The biggest sources of O^{2-} *in vivo* in most aerobic cells are probably the electron transport chains of mitochondria and endoplasmic reticulum.

1.4.2 Mechanism of free radical propagation

1) Addition: $X^{\bullet} + Y \rightarrow [XY]^{\bullet}$

For example: Addition of OH·to guanine in DNA

2) Reduction: $X^{\bullet} + Y \rightarrow Y^{-\bullet} + X +$

For example: Reduction of O2 to $O2^{-}$·by paraquat radical

3) Oxidation(electron acceptance): $X^{\bullet} + Y \rightarrow X^{-} + Y + {}^{\bullet}$

For example: Oxidation of ascorbic acid

4) Oxidation(Hdyrogen atom transfer): $X^{\bullet} + YH \rightarrow XH + Y^{\bullet}$

For example: Reaction of alfa -tocopherol with lipid peroxy radical

Most of the molecules in the biological systems are non-radicals, however, in the presence of even one free radical in the system may cause to initiate new radicals from the non-radicals and usually proceeds to radical chain reactions.

1.4.3 Biological disorders induced by free radicals

Biological molecules have three dimensional structures and their functionalities also depend on their structures. The compsition of intracellular medium is hihgly different than the surronding tissues and this difference is

maintained by the cell memebrane structures is great importance of the hemeostasis. However, peroxidation of lipids, can lead to severe damager to lipid membranes and as a a result to the cell's inner composition. There are at least two well known mechanisms which bring about such damages through an unrepairable damage in the anatomical integrity of membranes and the other is through the prduction of toxic compounds which can diffuse through membranes (Poli et al., 1975). The most important substances of this type are aldehydes that are produced in large amounts from peroxidizing lipids and are secreted from their site of production (Diazani, 1982). Studies suggest that high concentrations of aldehydes may accumulate in certain areas and then block an essential sulphydrly group in an enzyme molecule. These toxic compounds are metabolized and detoxified through some mechanism with in the cells anerobic removal of aldehydes is catalyzed by aldehyde dehydrogen uses. Enzymes such as aldehyde reductase, alcohol dehydrogenase and glutathione transferase play important roles with some other compounds containing sulphydryl in this removal.

The production of radicals increased proportionally with the abundance of iron. Iron is a redox-capable catalyst and this causes an increase in the number of radicals produced. If iron is not strictly compartmantalized and bound to chelators it causes, more reactive and damaging species to be created.

Studies suggest that free radicals are highly responsible for aging and tumor production. A theory asserts that free radical reactions in the cell can lead to the cross-linking of major macromolecules such as nucleic acids and proteins, causing functional damage (Penzes *et al.,* 1984).

Some other approaches tried to explain the aging process on the basis of leakage, which occurs during the operation of mitochondrial respiratory chain. Excessive leakage of O^{-2} is believed to have a possible cumulative effect on mitochondrial functions because the effeciency of protective system decrease with age. When the amount of reducing agents transported to the mitochondrial respiratory chain is reduced, less oxygen is needed, leading to decreased basal

metabolism (Harman, 1995). The fact that small animals with high respiratory rate live shorter than larger animals with lover respiratory rates, is very reasonable in this sense. There has also been some other experimental evidence recently, which indicates of some sort of relation between aging, carcinogenesis and mutagenesis.

1.5 Antioxidants for the prevention of free radicals

1.5.1 Antioxidant enzymes

Preventive antioxidants suppress the formation of free radicals enzyme such as superoxide dimutase (SOD), glutatione peroxidase and catalese could be the most important defense to remove O^{-2} and H_2O_2 before they come close to iron promoters.

There is a variety of enzymes such as glycosylases and nucleases which repair damaged DNA.

There are also proteolytic enzymes, proteinases and peptidases present in the cytosol and in the mitochondria of mammalian cells recognize, and degrade oxidatively-modified proteins therefore prevent acculamation (Davis, 1998).

1.5.2 Radical scavenging (chain-breaking) antioxidants

This is the second line of defense against free radicals. They scavange the active radicals to suppress chain initiation and/or to break chain propagation reactions. Vitamin C, uric acid, bilirubin, albumin and thiols are hydrophilic scavengers. Kim *et al.*, (2000) stated that, esculetin and esculin are other strong radical scavenger.

Caroteneids such as β- caratone amd lycopene are also accepted as effective antioxidants. They quench singlet oxygen species rapidly and inhibit superoxide formation (Niki, 1993).

1.6 Antioxidant compounds in plants

1.6.1 Phenolic compounds

Phenolic compounds are synthesized by plants during their normal course of development (Harborne, 1982; Pridham, 1960) which are considered to be secondary metobilites synthesized in this development and in phenomenons such as, response to stres conditions as in infections, wounding, and UV radiation among others (Beckman, 2000 Nichelson and Hammerschmidt, 1992). These compounds are found in plants very frequently (Harbonne abd Turner 1984) and have a great range of variety and diversity as a group of phytochemicals, which are derived from phenylalanine and tyrosine (Shahidi, 2000). As an example of diversity of these compounds, we can mention plant phenolics, which include simple phenols, coumarins, stilbenes, phenolic acids (both benzoic and cinnamic acid derivatives), flavonoids, hydrolyzable and condensed tannins, lignans, and lingins as shown in Figure 1.1. Phenolics contribute in various processes in plants as phytoalexins contributors to the plant pigmentation, protective agents UV light, antifeedants antioxidants, attraction for pollinators and so on. In foods, they also participate in the taste of bitterness, flavor, astringency, color. Moreover, some plant phenolics have health-protecting capacities, and oxidative stability. These are advantageous for producers, processors, and consumers (Shahidi and Nazck, 2004).

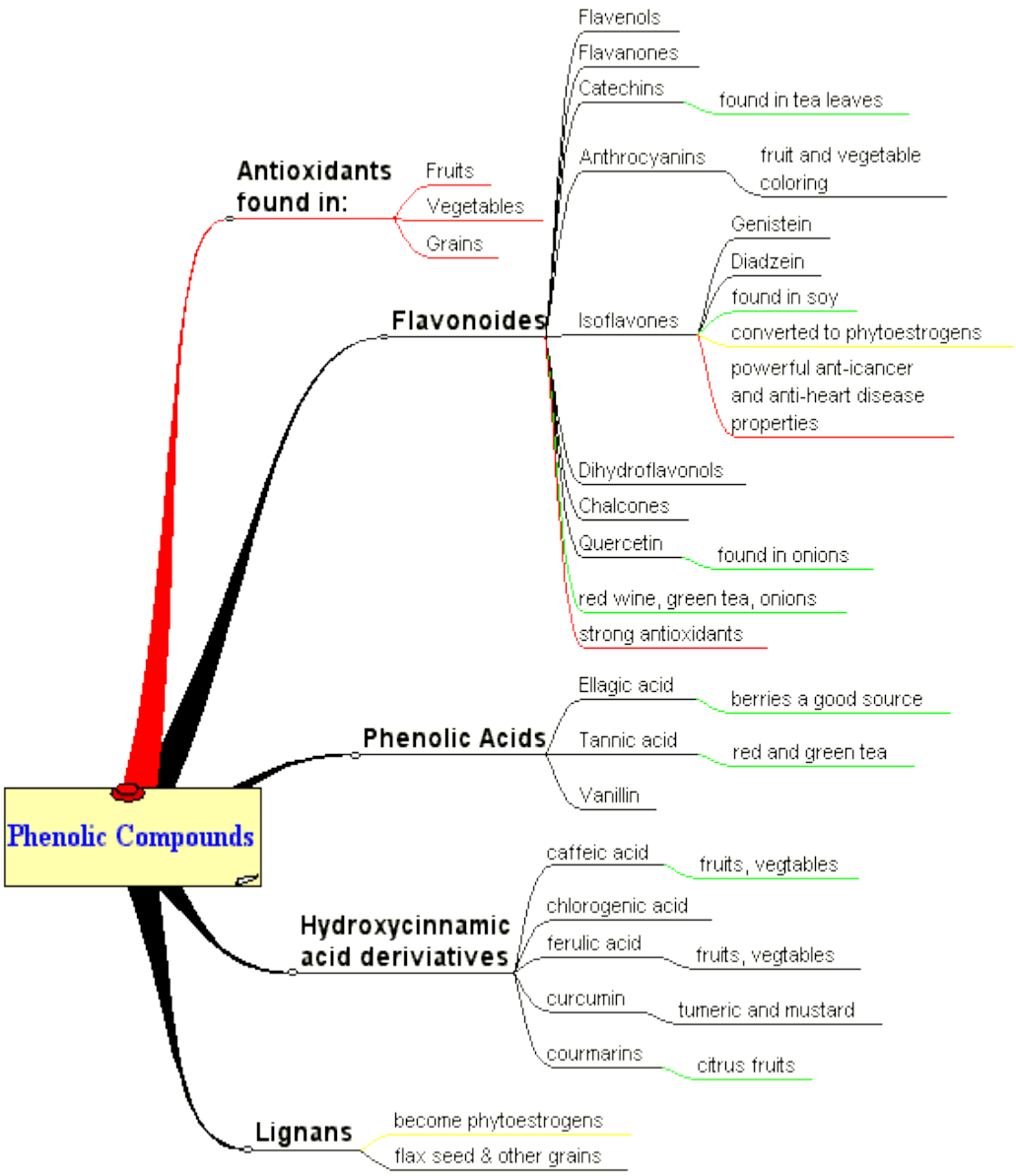


Figure 1.1: Phenolic compounds in food (quoted from Chi, 1991)

1.7 Cell based assay

1.7.1 Cancer cell proliferation studies of phenolic compounds

Plants have many phytochemicals with various bioactivities, including antioxidant, anti-inflammatory and anticancer activities. For example, some studies have reported that extracts from natural products, such as fruits, vegetables and medicinal herbs, have positive effects against cancer, compared with chemotherapy or recent hormonal treatments (Pezzuto, 1997; Wu et al., 2002). Therefore, many plants have been examined to identify new and effective antioxidant and anticancer compounds (Pietta et al., 1998; Kim et al., 1998; Swamy and Tan, 2000). The extracts of natural products may certainly have interactions with cell proliferation, cytotoxic effect, survival and apoptosis.

This study was designed to investigate the antioxidative properties and cytotoxic effect of *A. hippocastanum* bark extract.

1.7.2 HL-60 (Acute Promyelocytic Leukemia)

Human leukemia promyelocytic cell line HL-60, as a cancer cell line, has an excellent potential as the source of dividing cells. In this study (HL-60) cell line was used for the cytotoxicity studies and all the related information for human leukemia cell line is given in the followings.

Morphology: lymphoblast-like

Species: human, Caucasian female 36 years old; Tissue: peripheral blood; Tumor: leukemia;

Validated by isoenzymes: confirmed as human with NP, G6PD, AST, LD

Depositor: obtained from American type culture, UK (ATTCCCCL240)

1.7.3 Cytotoxicity

Cytotoxicity refers to properties which caused poisonous effects to cells by chemical substance, or by an immune cell.

In cytotoxicity assays, drug-induced changes in structural integrity or in metabolic pathways, are measured which can be related to cell death, although in survival assays, ultimately metabolic perturbations that are measured depending on the cell recovery or cell death.

There are many techniques such as XTT, MTT, dye exclusion test, available to monitor the cytotoxic effects of compounds of interest on the cell viability.

1.7.4 XTT assay principles

In the 1950's, the use of tetrazolium salts (such as XTT or MTT) were first rendered as a new technique. It was based on the fact that live cells converts tetrazolium salts into colored formazan compounds. In determining the viability of cells the technique was found to be very helpful. Activity of mitochondrial enzymes which are inactivated shortly after cell death confirms the basis for this biochemical procedure.

A colorimetric method based on the tetrazolium salt, XTT (sodium 3,3'-{1-[(phenylamino) carbonyl]-3,4-tetrazolium}- bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate), was first manifested in 1988 by P.A. Scudiero. The application of MTT caused the formation a non-soluble formazan compound which is required dissolving the dye for its measurement. However the application of XTT produces a soluble dye, it simplifies the procedure of measuring proliferation in a wide range of concentration. Therefore it is a perfect solution for finding the quantities about cells and determining their viability without requring the use of radioactive isotopes.

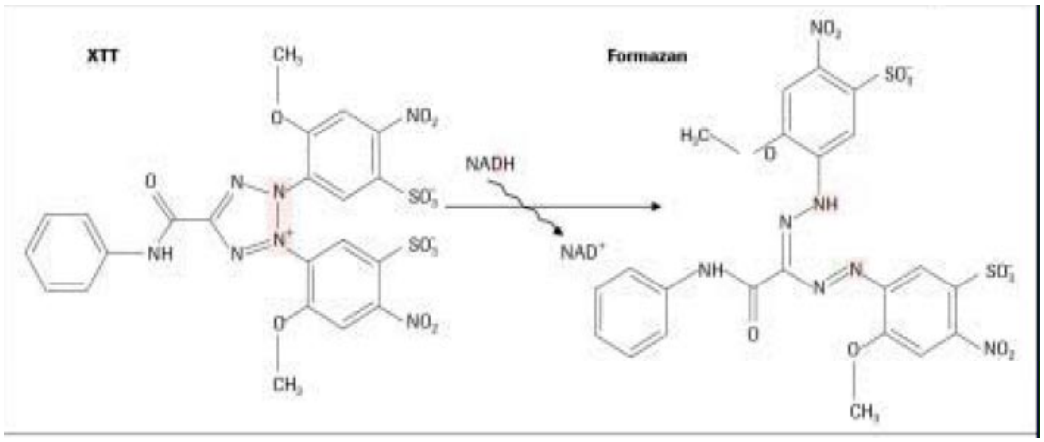


Figure 1.2: Reduction of XTT compound to formazan compound (quoted from www.sinozhongyuan.com/files/**roche**/manual_**apoptosis**.pdf)

1.8 Aim of the study

This study was designed to investigate the antioxidative, and cytotoxic properties of A. *hippocastanum* bark extracts. Isolation of bioactive compounds from the bark was carried out by utilizing the HPLC-DAD analysis. Isolates then were identified and characterized. Furthermore, the isolated compounds were examined for their antioxidative activity and for their inhibitory or cytotoxic effects on the HL-60 cells by using XTT assay and trypan blue dye exclusion test .

CHAPTER II

MATERIAL AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

Preperative chromatography grade methanol used in the preparation of extracts was purchased from Merck (Darmstadt, Germany). Water which was distilled twice, was purified using a Milli – Q system (Millipore, Bedford, MA, USA).

Ethyl acetate and ethanol of analytical grade were employed. Reagents and solvents were purchased from Merck (Darmstadt, Germany), and were of analytical or HPLC grade.

2,2-diphenyl-1-picrylhydrazyl (DPPH), esculetin, esculin hydrate, gallic acid, sodium carbonate (Na_2CO_3), were purchased from Sigma Chemical Company, (St.Louis, MO, USA).

Dimethyl sulphoxide (DMSO) and Folin Ciocalteu's phenol reagent were purchased from Merck (Darmstadt, Germany). RPMI 1640 medium, fetal bovine serum(FBS), L-glutamine, gentamicine, sodium 3,3'-{1-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT), phenazine metho-sulfate (activator), tryphan blue were purchased from Biological Industries, ISRAEL.

Promyelocytic leukemia cell line (HL-60 cells) were obtained from American Type Culture Collection, UK. Disposable syringe filter (pore size:0.22 µm and 0.45 µm Diameter: 33mm) is purchased from Millipore Corporation (Bedford, MA USA).

2.1.2 Apparatus

HPLC analysis was performed on a Waters apparatus (multisolvent delivery system model 600E, 20mL and 100μl sample loops were used for semi-preperative and for anaytical HPLC sample injections respectively and manual sample injectors for semi-preperative (Hamilton 1005 LTN) and analytical (Hamilton model 700 Nr) used seperately. Detection in HPLC was employed by either using a multi wavelength fluorescence detector (model 2475) or by using a photodiode array (2996 Waters) detectors. HPLC was equipped with an online degasser unit. Stationary phases used for an analytical methods was a Symmetry C18 RP column, 5 μm, 4.6 x 150mm (Part No: WAT045905) although for the semi-preperative method a Symmetry Shield 18 RP column, 7 μm, 19 x 300mm (Part No: WAT186001840) was utilized. Data acquisition and quantification were carried out with Waters Empower Software.

All the spectroscopic data were obtained in Cary 50 Bio UV-VIS spectrophotometer (Varian). Other instruments used for the experiments are listed as followings: Bandelin Sonorex (ultrasonic bath); Optic Ivymen System (incubator); rotary evaporator (Heidolph Laborota 4000); lyophilizator (Heto-Holten Model Maxi-Dry Lyo); blender: Waring model 32BL80 (New Hartford, CT, USA); NMR (Bruker-Spectrospin 400 ultrashield) 100 MHz C-NMR and 400 MHz H-NMR); FT-IR vertex 70 (Bruker)

Equipment used for cell culture:

Laminar Flow: Hera safe type KS12, Class II (Kendro), hemocytometer: Naubauer (use for counting blood cells), phase-contrast microscopy: Olympus CKX 41, microscopy: Olympus CX 31, CO_2 incubator: Hera Cell 150 (Kendro): ELISA Reader: Elx808 (Bio-tek, GERMANY).

2.2 METHODS

2.2.1 Collection and preparation of A. *hippocastanum* bark

A. hippocastanum barks were first collected in winter of 2005 from plants in Metu campus (near the sports center, different individuals were marked and bark collected from their southern parts of the tree). Crude bark material were washed, and they were placed on filter papers, so that they were dried under air flow at room temperature, kept in a dark. These cleaned dry samples of bark were ground to 3-5 mm particle size by Waring (model 32BL80) commercial blender at a high speed for at least 3 minutes.

2.2.2 Extraction of A. *hippocastanum* bark

A sufficient quantity of bark were ground to obtain a 3-4 mm particle size with Waring blender. Bark samples (50 g) were extracted in 1:6 ratio of metanol, etanol, ethyl acetate and distelled water for 24 hours in rotatinal incubator at 25 °C temperature. Then mixtures were filtered through a double layered cheese cloth or a rough filter paper and solvents of filtrate were evaporated with help of rotary evaporator (Heidolph Laborota 4000) at 25 °C until drying. Dried extracts were weight out the percent yield of extraction was calculated and stored at 4 °C in refrigerator.

2.3 Evaluation of antioxidant activity

2.3.1 Free radical scavenging activity by DPPH method

DPPH method was applied as proposed by Blois (1958) for determining the free radical scavenging activities extracts. DPPH is the purple-colored stable free radical which is reduced into the yellow colored diphenylpicryl hydrazine compound by subtracting a hydrogen from the phenolic compounds found in extracts. Reaction pathway is given in the Figure 2.1.

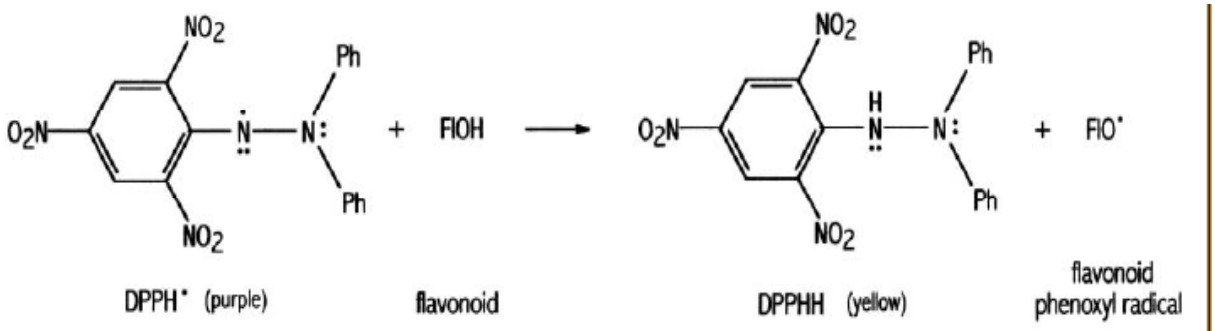


Figure 2.1: Scavenging of DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical by a flavonoid (Dragan, 2003)

A 0.05 mg/mL of DPPH ethanol solution which absorbs at 517 nm, produces nearly 1.3 unit of absorbance. Dry extracts were dissolved in ethanol and 0.1 mL of extract solutions was added to 1.4 mL of DPPH solution. With this process a series of extract solutions with varying concentrations have been prepared. Then the absorbance at 517 nm was recorded after 5 min of incubation at room tempature. Absorption of blank sample containing the same amount of ethanol and DPPH solution was prepared and measured. These experiments were carried out in triplicates. Radical scavenging effect of extracts were calculated as

$$DPPH\ radical\ scavenging\ (\%) = [(A_0 - A_1)] / A_0 \times 100$$

After 5 minutes reaction time; A_0 is the absorbance of the control with ethanol and A_1 is the absorbance of the sample in the presence of the extracts dissolved in ethanol.

50 % effective concentration (EC_{50}) values were calculated after constructing the percent *radical scavenging* versus log (extract concentration) plots.

2.3.2 Determination of total phenolic contents

Total concentration of phenolic compounds in extracts was determined as the method of Singleton and Rossi (1963). This process involved some modifications. A 0.1 ml of each extract solution was mixed with 2 ml aqueous solution of 2% Na_2CO_3 and vortexed vigorously. The same procedure was also applied to the standart solutions (0.05-0.3 mg/mL) of gallic acid. After 3 minutes incubation time a 0.1 mL of 50 % Folin–Ciocalteu's phenol reagent was added and each mixture was vortexed again and waited for a 30 minutes of incubation time at room temperature before the measurements of absorbance. At the end of the 30 minutes of incubation time, absorbance of each mixture was monitored at 750 nm and blanks were subtracted using ethanol at the same wavelength. Results were recorded as miligrams of total phenolics contained in miligrams of extract as the gallic acid equivalents (GAE).

2.4 HPLC ANALYSIS

2.4.1 Preperation of *A. hippocastanum* bark sample for HPLC analysis

The bark samples of *A. hippocastanum* was prepared prior to HPLC analysis by resuspending dried crude extracts from refrigerator (4°C) in HPLC grade methanol. First a stock solution was prepared from that diluted solutions were prepared and filtered through a 0.20 μm syringe filter (Minisart RC 4 single use syringe filter, Sartorius). The filtered solution then was injected to the injection loop of HPLC instrument.

2.4.2 Analytical HPLC

Analytic HPLC–DAD was utilized to develop a method for the separation, and identification of bioactive compounds from crude methanol extracts. The devoloped optimal elution program resulting in good resolution was composed of the following conditions:

Mobil phase: The mobil phase consisting of 1% acetic acid in water and methanol (84:16, v/v). HPLC analysis were carried out at a constant column temperature of 30 °C.

Stationary phase: Reversed Phase Symmetry column – C 18.

4.6 x 150mm, 5µm (4.6mm: column internal diameter; 150mm: column length; 5 µm: column dimension)

Flow rate: The flow rate of the solvent was 1 mL/min.

Injection volume: Injection volume was 10 µL

Absorption spectra were observed in the range of 200-500 nm by diode array detector. Chromotograms were obtained using following maximal absorption wavelengths in nm: 254, 285, 335 and 345

2.4.3 Semi-Preperative HPLC

Semi-preperative HPLC method was used to make high volume injections and to load higher concentration then the analytical HPLC method. From the given crude extracts, in order to isolate different compounds, and to collect them individually for further characterization procedures, type of stationary phase and the detection system was kept the similar to the one used in the anaytical HPLC method. Detection was performed at about 340 nm and each analyte was collected automatically by Waters fraction collector III according to time windows. Injection volume was 200 µL with the manual injector, each experiment was repeated 15-20 times. The fractions for each analyte were dried using a lyofilizator at -110 C. Four analytes were isolated from the semi-preperative analysis which were carried out for further characterization procedures. The optimal elution program for the best resolution was composed of the following conditions:

The mobil phase consisting of 1% acetic acid in water and methanol (84:16, v/v) HPLC anaylsis were carried out at a constant column temperature of 30 °C .

Stationary phase was consist of a Symmetry Shield (Waters) Reversed Phase 18, 7µm, 19 x 300mm, HPLC column. (19mm: column internal diameter; 300mm: column length; 7µm: column dimension)

The flow rate of the solvent was 17.06 mL/min.

Injection volume was 200 µL.

2.5 Instruments for the characterization of HPLC isolates

2.5.1 Ultra violet-visible spectrophotometer (UV-VIS)

Ultraviolet-Visible (UV-VIS) spectroscopy supplies information about structure of the materials in solution. UV-VIS absorption spectra are the result of light and matter interaction at the electronic structural levels in ultraviolet or visible spectral regions. The intensity of the absorption is proportional to the number, type and location of structures that are chromophores in the molecule.

UV-VIS absorption spectra for the samples of barks extracts of *A. hippocastanum* dissolved in methanol were recorded with help of a Varian Cary 50 (split beam) Spectrophotometer from Varian, Inc.

2.5.2 Nuclear magnetic resonance (NMR)

Proton and carbon NMR used to characterize the structure of organic molcules or in our case the phytochemical compounds obtained as HPLC analytes from the crude bark extracts.

Each fraction of analytes were separated as pure as possible and then completely dried by using lyphilizator. Samples were dissolved in dimethyl sulphoxide (DMSO). Nuclear magnetic resonance (NMR) spectrometer was used Bruker-Spectrospin 400 ultrashield (100 MHz for ^{13}C-NMR and 400 MHz for ^{1}H-NMR).

2.5.3 Fourier- transform Infrared spectrometer (FT-IR)

FT-IR can be used to quantitate some component of an unknown compounds. Dry samples of analytes were pelleted with potassium bromide (KBr)which is transparent in the infrared region. IR spectral data were recorded by FT-IR vertex 70 (Bruker).

2.6 Cell culture techniques

2.6.1 Culture conditions for HL-60 cell line

HL-60 cells, which constitute a human acute promyeloblastic leukemia cell line, were grown in medium RPMI-1640 with 10% heat-inactivated PBS, 2mM L-glutamine and 0.2 % (50mg/ml) gentamicine in a 5% CO_2 humidified incubator at 37 ^{o}C. The master cell bank was tested for sterility and viability. The working cell bank is prepared. prior to exposure to the extracts, In this study, HL-60 cell line used for working cell bank.

2.6.2 Cytotoxicity on cancer cells

Cell proliferation assays are frequently employed in such fields as cell growth factors, cytokines and for the screnning of cytotoxic agents and for the search of lymphocyte activation. Some dyes were developed for the analysis of large number of samples, which would help us to perform reliable and sensitive quantitive assays.

2.6.3. Cell culture treatments

A. hippocastanum bark extract prepared in methanol and a standard esculetin were used as cytotoxic agents in HL-60 proliferation studies. 10 mg dry extract and esculetin were redissolved in 1 mL of pure DMSO as the initial stock solutions and then used for the cell culture treatments. Experiments were carried out in thel 96-well flat-bottom plates by suspending cell cutures and extracts in a total volume of 100 µL. Each well contained approximately 10,000 cells in

medium with or without cytotoxic agents. Cells and medium were used as the control. Each time 50 µL cells in culture medium placed in the wells and 50µL extract solution. Concentrations of the extracts or standards were prepared from 5 to 100 µg/mL as final concentrations with less than 1% of DMSO. Each experiment was studied in two independent sets of fourtiplets. Cells with the extract solutions were incubated for 24 hours at 37 °C in 5% CO_2 incubated.

2.6.3.1 XTT assay

Metabolic active cells are able to reduce the tetrazolium salt of XTT to orange colored compounds of formazan. This ability provides a basis for our assays. Initially, 100 µL of phenazine metho-sulfate is added to 5 mL of XTT reagent before use, and 50 µL of this XTT solution is added to the cells cultivated already in 96 -well plate for 24 hours, which are then incubated for 5 hours. During the 5 hour incubation formazan is formed with a purple colour dye. The resultant dye's intensity, is proportional to the quantity of metabolically active cells due to activity of mitochondrial enzymes (dehydrogenase), which can be measured at a given wavelength (OD_{415}) with a multiwell plate reader ELISA instrument. A large number of samples can be tested easily, and rapidly with a multi-well plate reader. Percent proliferation effect of the extract or standard concentration is given in the following:

Percent proliferation = $(D_1/D_o) \times 100$

D_1: concentrations of extract (or standard) and cell culture

D_o: cell culture only

Fifty percent Effective Dose (ED_{50}) values were calculated after constracting the % proliferation versus log (extract concentration) curve.

2.6.3.2 Cell Viability (Trypan blue dye exclusion method)

Cell viability was determined by the trypan blue dye exclusion test. Effect of methanol bark extract and standard esculetin were investigated on the cell viability of cells. Before addition of methanol bark extract of *A. hippocastanum*,

approximately 10,000 HL-60 cells were seeded in the each well of 24-well flat-bottom plates. Various concentrations of *A.hippocastanum* bark extracts in the range of 0 to 100 µg/mL were added to the HL-60 cell cultures and they were incubated for 24, 48 and 72 hours. These cultures were then harvested and monitored for cell number by counting cell suspensions with hemocytometer (Naubauer). Cell growth (1.0×10^5 cell/mL) and viability (1.0×10^6 cell/mL) were controlled before and after the treatment with A. *hippocastanum* bark extracts, in which trypan blue exclusion method and microscopy (Olympus CX 31) were employed for examination.

2.7 Statistical analysis

All results are expressed as mean ± standard deviation (SD). Differences among available data were determined using *t*-tests and noted to be significantly different where $p < 0.02$. For relationship plots, significance of the relationship was determined by regression analysis of variance using Minitab Release 14 software (Minitab Inc., State College, PA).

CHAPTER III

RESULTS AND DISCUSSION

3.1 Yield of plant extract

Air dried samples (50g) from bark samples were extracted in 300 mL of methanol, ethyl acetate, ethanol and water overnight at 25 °C. Extracts were then filtered through triple layers of cheese cloth. Solid bark samples removed and the filtrates were saved. Solvents of the filtrates were evaporated with the help of a rotary evaporator (Heidolph Laborota 4000) at 25°C. When the extracts were completely dry, the percent yield of extraction was calculated for each solvent system individually. Extraction yields were in the range of 4.8-6.2 (w/w) percentage for *A. hippocastanum* bark with selected solvents.

3.2 Optimization time and temperature for the preparation of bark extract

Radical scavenging studies were carried out for the optimization of extraction parameters, as the incubation duration and temperature. Fifty percent effective concentration (EC_{50}) in DPPH radical scavenging activity was monitored for the bark extracts that were prepared according to the various incubation temperatures, the results were presented in the Table 3.1.

Table 3.1. Optimization studies for the effect of extract incubation temperature on the DPPH radical scavenging 50 percent effective concentration (EC_{50}) value of methanol bark extract. Each temperature effect was carried over 24 hours in the incubation chamber. The results are presented as (mean ± S.D) of three independent experiments.

Temperature (°C)	DPPH scavenging EC_{50} (µg/mL)
25 °C	10.7 ± 0.084
50 °C	11.2 ± 0.205
70 °C	11.6 ± 0.068

Temperatures of 25, 50 and 70 °C were selected for the incubation however, the results were not significantly different. Therefore the incubation temperature was chosen as 25 °C for its convenience.

Periods of incubation were tried to select an optimal time period from 1 to 72 hours, and resutls were tabulated in the Table 3.2.

Table 3.2. Optimization studies for the effect of incubation duration on the DPPH radical scavenging 50 percent effective concentration (EC_{50}) value of methanol bark extract. Each incubation duration was applied at 25 °C. The results are presented as (mean ± S.D) of three independent experiments.

Incubation Duration (h)	DPPH scavenging EC_{50} (µg/mL)
1	12.68 ± 0.115
6	12.06 ± 0.127
12	11.94 ±0.133
24	10.32 ± 0.145
48	11.30 ± 0.151
72	11.50 ± 0.089

The effect of changes in the incubation periods of extract preparation were observed against fifty percent effective concentration of DPPH radical scavenging activity. Although, the results can not be considered significantly different than each other, a selection had to be done, and 24 hours of incubation period was prefered since its EC_{50} value (10.32 µg/mL) of radical scavenging was still the lowest . As a result, the prefered conditions which is for the most part was selected with respect to the convenience of use as 24 hours and 25 °C.

3.3 Determination of antioxidant capacities of bark extracts

Antioxidant activities of *A hippocastanum* bark extract was investigated by measuring both DPPH scavenging activity and total phenolic contents.

Measurements of DPPH percent scavenging activities were carried out for different concentrations ranging between the values 0.01–1.4 mg/mL of plant crude extracts, and the results for DPPH percent radical scavenging activity (% RSA) versus extract concentrations in mg/mL as shown in Figure 3.1, were given in Table 3.3, which displays EC_{50} values for DPPH percent scavenging activities of *A. hippocastanum* methanol, ethyl acetate, ethanol and water bark extract respectively.

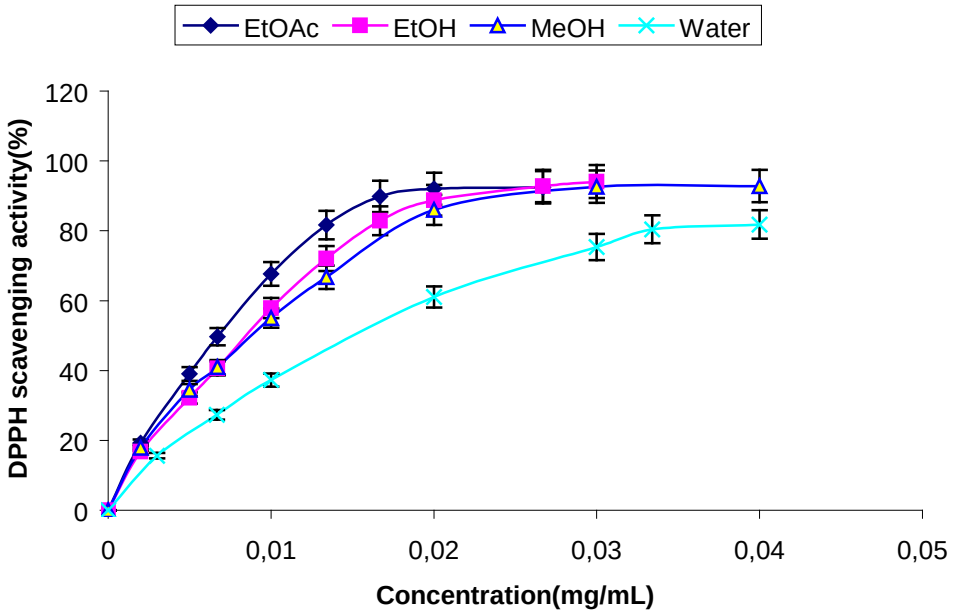


Figure 3.1. Percent DPPH scavenging activities of *A. hippocastanum* bark extracts prepared in methanol (MeOH), ethyl acetate (EtOAc), ethanol (EtOH) and water as extraction solvents. Each experiment was repeated 4 times (n=4) and standard deviation (SD) was found to be in the range of 0.00015 to 0.000315 mg/mL.

DPPH radical scavenging activity of extracts prepared in ethyl acetate, methanol, ethanol and water were monitored at 517 nm for 5 minutes. The highest DPPH radical scavenging capacity was obtained in ethyl acetate extracts , on the other hand the lowest capacity was in water extracts of *A. hippocastanum* as shown in the Figure 3.1. DPPH radical scavenging capacities of reference materials such as quercetin, rutin and esculetin were also determined since they were quite often used in the literature as phenolic standards, and displayed in the Figure 3.2. Meanwhile, fifty percent DPPH scavenging capacity of ethyl acetate extract and that of quercetin were nearly the same. On the other hand, ethyl acetate extract was more active than rutin but, less active than esculetin as shown in Figure 3.2. Comparison of EC_{50} values of EtOAc bark extract, esculetin and rutin for DPPH radical scavenging capacities were presented in the Table 3.3.

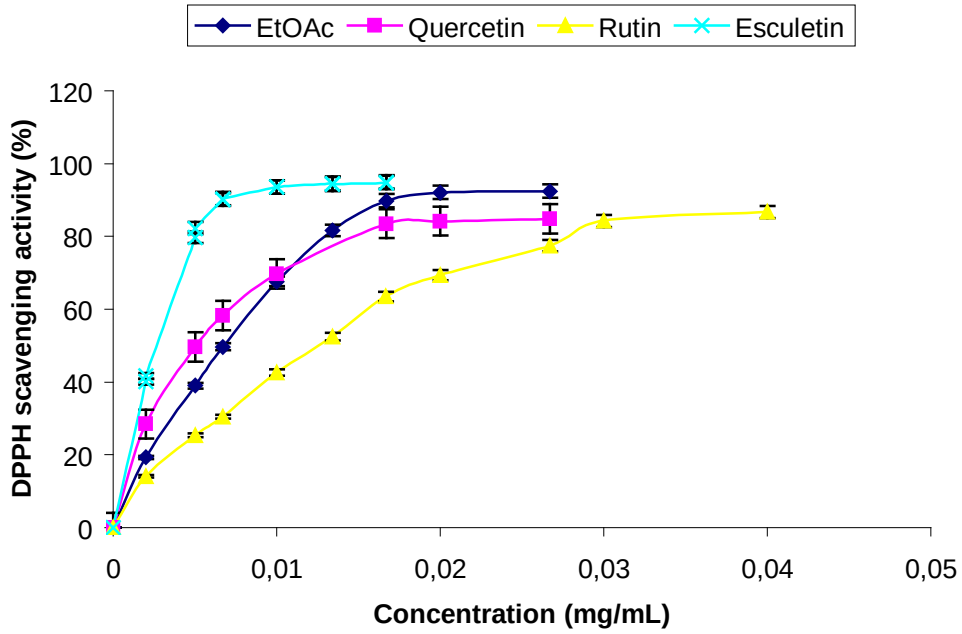


Figure 3.2. Comparison of percent DPPH scavenging capacities of A. *hippocastanum* ethyl acetate bark extract and the reference materials

3.4 Determination of total phenolic contents in *A. hippocastanum*

Total phenolic contents of bark extracts were determined by using gallic acid equivalents, which was virtually the method of Singleton and Rossi (1965). The results were presented as µg phenolic equivalents of gallic acid (GAE) in mg of extract given in the Table 3.3.

Table 3.3 : Comparison of 50 % effective concentrations for DPPH radical scavenging activity and total phenolic contents of extracts prepared in various polarity of solvents and standard materials

Plants and Standards	Solvent	EC_{50} (µg/ml) DPPH RSA SD	TP GAEs µg/mg of extract SD
A.hippocastanum	EtOAc	[a]9.0 ± 0.246	[b]54.23 ± 1.349
A.hippocastanum	EtOH	10.0 ± 0.182	53.30 ± 1.314
A.hippocastanum	MeOH	11.0 ± 0.150	50.52 ± 1.439
A.hippocastanum	Water	19.0 ± 0.315	28.98 ± 1.832
Quercetin	EtOH	9.0 ± 0.099	NA
Rutin	EtOH	15.0 ± 0.097	NA
Esculetin	EtOH	4.5 ± 0.059	NA

DPPH RSA EC_{50} : Effective concentration of plant extracts for 50 % of DPPH radical scavenging activity
TP GAE : Total phenolic contents µg equivalents of gallic acid/mg of plant extract
NA: not applicable
SD: standard deviation
[a] Mean of triplicate trials (three times)
[b] Mean of double trials (three times)

Total phenolic compounds of the bark extracts of *A.hippocastanum* prepared in ethanol, ethyl acetate, methanol and water were determined as microgram equivalents of gallic acid per mg of crude extracts. Ethyl acetate extraction of bark presented the highest total phenolic content, among the others, although it was still not significantly different than the ethanol and methanol. However the phenolic content of water extract was statistically lower than all. Considering the data available in Table 3.3 one can find a corretalion between the the values of phenolic contents and the 50 percent radical scavenging capacities (EC_{50}) of the extracts. Consequently, the lower the EC_{50} value means the higher the radical scavenging capacity and from the Table 3.3 it correlates with the higher the phenolic contents. Conceivably, high radical scavenging capacity of

bark extracts from A. *hippocastanum* could be due to high phenolic content of these extracts, which may include various phenolics, like coumarins, flavonoids, alkoloids, or terpenoids (Anne-Meri, 2002).

3.5 Analysis of the methanol bark extract of A. *hippocastanum* by analytic HPLC

Considering the results obtained from radical scavenging activity and also the total phenolic contents, metanol extract was one of the effective antioxidant extract, which displayed an EC_{50} value of about 11.0±0.15 µg/mL for DPPH scavenging. In this study, considering its lower cost and ease of use, methanol was selected as the extraction solvent for the further investigation.

3.6 Optimization of analysis conditions

3.6.1 Mobile phase

Some seperation systems for example methanol–H_2O-acetonitrile (20:70:10, v/v), methanol–H_2O-acetonitrile (15:85:5, v/v), methanol–1% acetic acid in water (16:84, v/v), acetonitrile–0.1% acetic acid in water (15:85, v/v) were tested in HPLC separation. The results demonstrated that the optimum seperation system was methanol–1% acetic acid in water (16:84, v/v). Also, isocratic elution compounds are eluted using constant mobile phase composition. The separation of compounds can be described using several conditions; retention time, bandwidth, resolution and capacity factor (Snyder, 1983). Consequently, an isocratic elution technique was selected in an attempt to acheive good peak resolution and a shortened total analysis time as shown Figure 3.3.

3.6.2 Stationary phase

Reversed-phase HPLC is now commonly used for seperation of complex mixtures of phenolic compounds and other natural products in plant extracts.

Reversed-phase chromatography was selected because polyphenols are insoluble in water but soluble in alcohols. So, the stationary phase was Symmetry C-18, 5μm, 4.6x150mm (HPLC column) was chosen, since a better separation of analytes were available with this column.

2.6.3 Injection volume

Injection volumes were optimised by using 10 to 100 microliters. During the analytical studies, 10 μL injections gave better seperations of the individual compounds of the extracts.

3.6.4 Column temperature

HPLC analysis were carried out at room temperature.

3.6.5 Flow rate

Optimization of flow rate was resulted in 1.0 and 17.06 (mL/min) for analytical and semi-preperative HPLC analysis, respectively.

3.6.6 Absorbance

Analyte absorptions were recorded in the range of 200-500 nm by using diode array detection in HPLC-DAD. In elution programs (chromatograms) peak absorptions were maximum at the following wavelengths : 254, 285, 335 and 345 nm's.

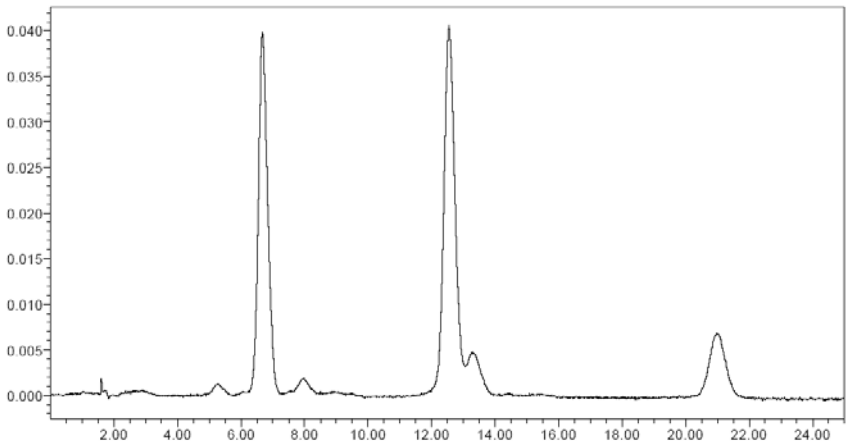
3.7 Retention times of analytes and standards in the analytical HPLC-DAD

The chromatographic profile obtained using waters empower software programme is displayed in Figure 3.3. The elution order of compound A (CPD_A), compound B (CPD_B), and compound C (CPD_C) were obtained with the retention times of 6.8; 12.4; and 21.02, minutes respectively. Small shoulders

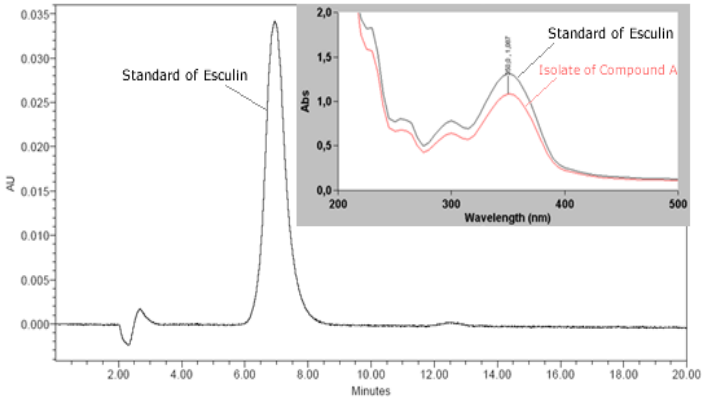
which appear around the peaks of analytes may be result of impurities from partially ionized hydroxyls of phenolic compounds (Vinas et al., 2000).

This method was intented for the seperation, identification and quantification of phenolic compounds in the methanol bark extract. Analytical and semi – preperative HPLC methods were applied to qualify and quantify the isolates obtained in the chromatography profiles respectively. An optimal solution was achieved in the seperation of these compounds by implementing various isocratic solution mixtures. Onces the conditions were optimized standard compounds were applied to the same column to find out the structures of matching isolates by comparing the matching retention times. Fortunately, two of the standards have revealed exactly the same retention times with the two of the isolates in the elution profiles, these results were confirmed also by the UV-VIS wavelength scans which were displayed in the Figure 3.3.

A)



B)



C)

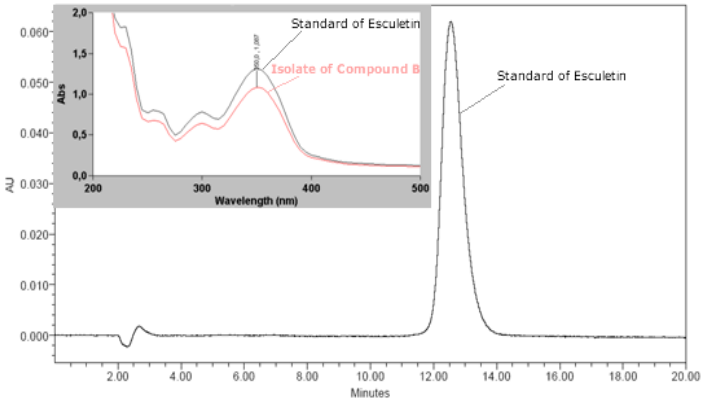


Figure 3.3: A) Result of analytical HPLC-DAD profile of *A. hippocastanum* total methanol bark extract. **B)** standard Esculin with the inlay of UV-VIS scan comparisons of esculin and matching isolate compound-A **C)** standard Esculetin with the inlay of UV-VIS scan comparisons of esculetin and matching isolate compound-B

3.8 Semi-preperative HPLC-DAD for the characterization of isolates from methanol bark extract

The organic solvents usually selected for preliminary experiments was methanol (MeOH) due to the high solubility of polyphenols in this solvent. In this manner, several optimation experiments were carried out to resolve the polyphenol mixture using different solutions of methanol-water, acetonitrile-water, methanol-formic acid and methanol-acetic acid. Methanol-acetic acid mixture was found to have the best separation between the isolatable compounds of extract. Use of acid will prevent the ionization of phenolic compounds therefore isolation of each compound will be succeded, this phenomenon was also reported by Vinas et. al., as an improvement in the chromatogtaphic efficiency of phenolic compounds was obtained in the presence of acetic acid (P.Vinas et al., 2000).

Mobile phase and stationary phase optimizations were completed and optimum conditions (as discussed in chapter II) for the mobil phase was set as methanol–1% acetic acid in water (16:84, v/v) in the preliminary studies, and for the stationary phase it was chosen as symmetry-shield reversed phase 18 column (7µm, 19 x 300 mm), to have matching format with the analytical column. Flow rate was used as 17.06 mL/min. Injection volume was optimized to 200 µL.

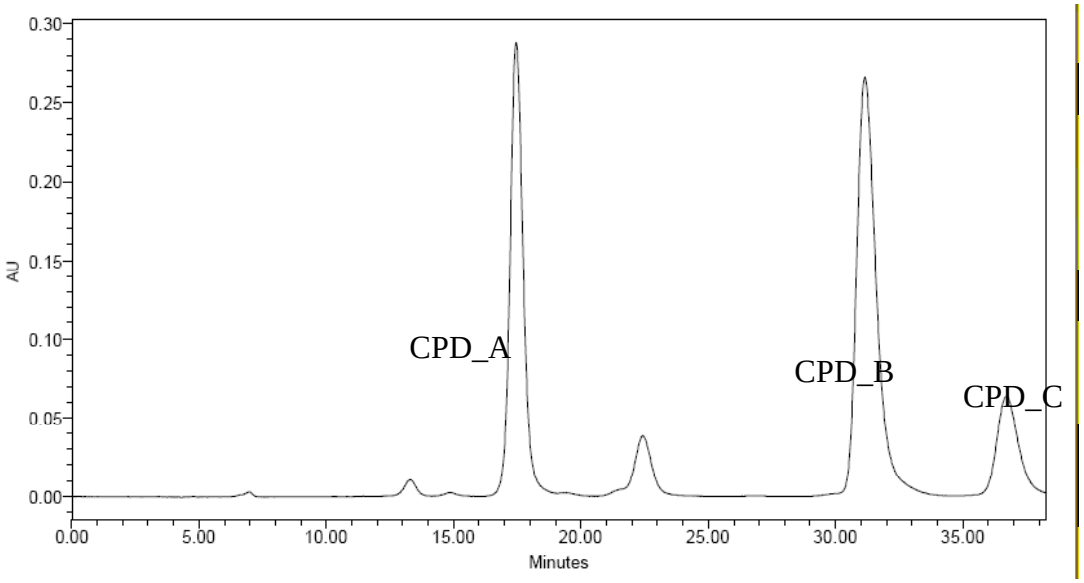


Figure 3.4: Result of semi-preperative HPLC-DAD profiles chromotogram of *A. hippocastanum* methanol bark extract [(SymmetryShield C18, 7 μm, 19 x 300 mm HPLC column), flow rate : 17.06 mL/min and injection volume : 200 µL]

Three phenolic compounds were isolated from the *A. hippocastanum* methanol bark extract 50 mg/mL by injecting 200µL for each run. Runs were repeated about 10 times and each fraction was collected seperately, and each repeat of the same fractions were combined and concentrated by evaporation and or freeze drying at the lyophizator (Heto-Holten Model Maxi-Dry Lyo).

Isolates were collected at the end of each run and after freeze-drying process they were weighed as 30, 50 and 15 miligrams of CPD_A, CPD_ B and CPD_C respectively. The fractions from each peak seperated through semi-preperative HPLC elution profiles were examined for their antioxidative activity by using DPPH radical scavenging capacity assay. Three isolated compounds exhibited antioxidative activity were as displayed in the Figure 3.5. Three isolated compounds having the antioxidant activity were further carried out their structural characterization by techniques of ^{1}H and ^{13}C NMR, FT-IR and UV-VIS

spectra, results were exhibited in the Appendix C and D. Characterization and comparison studies of isolated compounds (CPD_A, CPD_B) from HPLC elution were confirmed to be very same with the standart samples of Esculin, Esculetin.

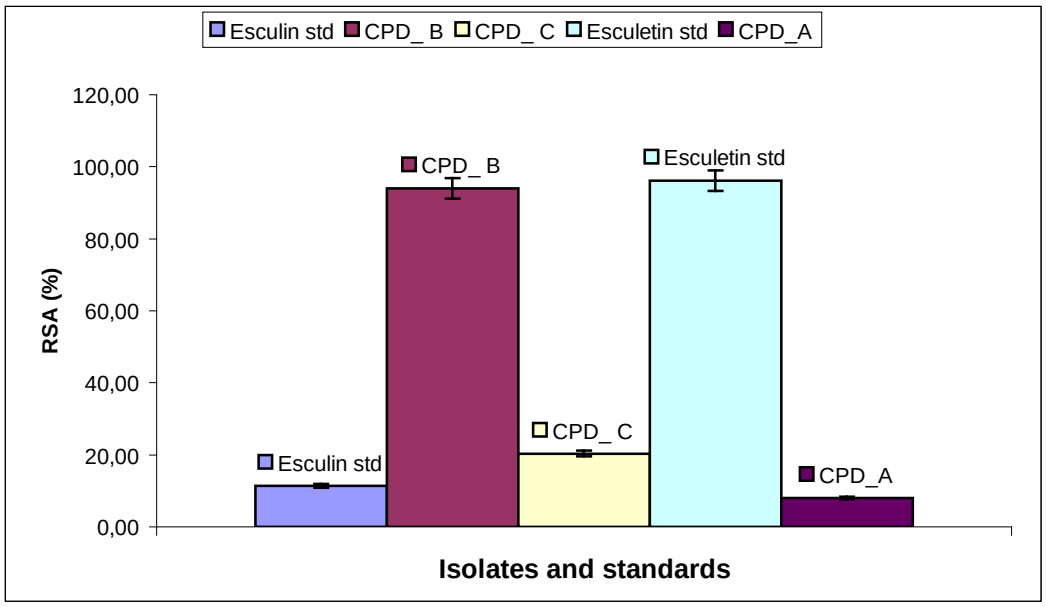


Figure 3.5: DPPH radical scavenging activity of the phenolic compounds isolated from bark extract of *A. hippocatanum.* 0.02 mg/mL of each isolates and standards were examined for their percent DPPH scavenging activities. Each experiment was carried out in double triplicates.

3.9 HPLC validation of esculin and esculetin

The HPLC method was validated for the specifity, linearity, and precision. The linearity of the method was tested with a series of standart solutions of esculin and esculetin. For esculin is type of y = ax + b with correlation coefficient r = 0.999 and for Esculetin y = ax + b with correlation coefficient r = 0.997

3.9.1 Calibration and precision using photodiode array

The linearity of the method was tested with a series of standard solutions of esculin and esculetin. Calibration lines obtained in the concentration range of 0.1 – 1.0 mg/mL are presented in the Appendix A. For esculin it is of type Y = 6.91e + 006 X - 1.62e + 005 with correlation coefficient r = 0.9981 and for esculetin Y = 1.45e + 007 X - 6.64e + 005 with correlation coefficient r = 0.9984.

3.9.2 Calibration and precision using fluorometric detection

Calibration and precision studies for fluorescence detector of HPLC was also carried out, in this case all the chromatographic conditions were the same with the analytical HPLC-DAD. Calibration results were shown in Appendix B.

Some of the phenolic compounds are known to have fluorescent properties, esculetin, and esculin are also fluorecent with emission wavelengts of 445 nm and 515 nm respectively. Two of the isolated compunds A and B has displayed fluorescent properties as given in the Figure 3.6. Analytic elution profile with diode array detector should be compared with the same elution that passes through the fluorescent dedector. In the fluorescence elution profile compound A seems to have higher quantum yield than the compound B, this is clearly observable in the absorbance spectrum. Fluorescing samples are much less than the absorbing samples in general, therefore this makes fluorescence a useful tool in identifying very specific compounds. Fluorescence is an environmentally sensitive detection system, consequently changes occuring in the environment of the fluorescent chromophore would project itself into the spectral data. Ionization of the phenolics or the doublet formation in the two ionized phenols would change the emission wavelength. Further investigations on the changes of the fluorescent properties of phenolic compounds must be persued upon free radical scavenging action.

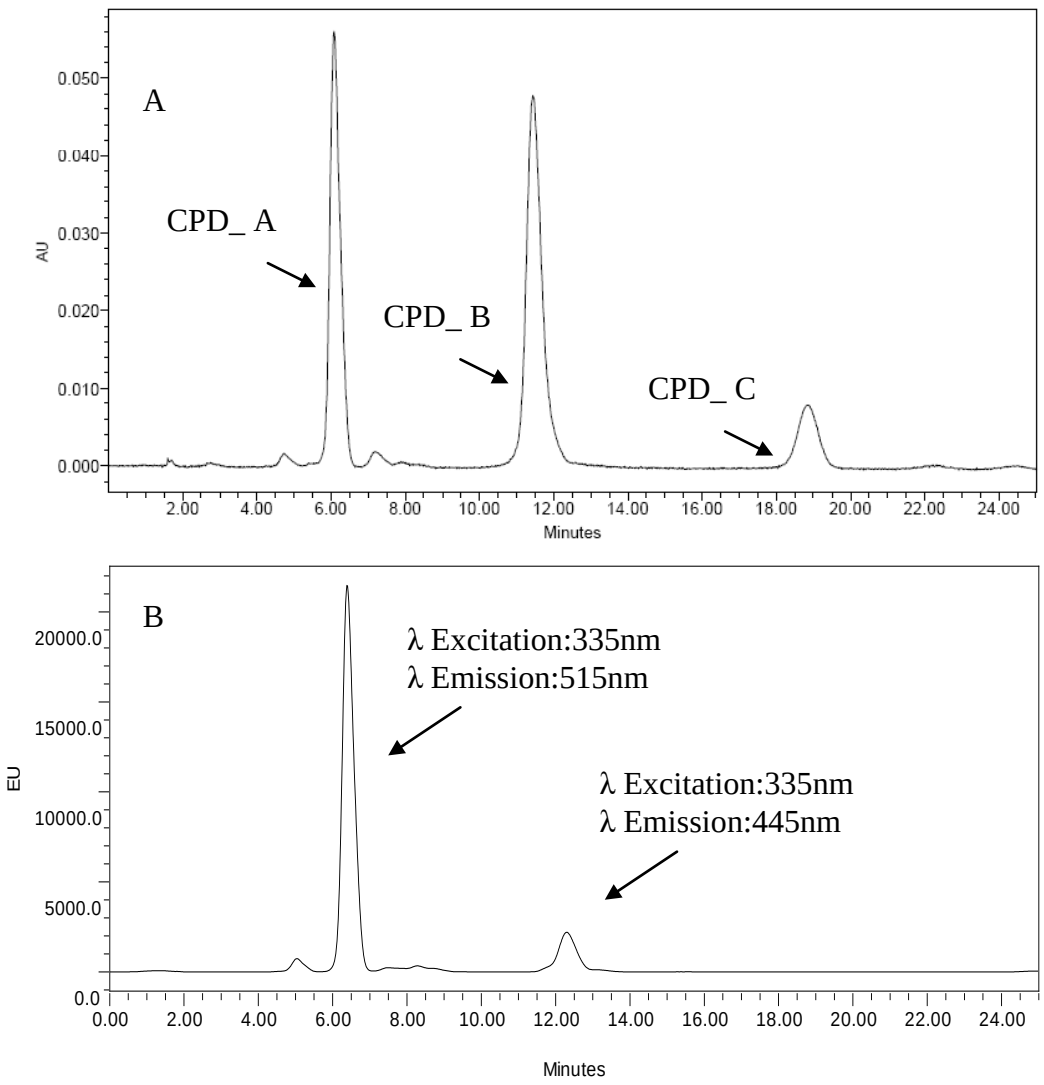


Figure 3.6: **A)** Result of analytical HPLC chromotogram of *A. hippocastanum* Methanol bark extract in diode array detector (U.V) and **B)** Flouorocent detector [(Symmetry C18, 5 μm, 4.6 x 150 mm HPLC column), flow rate:1ml/min and injection volume: 15µL]

3.10 Analysis of *A. hippocastanum* bark

Once the chromotographic conditions for the separation had been optimised, the procedure was applied for the determination of phenolic compounds from *A. hippocastanum* bark extract. These studies were carried out

using both photodiode array and fluorimetric detection and the results obtained were compared.

The elution profile obtained for *A. hippocastanum* bark sample with the photodiode array detector HPLC is shown in Figure 3.3. The peaks were identified by: (1) matching the retention times obtained from the isolates of *A. hippocastanum* methanol bark extract, and the standard compounds. (2) comparison of the fluorimetric characteristics of isolates and standard compounds. The following criteria were used to confirm the purity of the fractions from the elution profile:

a) The comparison of UV-VIS scan spectra of standard compounds with the result of the each fraction. Inlay spectra given in the Figure 3.3 demonstrates that isolates are as pure as the standard compounds.

b) Comparison of isolates and standards through hydrogen (^{1}H) and carbon (^{13}C) NMR spectroscopy

3.11 NMR results

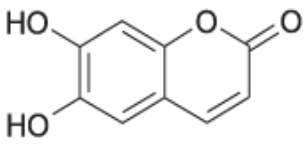
3.11.1 Structure determination of isolated compounds by NMR

CPD_A: ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.85 (d, j=9.5 Hz, 1H, H-4), δ: 7.4 (s, 1H, H-5) δ: 6.84 (s, 1H, H-8), δ: 6.2 (d, j=9.3 Hz, 1h, H-3), δ: 5.1 (d ,j=3.7 Hz 1H), δ: 5.05 (d, j=5.21 Hz 1H), δ: 4.8 (d, j=7.147 Hz, 1H), δ: 4.6(bs, 1H), δ: 3.75 (d, j=11.537, 1H), δ: 3.53 (m, 1H), δ: 3.2 (m, 1H),

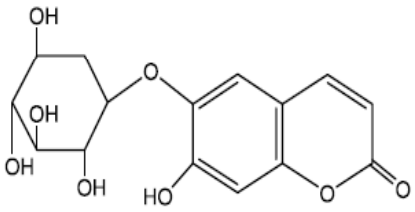
^{13}C NMR (400 MHz, DMSO-d_6) δ 160.4, 151.3, 150.4, 144.4, 142.6, 114.6, 112.0, 110.7, 103.1, 102.1, 77.2, 76.0, 73.2, 69.7, 60.6. CPD_A was confirmed as esculin

CPD_B: ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.9 (d 9.422 Hz, 1H, H-4), δ: 7.0 (s, 1 H) (H-5 or H-8), δ: 6.72 (s, 1H) (H-5 or H-8), δ: (d, 9.351 Hz, 1h, H-3),

^{13}C NMR (400 MHz, DMSO-d_6), δ 160.7, 150.6, 148.5, 144.3, 142.9, 112.1, 111.2, 110,5, 102.5. CPD_B was identified as esculetin



Esculetin($C_9H_6O_4$)



Esculin($C_{15}H_{16}O_9$)

Figure 3.7: Chemical structures of isolated compounds in *A. hippocastanum* bark: Esculin (CPD A) ; Esculetin (CPD B)

3.12 Cytotoxicity

3.12.1 Cytotocixity of methanol bark extract and esculetin on HL-60 cells (XTT assay).

Cytotoxicity of methanol bark extract and that of esculetin, as a standard, on the growth of HL-60 human leukemia cancer cell lines, in vitro, are exhibited in Figure 3.8. The methanol bark extract and esculetin showed relatively high antiproliferative activity on the HL-60 cell growth with a dose-dependent manner. The antiproliferative activities or cytotoxic effects of the extract expressed as the 50 percent effective dose (ED_{50}), lower the ED_{50} value means higher the antiproliferative activity. Cytotoxic effects of extract and esculetin were calculated with the ED_{50} value of 56.18, and 21.23 µg/mL, respectively, in the presence of increasing concentrations between 0-100 µg/mL, as shown Figure 3.8. According to the effective dose (ED_{50}) results, bark extract has shown less potential for cytotoxic effect than standard esculetin.

Esculetin is reported as a potentially cytotoxic agent in human leukemia (HL-60) cell lines (Chia Yih Chu, 2001)

The cytotoxic effect of *A. hippocastanum* methanol bark extract on HL-60 cells is due to its content of coumarins, this may result as a potential in the treatment of cancer.

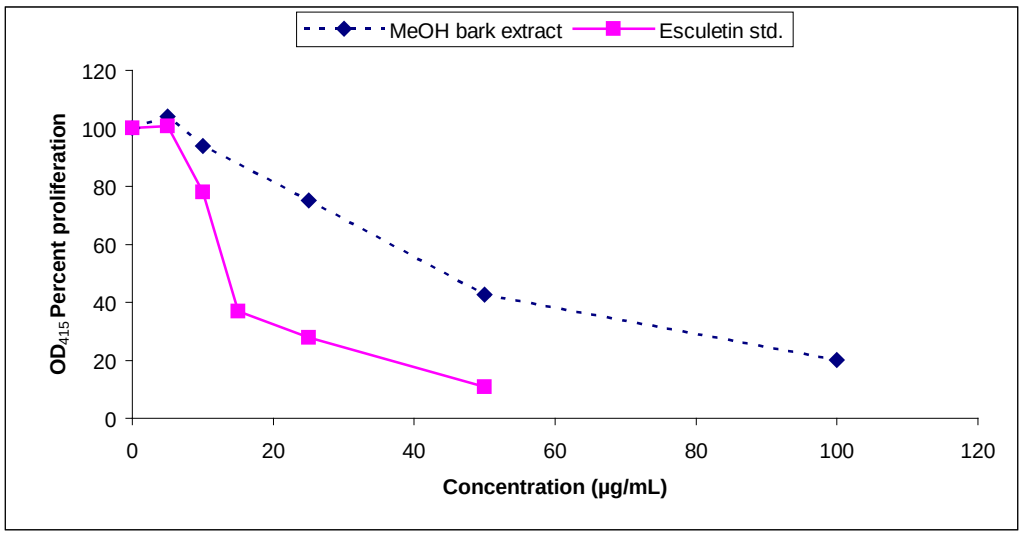


Figure 3.8: XTT results of *A. hippocastanum* methanol (MeOH) bark extract (◊--◊) and standard of esculetin (□-□) against human leukemia (HL-60) cells at various concentrations for 24 h, used DMSO concentration was lower than 1 % . $P<0.02$.

3.12.2 Cell viability on HL-60 cells by trypan blue dye exclusion methods.

The potential cytotoxic effect of methanol bark extract and esculetin was investigated, determining their effects on the viability of a human leukemia cell

line, HL-60. The cytotoxic effect of methanol bark extract on HL-60 cells is displayed in the Figure 3.9.

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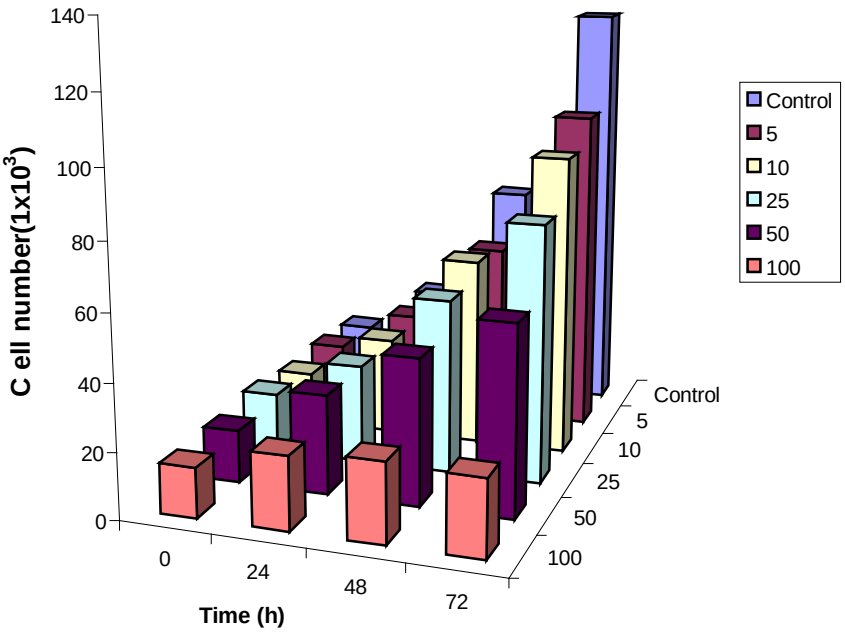

Figure 3.9: Cytotoxicity of methanol bark extracts on HL-60 cells at various concentrations (0-100 µg/mL) for 0, 24, 48, 72 h by trypan blue exclusion. Growth curves of bark extracts cells plotted by trypan blue. The HL-60 cells treated with methanolic bark extracts displayed much slower growth than the cells from the negative control group (blue).

In the early stages of the growth curve of HL-60 cells, low concentrations of the bark extract seems to have an insignificantly low effect. However, starting with the 48 hour and 10µg/mL of extract concentration the cytoxic effect is observable in a dose- and time- dependent manner on HL-60 cells proliferation.

Esculetin as a standard material was applied in the cytotoxicity assays and its concentration dependence was examined on the viability of HL-60 cells and on growth curves were exhibited in the Figure 3.10.

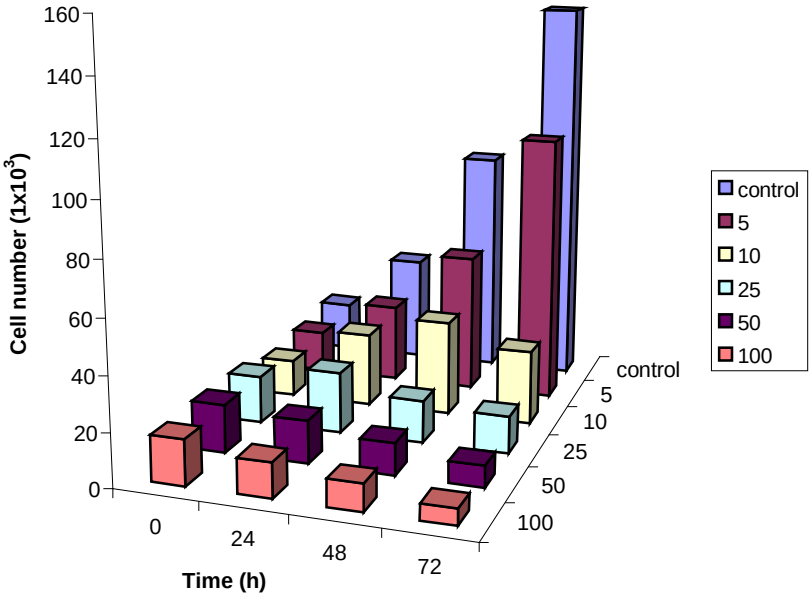

Figure 3.10: Cytotoxicity of Esculetin on HL-60 cells at various concentrations (µg/mL) of esculetin and at different incubation times by application of trypan blue dye exclusion method.

At low concentrations of esculetin (5µg/mL) showed an insignificant effect on growth of the cells. On the other hand, when esculetin concentration is reached to 25 µg/mL, a sharp decrease in the growth is observed compared to the control as shown Figure 3.10.

Considering the cytotoxic effects of methanol bark extract and esculetin on the HL-50 cells it is easily distinguishable that the effect of esculetin on the

growth curve is higher than the crude bark extract. Esculetin can be considered as a better cytotoxic agent than crude methanol bark extract.

In addition, dead cells of HL-60 were monitored with the trypan blue dye exclusion method and counted daily at scheduled times, with an anticipation of an increase in the number of dead cells. The effect of methanol bark extract determined on human leukemia (HL-60) cells. Cells were exposed to increasing concentrations (0, 5, 10, 25, 50, 100 µg/mL) of the extract for 0, 24, 48, 72 hours and the numbers of viable and dead cells were counted. Although the growth rate decreased in the presence of methanol bark extracts but there was no significant change in the number of dead cells as shown in the Table 3.4.

Table 3.4: Effect of methanol bark extract on human leukemia (HL-60) cells, upon increasing concentrations of the extract for incubation periods

Parameter	Cell number ($1x10^{3}$)							
	Incubation time (h)							
Concentration (µg/mL)	**0**		**24**		**48**		**72**	
	*L	**D	L	D	L	D	L	D
0	16	2	35	3	73	4	160	3
5	17	3	27	2	55	1	100	3
10	16	1	32	2	58	2	92	2
25	18	2	30	3	53	2	78	1
50	16	2	25	1	44	3	57	3
100	15	1	22	4	24	3	23	2

*L: live cell
**D: dead cell

The effect of esculetin determined on human leukemia (HL-60) cells. Cells were exposed to increasing concentrations (0, 5, 10, 25, 50, 100 µg/mL) of esculetin for 0, 24, 48, 72 hours and the numbers of viable and dead cells were caculated. Although the growth rate decreased in the presence of esculetin

nevertheless, there was no significant change in the number of dead cells as shown in the Table 3.5.

Table 3.5: Effect of esculetin on human leukemia (HL-60) cells, upon increasing concentrations for varying incubation periods.

Parameter	Cell number (1x10$^{3)}$							
	Incubation time (h)							
Concentration (µg/mL)	**0**		**24**		**48**		**72**	
	*L	**D	L	D	L	D	L	D
0	20	3	39	4	77	3	140	5
5	16	1	29	3	51	1	82	3
10	18	2	30	2	50	1	71	1
25	15	3	27	3	45	3	58	3
50	16	2	26	1	24	2	15	3
100	18	2	25	4	15	3	8	4

*L: live cell
**D: dead cell

This result may be interpreted as that the extract and esculetin could stop of the proliferation of HL-60, but they may not cause their deaths per se. In further and more extensive studies, the cytotoxicty mechanisms can be explained.

CHAPTER IV

CONCLUSIONS

A. hippocastanum bark extracted in solvents of different polarity such as, ethanol, methanol, ethylacetate and water. Highest antioxidant effect among the extracts was found in ethlyacetate *A. hippocastanum* bark by monitoring the DPPH radical scavenging activity with an EC_{50} value of 0.009 mg/mL. Ethylacetate extract also has highest phenolic content as anticipated.

Another high radical scavenging activity extract was methanol extract of bark and it was further isolated for its content of bioactive compounds using a HPLC-DAD method.

Merely pure isolates from HPLC elutions were collected and carried out to further investigations for their antioxidant capacity and charecterization. Meanwhile, three isolates were eluted, two of them having retention times matched with the standards of esculin and esculetin respectively.

Finally, cytotoxic studies were carried out by using HL-60 cells. *A. hippocastanum* bark extract prepared in methanol and esculetin as standards were applied at different concentartions to the feeding environment of the cells. Fifty percent effective dose of cytotoxicity were calculated with ED_{50} values of 56 and 21 µg/mL, for methanol extract and esculetin, respectively.

Consequently, both esculetin and methanol extracts could be considered as powerful cytotoxic agents, and at the same time they were efficacious antiproliferative agent and antioxidants.

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APPENDIX A

Table A.1: Calibration data of standards by using HPLC- DAD

Compound	λ (nm)	İntercept	Correlation Coeffiecent	RSD (%)	LOD (µg/ml)	LOQ (µg/ml)
Esculin	340	-1620	0.998057	90.147	0.001	0.004
Esculetin	340	-6640	0.998288	94.852	0.0005	0.001

Table A.2: Calibration data of standards by using Fluoresence dedector

Compound	λ (nm)	İntercept	Correlation Coeffiecent	RSD (%)	LOD (µg/ml)	LOQ (µg/ml)
Esculin	340	1780	0.999267	96.457	0.001	0.0033
Esculetin	340	3970	0.998976	95.609	0.0028	0.008

APPENDIX B

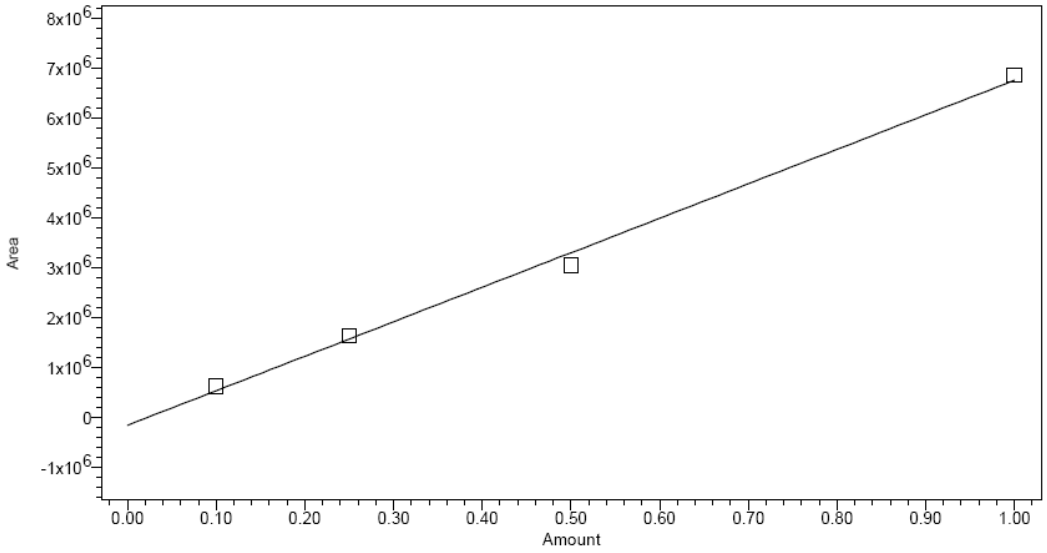

Figure B.1: Esculin calibration curve

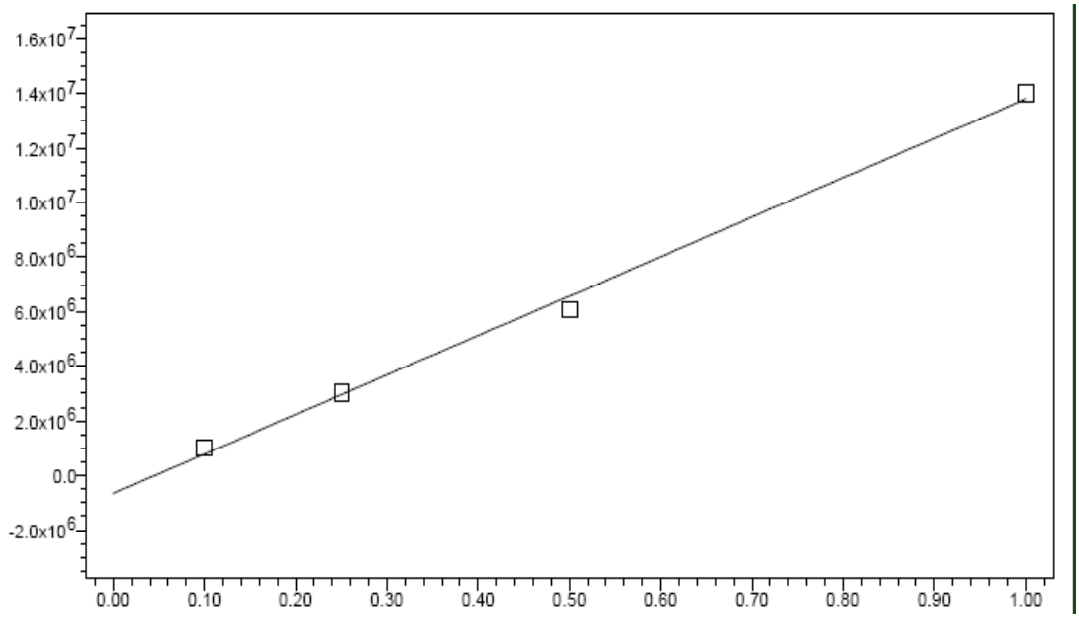

Figure B.2: Esculetin calibration curve

APPENDIX C

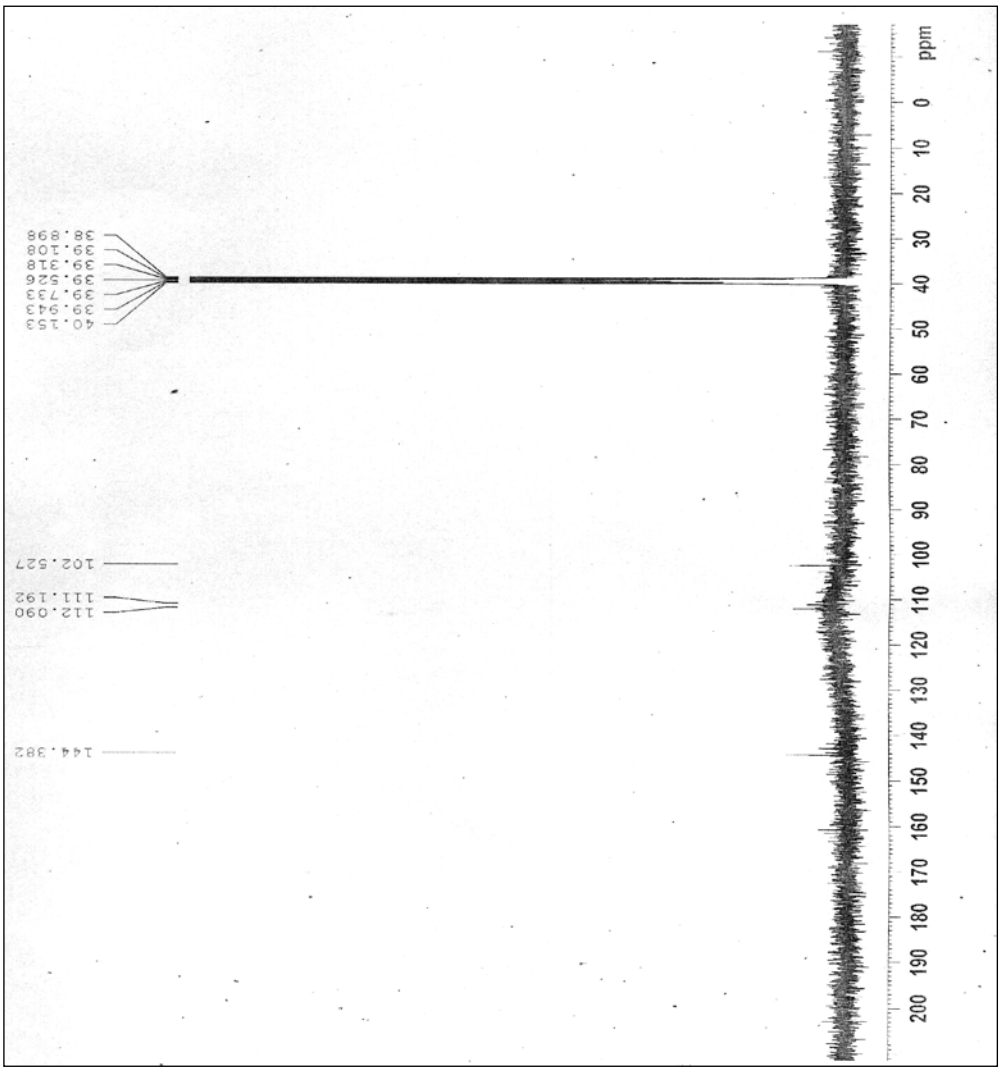

Figure.C .1: ^{13}C-NMR spectrum of isolate esculetin

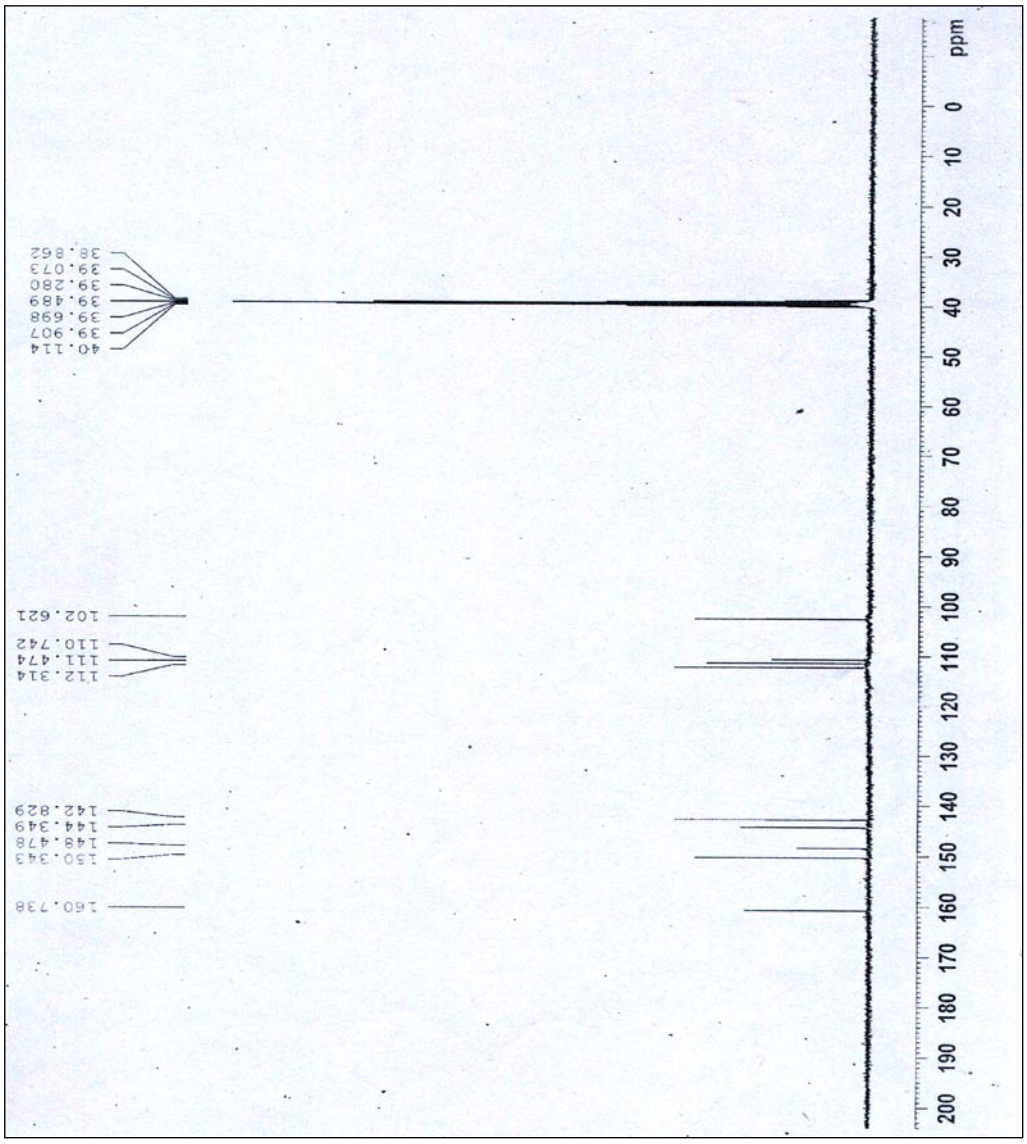

Figure.C.2: ^{13}C-NMR spectrum of standard esculetin

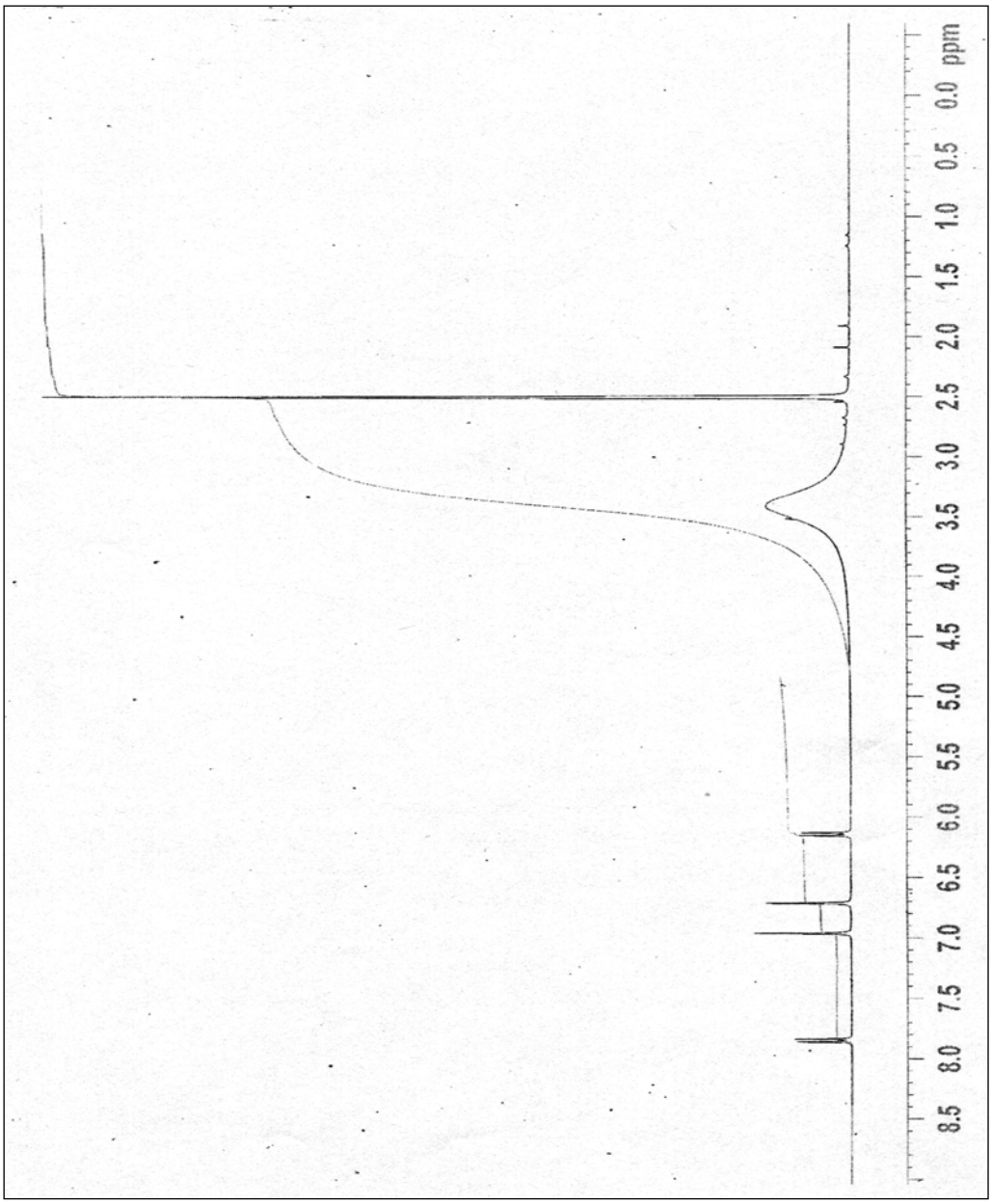

Figure.C.3:[1]H NMR spectrum of isolate esculetin

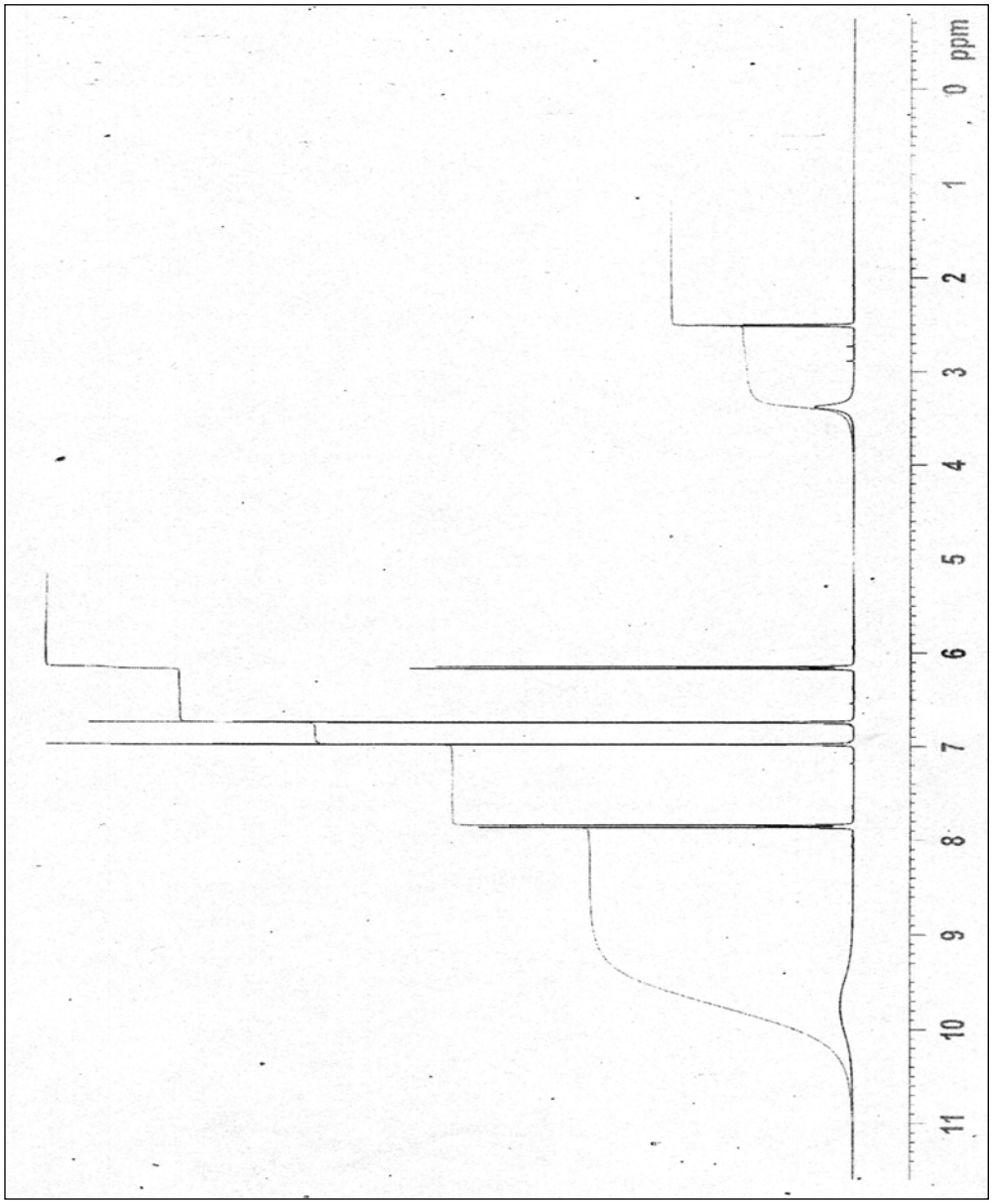

Figure.C.4: :^{1}H NMR spectrum of standard esculetin

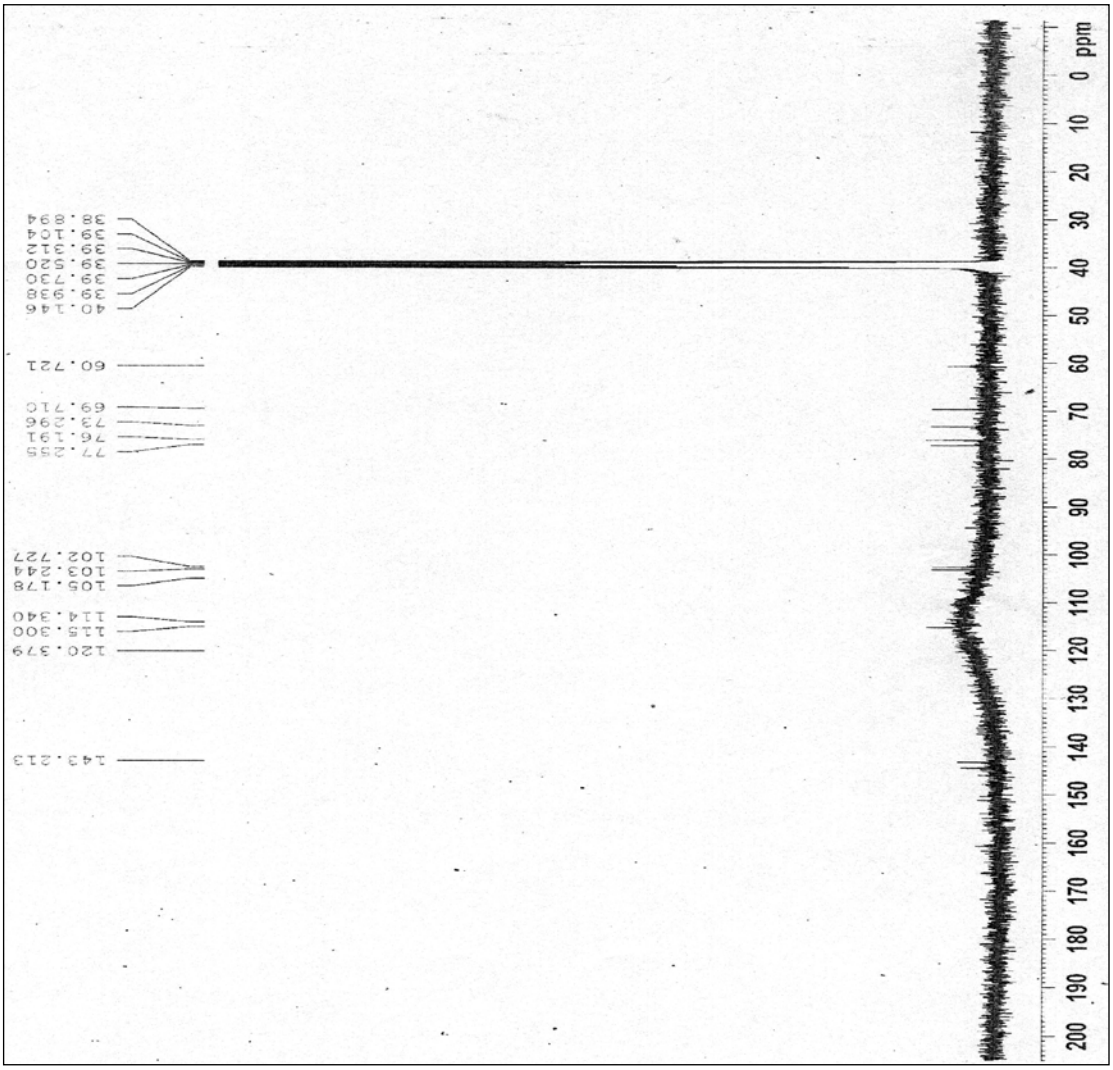

Figure.C.5: ^{13}C-NMR spectrum of isolate esculin

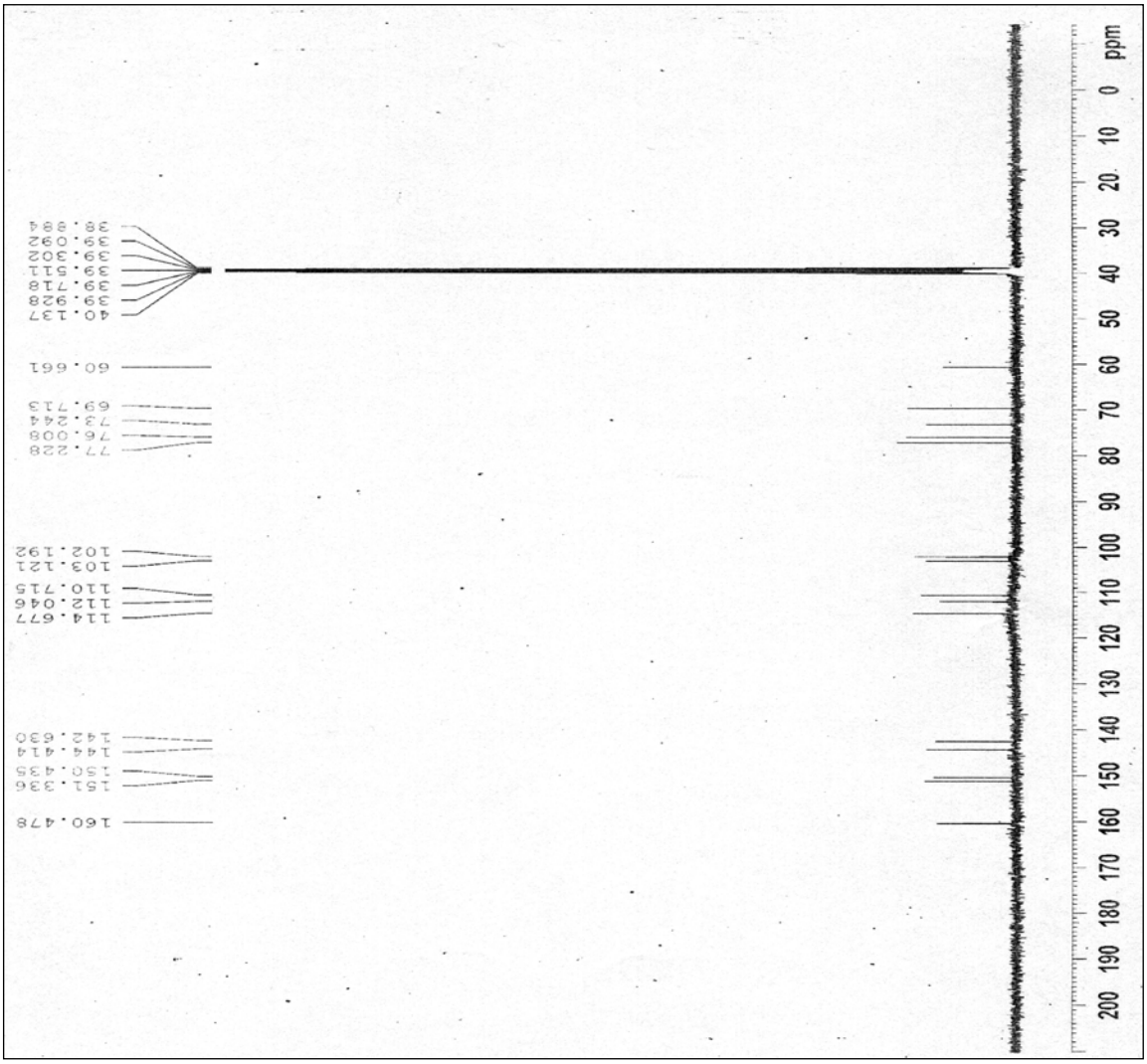

Figure.C.6: ^{13}C-NMR spectrum of standard esculin

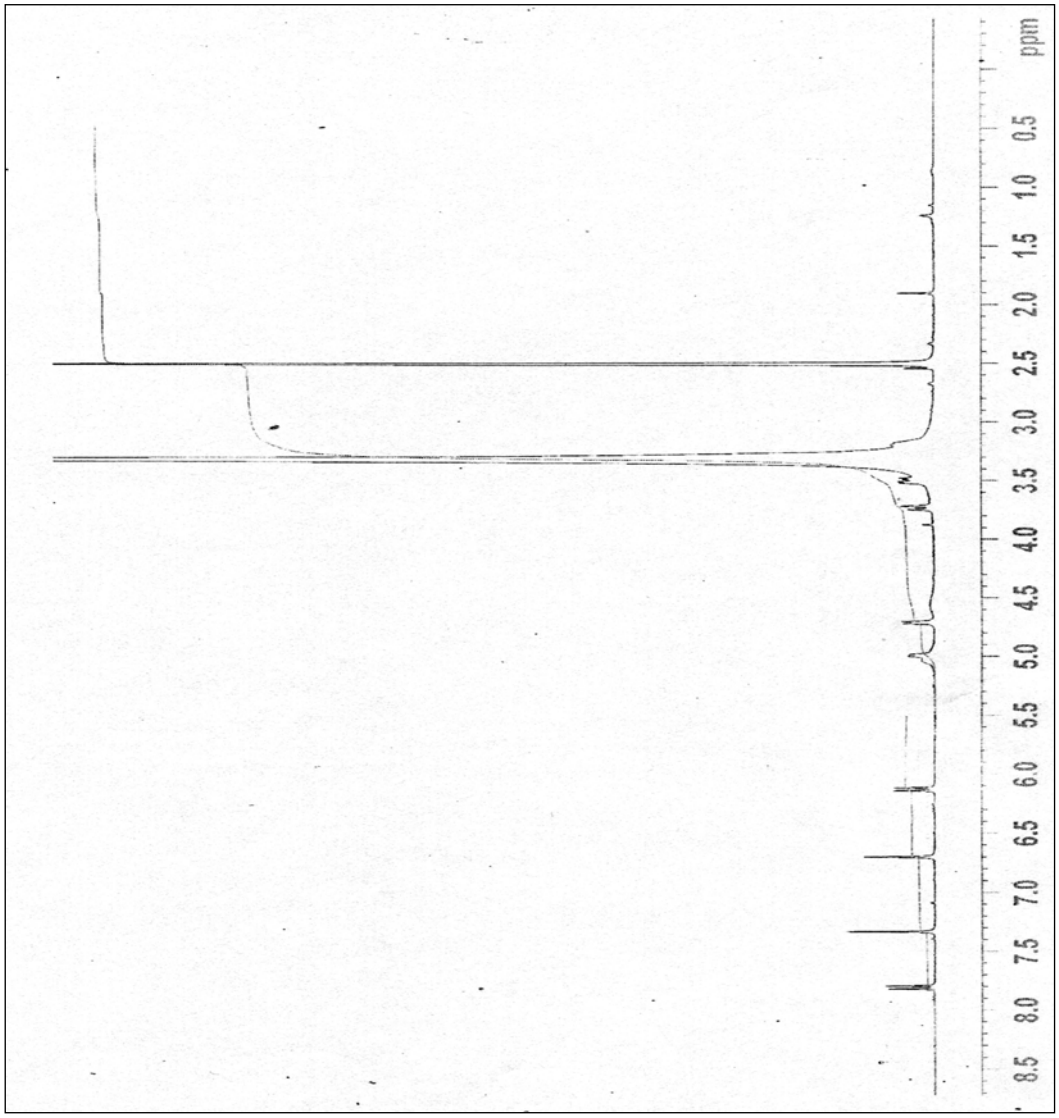

Figure.C.7: ^{1}H NMR spectrum of isolate esculin

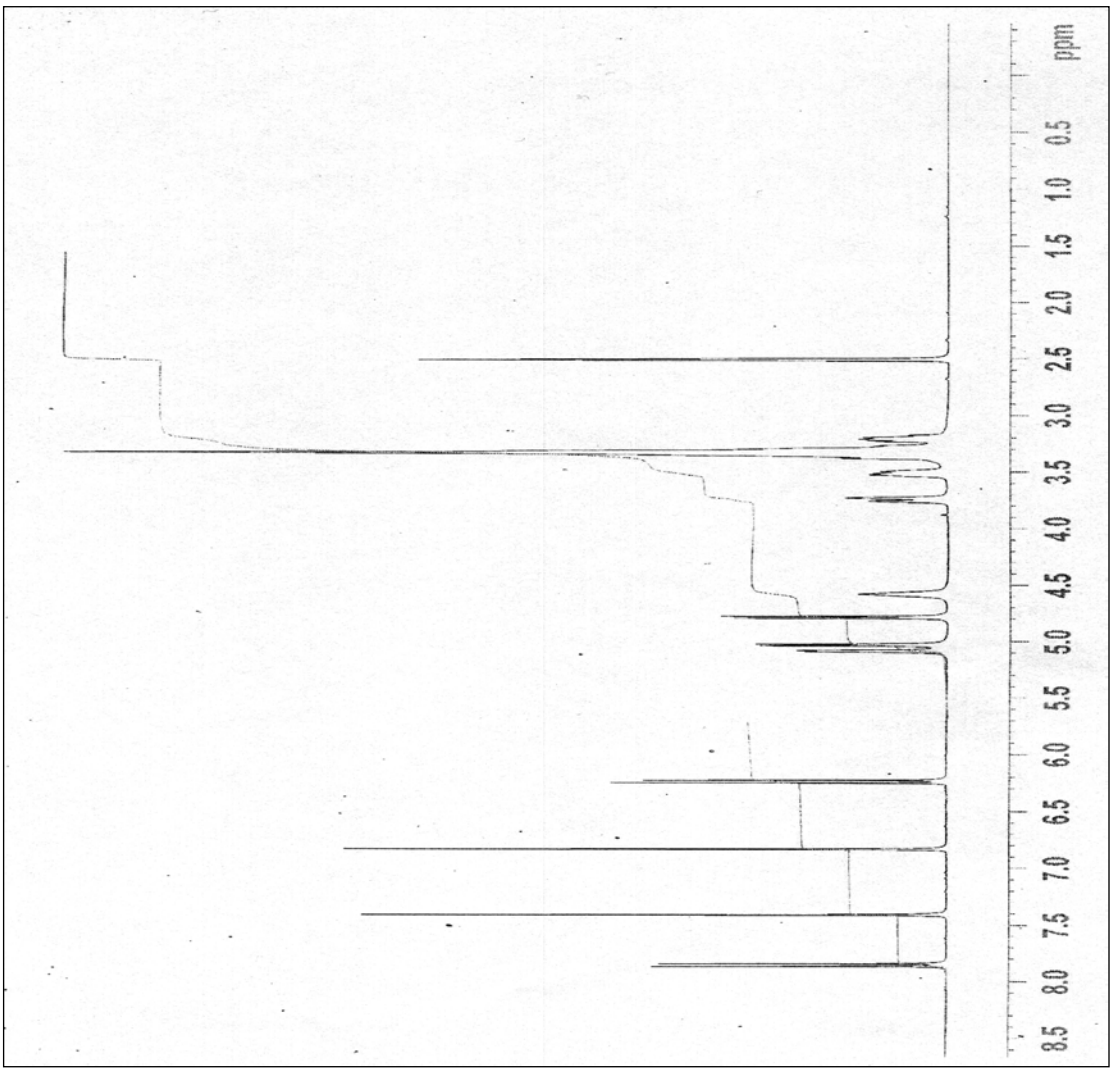

Figure.C.8: ^{1}H NMR spectrum of standard esculin

APPENDIX D

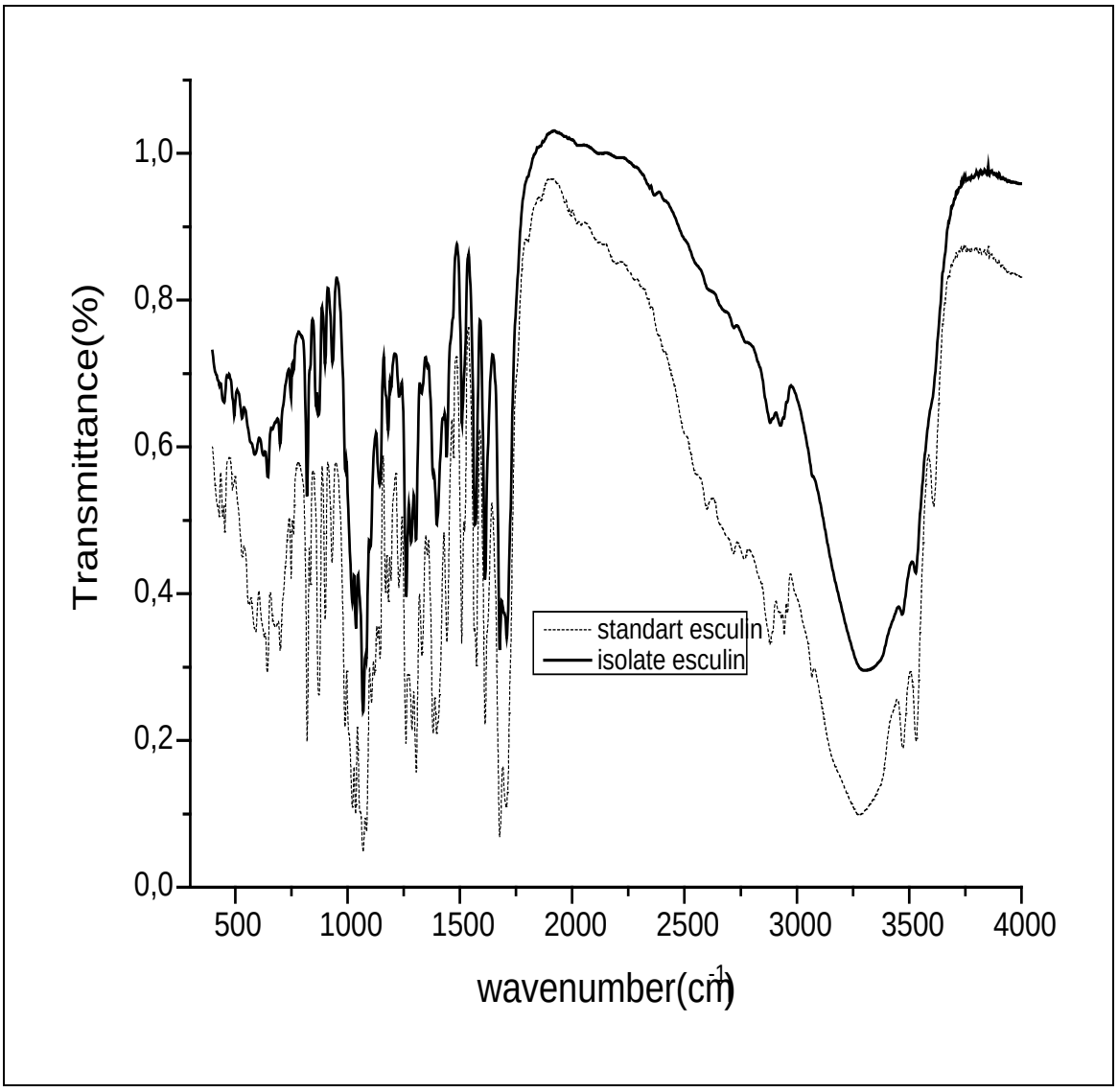

Figure D.1: Comparison of isolate and standard esculin spectrum by using FT-IR.

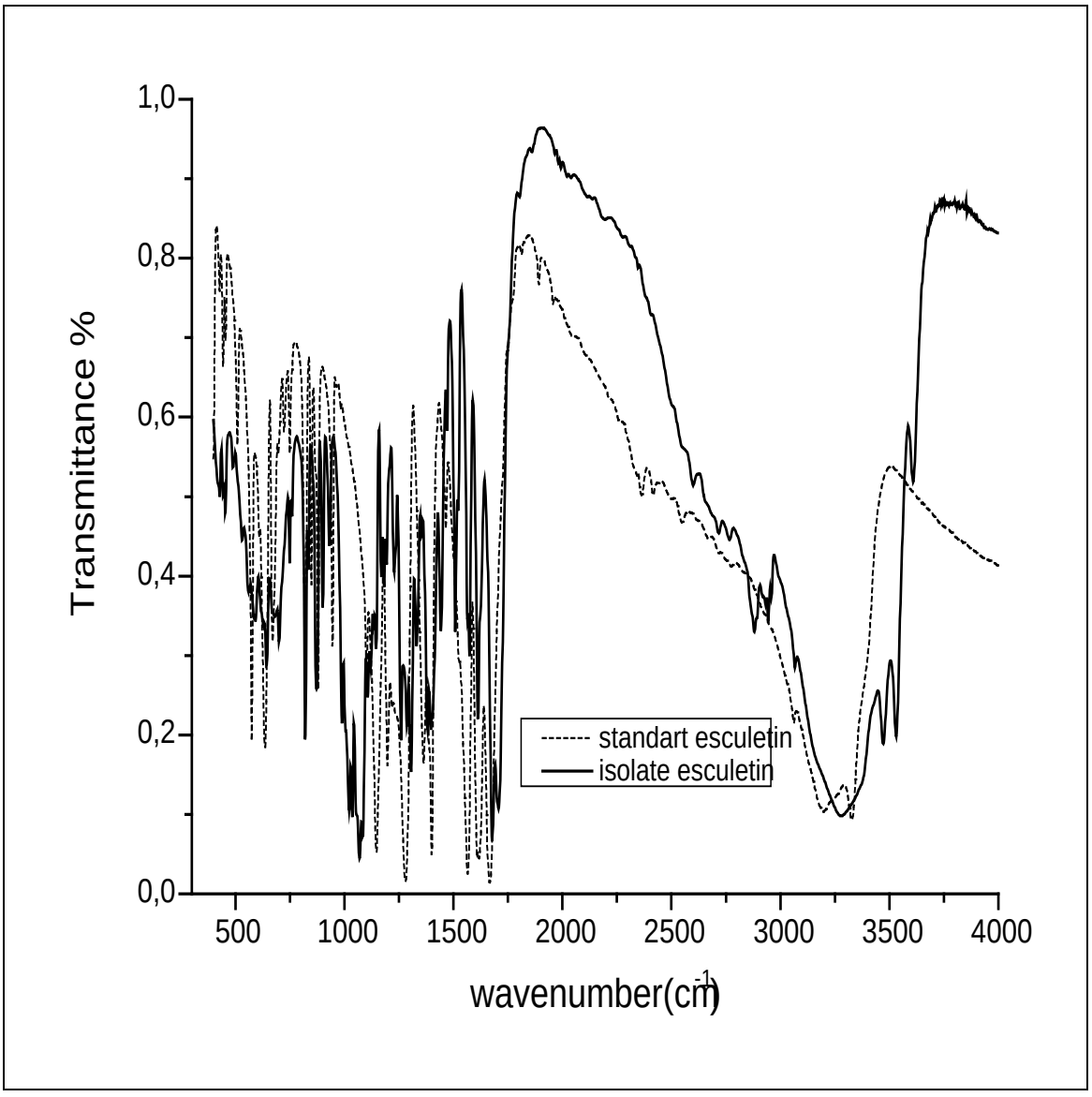

Figure D.2: Comparison of isolate and standard esculetin spectrum by using FT-IR