THE EFFECTS OF PLANT ORIGINATED BIOACTIVE COMPOUNDS ON MELANOGENESIS AND TYROSINASE

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ŞULE ŞAHİN

IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
BIOCHEMISTRY

DECEMBER 2016

Approval of the thesis:

THE EFFECTS OF PLANT ORIGINATED BIOACTIVE COMPOUNDS ON MELANOGENESIS AND TYROSINASE

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ABSTRACT

THE EFFECTS OF PLANT ORIGINATED BIOACTIVE COMPOUNDS ON MELANOGENESIS AND TYROSINASE

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Aralık 2016, 88 pages

Melanin is a dark pigment, found in skin, hair and eye. Its main function is to protect skin from the damaging ultraviolet rays of the sun. The skin color irregularity is caused by abnormalities in melanocytes, where the melanin synthesis is catalyzed by tyrosinase enzyme responsible for the pigmentation. Against these skin imperfections, unfortunately, an effective medication has not been available, yet. Nevertheless, in folk medicine, plants have been used effectively for the treatment of many skin diseases as well as pigmentation related skin disorders. As the primary plant material used in this study commonly known as "tumbleweed" was selected among the plants used in folk medicine. In this study, *Gundelia tourneforti* L. (tumbleweed) methanol extract and solvent-solvent fractions were applied to examine their effect on melanin synthesis and tyrosinase activity in the primary human melanocytes and melanoma cells. The intracellular and secreted melanin amount as well as tyrosinase activity were examined via biochemical assays and monitored by ELISA reader. The treatment of the extracts had revealed an increasing effect on the tyrosinase activity and melanin synthesis. Tyrosinase

and tyrosinase related proteins 1 and 2 (TYR, TYRP1, TYRP2), taking place in the melanin synthesis, and as well as their transcription factor microphthalmia-associated transcription factor (MITF) were also examined at the mRNA expression level by real time q-PCR. The q-PCR result proven that the up regulation of mRNA expression of those proteins was responsible for the melanin synthesis promoting effect of *G. tournefortii* L. in melanocytes.

Keywords: Melanocyte, Melanin, Melanogenesis, Tyrosinase.

ÖZ

BİTKİ KÖKENLİ AKTİF BİLEŞENLERİN MELANOGENEZİS VE TİROZİNAZ ENZİMİNE ETKİSİ

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Aralık 2016, 88 sayfa

Melanin deri, saç ve gözde bulunan koyu renkli bir pigmenttir. Temel görevi, deriyi güneşten gelen zararlı ultraviole ışınlardan korumaktır. Cilt rengindeki bozukluklar, pigmentasyondan sorumlu olan tirozinaz enzimi tarafından katalizlenen melanin sentezinin gerceklestigi melanositlerdeki anormalliklerden kaynaklanır. Malesef, bu cilt kusurlarına karşı etkili bir ilaç henüz mevcut değildir. Bununla birlikte, halk hekimliğinde bitkiler birçok deri hastalığının yanı sıra pigmentasyona bağlı cilt bozukluklarının tedavisinde etkili bir şekilde kullanılmaktadır. Halk hekimliğinde kullanılan bitkilerden olan ve genellikle "Kengel" olarak bilinen bitki örnekleri bu çalışmayı yürütmek için birincil materyal olarak seçildi. Bu çalışmada, *Gundelia tourneforti* L. (Kengel) metanol özütü ve çözücü-çözücü fraksiyonları melanin sentezi ve tirozinaz aktivitesi üzerindeki etkilerinin incelenmesi amacıyla primer insan melanositleri ve melanoma hücrelerine uygulanmıştır. Hücre içi ve salgılanan melanin miktarı ve tirozinaz enzim aktivitesi biyokimyasal testlerle incelendi ve ELISA okuyucu ile gözlemlendi. Uygulanan ekstraktların, tirozinaz aktivitesini ve melanin sentezini artırdığı görülmüştür. Melanin sentezinde yer alan tirozinaz ve tirozinazla ilişkili protein 1 ve 2 (TYR, TYRP1, TYRP2) ve bunların transkripsiyon faktörü mikroftalmiye ile ilişkili transkripsiyon faktörü (MITF) mRNA ekspresyonu

düzeyinde gerçek zamanlı q-PCR ile incelendi. q-PCR sonucu *G. tournefortii* L.'nin melanositlerdeki melanin sentezini teşvik edici etkisinden bu proteinlerin mRNA ekspresyonunundaki artışın sorumlu olduğunu kanıtladı.

Anahtar kelimeler: Melanosit, Melanin, Melanin Sentezi, Tirozinaz.

To my family

ACKNOWLEDGMENTS

First and foremost, I would like to express my deepest appreciation to my dear supervisor Assoc. Prof. Dr. Nursen Çoruh for her generous guidance, continuous support and understanding. Her enlightening guidance has been priceless to me both on my research area and academic career. This thesis would not have been completed without her constant trust and motivation.

I would like to express my gratitude to my co-supervisor Prof. Dr. Orhan Adalı for his valuable advices. I would also acknowledge my committee professors Musa Doğan, Tülin Güray, Pembegül Uyar Arpacı and Nizamettin Özdoğan for their contributions and criticisms on my thesis work. I would like to thank Professor Fevzi Özgökçe for the identifying and collecting the plant samples in my thesis study.

I am greatly indebted to Prof. Dr. Glynis Scott for the opportunity she gave me in her research laboratory for one year. It was an amazing experience and I am always grateful for her support, understanding and patience. Under her guidance I successfully overcame many difficulties and learnt a lot.

Special thanks to my dearest friend and research partner Yeşim Kümbet for her faithful friendship, help, limitless support. This study never would have been came to this stage without her. I cannot thank her enough for the sleepless nights of experiments and for all the fun we had while working together. I will remember those hard and stressful days during my life with a big smile because of her accompany.

Many thanks to my lovely lab-mates Sepideh Fazelii and Azra Rafiq. I am grateful to my friends Elif Aşık, Özlem Yıldız, Viviane Sanfelice and Emrah Sağır who

motivated me when I felt hopeless. I am really thankful for their friendship and encouragement.

I would like to thank my fiance, Bora Karaoğlu for his unfailing love, support and understanding. I am lucky to have him in my life. I love you and look forward to our lifelong journey.

The last but not least, I wish to thank Şahin family, my dad Mustafa, my mom Saime, my brother Ahmet and my sister Melike for their endless love, continued trust and patience. My parents always encouraged me and taught to believe in myself throughout my life. Words cannot express how I am grateful for the way they raised me. I never would have been able to achieve my goals without their continuous support. Also many thanks to my brother and sister for listening and giving advice to me at everytime and any situation.

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LIST OF ABBREVIATIONS

Mg	Milligram
mL	Milliliter
μL	Microliter
mm	Millimeter
nm	Nanometer
ABTS	2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
CAE	Catechin Equivalent
DPPH	2,2-diphenyl-1-picrylhydrazyl
GAE	Gallic acid equivalent
DMSO	Dimethyl sulfoxide
EC_{50}	Effective Concentration
PBS	Phosphate Buffered Saline
RSA	Radical scavenging activity
ROS	Reactive Oxygen Species
SD	Standard deviation:
TEAC	Trolox Equivalent Antioxidant Capacity
TYR	Tyrisinase
TYRP	Tyrosinase related protein-1
TYRP2	Tyrosinase related protein-2
MITF	Microphthalmia-associated transcription factor
CE	Crude extract
CH	Chloroform fraction
EA	Ethyl acetate fraction
UV-Vis	Ultra violet visible spectroscopy
α-MSH	α-melanocyte-stimulating hormone
IBMX	Isobutylmethylxanthine
ERK	Extracellular Signal-regulated Kinase

CREB	cAMP related binding protein
DOPA	L-3,4-dihydroxyphenylalanine
DHI	5,6-dihydroxyindole
DHICA	DHI-2-carboxylic acid

CHAPTER 1

INTRODUCTION

The human skin is composing of distinctly separated two layers named dermis and epidermis. The cell components of skin such as endothelial cells, melanocytes, fibroblasts, as well as keratinocytes are in contact with the extra cellular matrix, kept in a dynamic equilibrium. (Perlis, 2004) (Figure1.1).

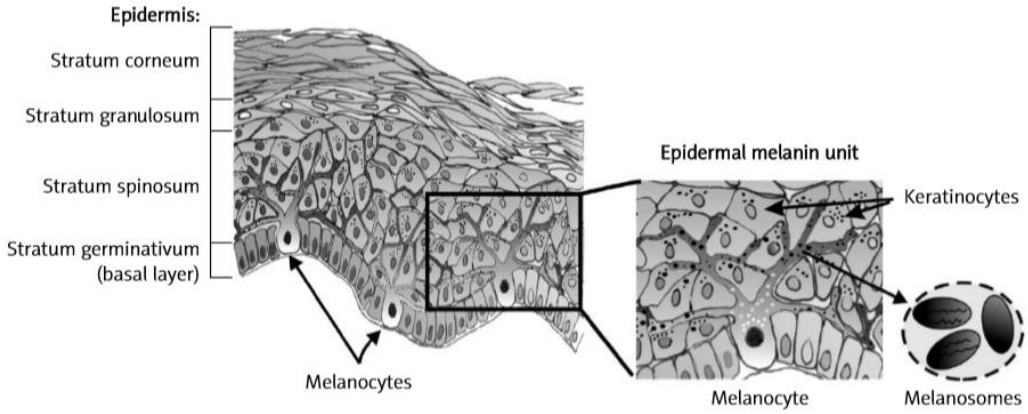

Figure 1.1: Structure of the epidermis (Cichorek, 2013).

Keratinocytes, the outermost part of the epidermal layer, are able to renew themselves as part of the defense mechanism against environmental conditions. Many of the processes like the differenciation and proliferation of the basic cells and the multilayer tissue formation as well as the defence and cell growth maintenance are carried out by the keratinocytes (Hsu, 2002).

The pigment synthesising skin cells which are called melanocytes are part of the neural crest lineage. After epithelial-mesenchymal conversion the neural crest lineage transfers to the skin layer where the melanocytes would be formed (Chin, 2003).

Melanocytes during their growth periods are spread through keratinocytes with a conserved ratio of keratinocytes to melanocytes as 35: 1. Melanocytes are able to spread out and contact with the keratinocytes by their dendritic extentions. The melanosomes are, melanin containing organelles of melanocytes, transferable through dendritic extentions of melanocytes. Melanin pigment content of melanosomes is responsible for the protection from hazardous effects of UV radiaton (Kincannon, 1999).

1.1 Melanogenesis

Melanogenesis, the process of melanin synthesis, takes place in melanosomes which are cytoplasmic organelles of melanocytes (Seiji, 1961). Pheomelanin and eumelanin are the two main melanin types with different colors synthesized accordingly during this process. The types of melanins produced are shaped according to the presence of enzymes and suitable substrates in the melanosomes. Tyrosine is converted to L-3,4-dihydroxyphenylalanine (DOPA) by tyrosinase (TYR) hydroxylation and the resulting product turnes to DOPAquinone by going through an immediate oxidation (Slominski, 2004; Fitzpatrick, 1967). In case of cysteine availability, DOPAquinone is converted into 3- or 5-cysteinyl DOPAs,

then yellowish red colored pheomelanin is formed (Hearing, 2011). Brown-black eumelanin is produced in the deprivation of cysteine, glutathione or thioredoxin type thiols.

Cyclization of DOPAquinone results in DOPAchrome formation (Sugumaran, 1991). When DOPAchrome goes through an spontaneous carboxylic acid elimination product is 5,6-dihydroxyindole (DHI). Then the resultant product rapidly polymerizes and forms DHI-melanin. Then again, in case of DOPAchrome tautomerase (TYRP2/DCT), DOPAchrome is goes to DHI-2-carboxylic acid (DHICA) (Del Marmol, 1996). Where, the availability tyrosinase and TYRP1 enzymes could catalyze the DHICA-melanin conversion. The ratio of melanin types determines visible pigmentation of human skin. Diversity of skin color is set by the ratio of eumelanin to total melanin content among the different ethnic groups in worldwide (Yamaguchi, 2007; Slominski, 2004). Since eumelanin giving characteristics to the darker skin which has higher protection against uv irradiation than pheomelanin, therefore the skin cancer risk in the lighter skin people is almost 40-fold higher (Abdel-Malek, 2010; Hearing, 2011).

Endoplasmic reticulum originated melanosomes production necessitates the presence of tyrosinase (TYR) and tyrosinase-related proteins (TYRP1, TYRP2). The synthesis of tyrosinase takes place on the ribosomes before it goes through an essential glycosylation step in the Golgi complex. (Park, 2009; Watabe, 2008). Melanosomes with an amorphous matrix at the first stage is called as Premelanosomes. Although the pigment synthesis does not take place, tyrosinase enzyme is present in the melanosome with a fibrillary matrix at the stage II. The melanin synthesis and the deposition of pigments start at the stage III. The mature melanosome is formed at the final stage IV (Schiaffino, 2010). Melanosomes lose tyrosinase activity after fulfillment with melanin and they are carried to keratinocytes with dendritic extentions of melanocytes (Schallreuter, 1998) (Figure 1.2).

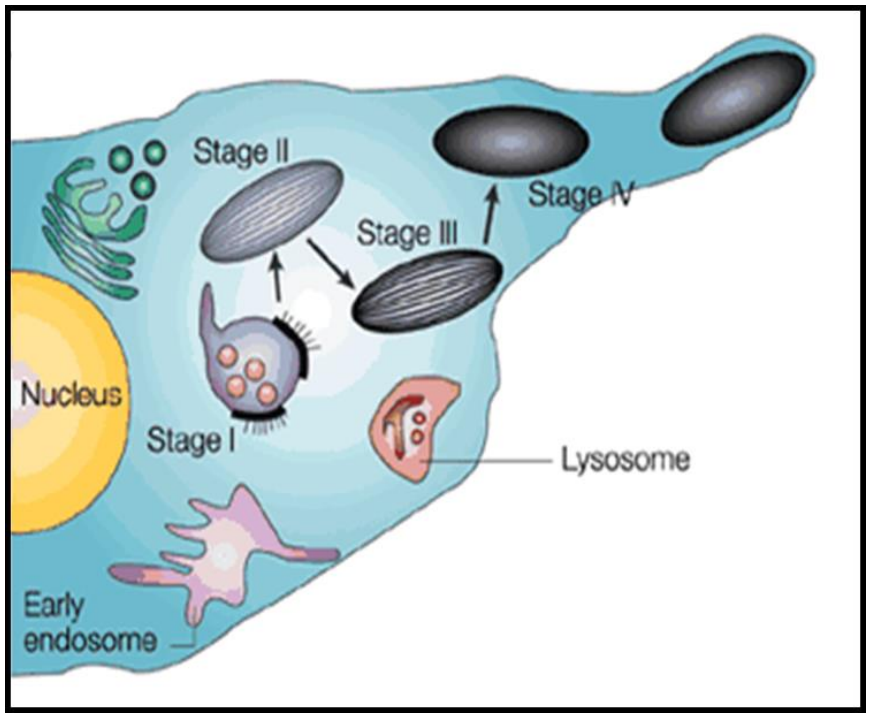

Figure 1.2: Stages of Melanosome development (Marks, 2001).

1.2. Melanogenic Enzymes and MITF

The all three types of melanin (eumelanin, pheomelanin and neuromelanin) found in skin are derived from tyrosine amino acid (Ito, 2003). The most important enzyme of melanin synthesis is tyrosinase which catalyses the rate limiting step of pathway. It is 60-70 kDa, copper containing glycosylated enzyme which is responsible for the oxidation of L-tyrosine to L-DOPA. Tyrosinase consists of internal, transmembrane and cytoplasmic domain and resides in the membrane of melanosomes (Slominski, 2004; Schallreuter, 2008). The cytoplasmic and transmembrane domains are critical to direct enzyme to the melanosome (Jimbow, 2000; Selaturi, 2000).

Due to the catalytic responsibility of tyrosinase in three reactions of melanin synthesis pathway the activity of enzyme is proportional to the synthesized melanin amount (Tripathi, 1992). In addition to tyrosine hydroxylation to L-DOPA, oxidation of DOPA to dopaquinone and further conversion to dopachrome is catalyzed by tyrosinase. Dopachrome is further converted to indole 5,6-quinone-2-carboxylic acid (DHICA) or dihydro-indolizine (DHI) (Figure 1.3). While oxidation of DHICA is catalyzed by TYRP-1, TYRP-2 catalyzes formation of DHICA (Hasegawa, 2010; Chadwick, 2012).

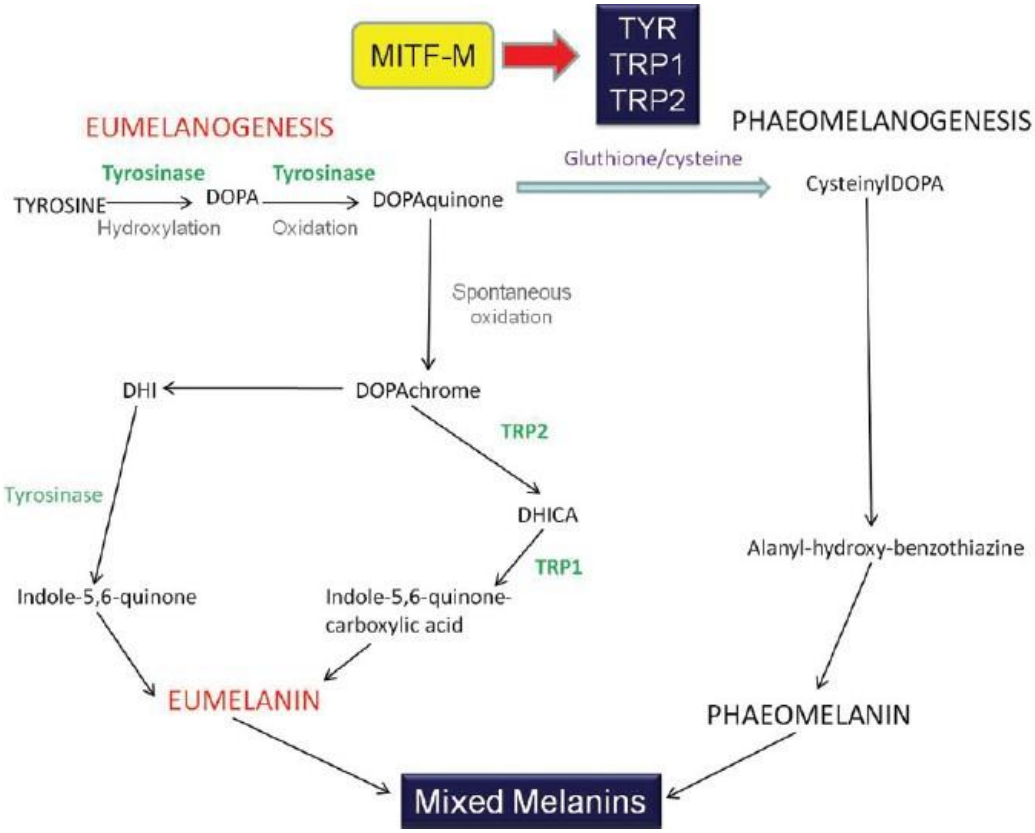

Figure 1.3: Melanin Synthesis (Chadwick, 2012).

As the transcriptions of those three enzymes are under control of microphthalmia-associated transcription factor (MITF), this basic-helix-loop-helix/leucine-zipper (bHLHZip) shaped transcription factor would be suggested as a critical regulator of the melanogenesis (Widlund, 2003) (Figure 1.3). Defects in mouse and human melanocytes were reported because of mutations of this transcription factor (Silvers, 1979; Tassabehji, 1994).

1.3. Modulation of Melanogenesis

A numerous factors endogeneous or exegeneous such as ultraviolet radiation, α-melanocyte-stimulating hormone (α-MSH) and isobutylmethylxanthine (IBMX) can easily promoted melanogenesis. An increased expression of tyrosinase and its related enzymes initiated when the increased MITF expression which can be accomplished via α-MSH and its receptor complex and by activation of Extracellular Signal-regulated Kinase (ERK) phosphorylation. Furthermore, MITF gene expression can also be increased by the activation of cAMP related binding protein (CREB) via phosphorylation by ERK (Kim, 2009). Some of the cosmetic and chemicals agents can moderate the synthesis of melanin by shifting tyrosinase activity. Several cosmetic agents used for skin paling such as kojic acid, linoleic acid, and arbutin show anti-melanogenic activity (Lei, 2000). However, these agents which have been used to modulate melanin synthesis can cause side effects and toxicity. In order to be conscious about those negative effects, natural sources must be explored seriously. Many plant extracts have been included in cosmetic formulas to promote melanogenesis for the treatment of skin color (Nath, 1994; Grimes, 2005). Piebaldism is the deficiency of melanocytes in the skin which happens as a birth defect, while vitiligo is a progressing disease with increasing paled patches on skin. There are several diseases, related to the irregularity in pigment dissemination, can be jammed under the name called leukoderma (Nath, 1994). Therefore these melanogenesis promoting extracts may have a potential for the treatment of the hypopigmentation disorders.

1.4. Phenolics and Melanogenesis

Flavonoids are common constituents found as pigment compounds and main part of human diet as vegetables, fruits and grains. Because of their several biological activities they have a wide range of cosmetic and medical application. The flavonoids have two phenyl groups which are bound with carbon atoms.Flavonoids because of their hydroxyl group location and side chains have different chemical structures and biological activities including melanin synthesis modulation. The melanogenesis-promoting actions of different plant extracts and containing flavonoids were investigated (Moreira, 2012; Takekoshi, 2014).

1.5. *Gundelia Tournefortii* L.

Tumbleweed (*Gundelia tourneforti* L.) which is from Asteraceae (Compositae) family grows on its own motion in several locations such as Israel, Iran, Turkey, Azerbaijan, Jordan, Turkmenistan and Syria. It is a perennial plant with thistle like flowers and spikes (Davis, 1975; Karis, 2001; Karabulut, 2006; Coruh, 2007). Tumbleweed needs humidified soils and sunny places and grows diverse locations in Turkey (Hedrick 1972; Jeffrey 2007). The aerial parts and the stem of this plant have different application areas in Eastern Anatolia. In addition to usage for the the treatment of vitiligo and as diuretic in folk medicine it is one of the food sources of people in that region (Hedrick 1972; Lew-Yadun 1999; Ertug 2000; Çoruh, 2007). Because of that in this study the effects of *G. tourneforti* extracts and fractions on melanogenesis was investigated.

Figure 1.4: *Gundelia tournefortii* L. var. *tournefortii* (Van Yuzuncu Yil University Herbarium)

1.6. Scope of the study

The impairment of melanin synthesis results in failed pigmentation and undesired pale patches appear on skin which has psychological effects such as social anxiety, shyness and loss of self-confidence. For the purpose of retrieval of the skin color, the modulation of melanogenesis and targeting synthesis pathway are considered as key factors. The potential effects of *Gundelia tournefortii* L. **(***G. tournefortii*) was investigated in regulation of melanin synthesis in connection with the its usage in folk medicine for the recovery of skin color.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Plant material

Aerial parts of *Gundelia tournefortii* L. were collected on the month of June or July 2013, in East Anatolia region, Van, Turkey. Plant samples were pressed according to the herbarium rules with the voucher number of F11174.

The plant materials were identified by Associate Professor Fevzi Özgökçe, Deparment of Botany, Faculty of Biology, Yüzüncü Yıl University.

2.1.2. Primary Epidermal Melanocyte cell line

Normal, human, adult melanocytes (ATCC PCS200-01) was purchased from ATCC (American Type Culture Collection).

2.1.3. WM-115 Human Melanoma cell line

WM-115 (ATCC CRL-1675) human melanoma cell line was purchased from ATCC (American Type Culture Collection).

2.1.4. Chemicals and other materials

Preperative chromatography grade methanol used in the preparation of extract was purchased from Merck (Darmstadt, Germany). Water was purified by Milli – Q system to ultra-pure water (Millipore, Bedford, MA, USA).

For the fractionation process, chloroform and ethyl acetate were purchased from Merck (Darmstadt, Germany) and magnesium sulfate was purchased from Sigma Chemical Company (St.Louis, MO, USA).

Disposable syringe filters in pore size of 0.45 μm and 0.22 μm were purchased from Millipore Corporation (Bedford, MA USA) and syringe filter (0.20 μm Minisart RC 4), from Sartorius (Gottingen, AG Germany).

2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), sodium carbonate (Na_2CO_3), and the reference compounds were all purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Folin Ciocalteu's phenol reagent, Aluminium chloride ($AlCl_3$) were purchased from Merck (Darmstadt, Germany).

Dimethyl sulphoxide (DMSO) purchased from AppliChem (Saxony-Anhalt, Germany). Dulbecco's phosphate buffered saline (PBS) purchased from Biological

Industries (Haemek, Israel). Eagle's Minimum Essential Medium (EMEM) ATCC-30-2003, Dermal Cell Basal Medium ATCC-PCS-200-030 and Adult Melanocyte Growth Kit ATCC-PCS-200-042 were purchased from ATCC. Fetal Bovine Serum (heat-inactivated) were purchased from Biochrom Ltd. (Cambridge, UK). Trypan blue stain 0.4 % and counting slides appropriate for Juli™ Br was purchased from NanoEnTek (Guro-gu, Seoul, Korea). Tyripsin-EDTA solution, antibiotic-antimycotic solution (100x) and BCA assay kit were purchased from Sigma Chemical Company (St.Louis, MO, USA). Cell Proliferation XTT Kit was

purchased from Biological Industries, Israel. Life Science innuPREP RNA mini kit was purchased from AnalytikJena, Germany. Transcriptor First Strand cDNA synthesis kit version 8 and The LightCycler 480 SYBR Green I Master were purchased from Roche, Germany. 3,4-Dihydroxy-L-phenylalanine, synthetic melanin and Triton X-100 were purchased from Sigma (MO, USA). Sodium Hydroxide was purchased from Merck (Darmstadt, Germany).
Cell culture grade chemicals and reagents were used for the whole step of cell culture experiments.

2.1.5. Instruments

The instruments used for the experiments are listed as followings: Bandelin Sonorex (ultrasonic bath); rotary evaporator (Heidolph Laborota 4000); blender: Waring model 32BL80 (New Hartford, CT, USA). Microplate reader (Multiskan GO-Thermo Scientific, USA).

Equipment used in cell culture experiments:
Airstream Class II Biohazard Safety Cabinet (ESCO, Thailand),) was used for cell culture assays. Other instruments: JULI-Br and FL Station Live Cell Movie Analyzer (NanoEnTek Inc.), Nüve EC160 CO_2 incubator, NF615 Hettich Universal 32 R Centrifuge, FLUOstar OPTIMA ELISA reader (BMG Labtech), Mettler TOLEDO, MPC227 pH/conductivity meter, WiseStir® MSH20A (Wisd Laboratory Instruments) Magnetic stirrer, Memmert WB14 waterbath, Precisa XB 220A scale, Sartorius BL 1500 Sinbo Digicook**.** For RT- PCR analysis AlphaSpec Nanodrop (Alpha Innotech), Light cycler 480 (Roche) RT-PCR, GeneAmp PCR System 9700 (Applied Biosystems) were used in Central Laboratory of Biological Sciences.

2.2. METHODS

2.2.1. Extraction procedure

The plant material washed with water several times and was dried with the blotting paper without direct sunlight. A sufficient quantity of dried plants were grinded to get almost 4 mm particle size with Waring (model 32BL80) blender and stored in a dry and dark environment at 25 ^{o}C. The grinded plant material (200 g) was incubated in methanol at the ratio of 1:6 for 24 hours in the incubator with 180 rpm rotation at room temperature. Then, mixtures were filtered through a rough filter paper. All the extraction procedure was repeated three times with fresh methanol for each time. Collected filtrates were dried completely using a rotary evaporator (Heidolph Laborota 4000) with vacuum at 40 °C. The crude extract was weighed for the percent yield calculation.

2.2.2. Fractionation procedure

The fractionation of crude extracts was performed by using solvents with increasing polarity from non-polar to polar (**Table 2.1).** The fractionation method described by Naczk and Shahidi (2004) was performed with some modifications.

Table 2.1: Physical constants of solvents used in the study, listed in order of increasing solvent polarity.

	Formula	**Formula weight**	**Density**	**Boiling point**	**Polarity index**
Hexane					
Chloroform	$CHCl_3$	88.11	1.48	61.7	4.10
Ethyl acetate	$CH_3COOC_2H_5$	119.39	0.90	77.1	4.40
Methanol	CH_3OH	32.04	0.79	64.7	5.1
Water	H_2O	18,02	1.0	100	9.0

First, a 12.1 g of crude methanol extract was dissolved in 500 mL of methanol and water mixture in the ratio of 70:30 v/v and instantly 500 mL hexane was added and mixture was vigorously shaken and kept steady in a separator funnel until organic and aqueous phase were separated. Aqueous phase which remain at the bottom of the separator funnel was taken into a beaker for the further fractionation steps; while the organic phase (hexane) was dried with rotary evaporator. This separation procedure was carried out with the solvents in the order of increasing polarity until the organic phase was completely transparent.

The second fractionation step was performed by mixing chloroform and aqueous phase. At the last step ethyl acetate was applied and the fractions are collected. Each fractionation phase was dried by evaporation and kept at +4 °C. The yield of each fractionation step was calculated as percent of 12.1 g crude extract (w/w). **Figure 2.1** summarizes the extraction and fractionation procedures.

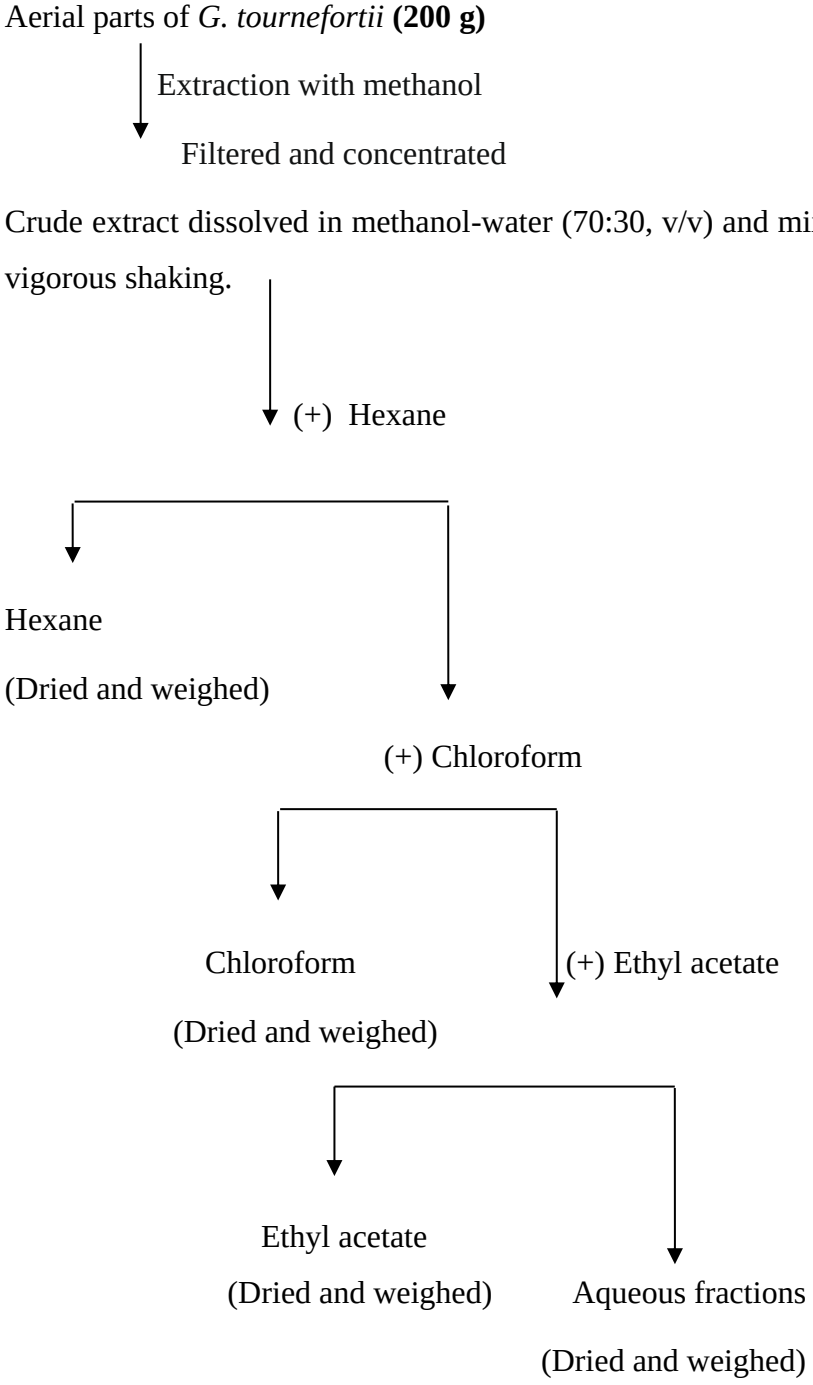

Figure 2.1: Sequential organic extractions, fractionation of *G. Tournefortii*

2.3. Determination of *in vitro* Antioxidant Capacity

2.3.1. Free radical scavenging capacity by DPPH method

A modified protocol of DPPH (2,2-diphenyl-1-picrylhydrazyl radical) procedure was carried out to decide DPPH radical scavenging capacity according to Blois' method (Blois, 1958) with some modifications. Basically, 0.05 mg/mL (0.127 mM) DPPH solution was prepared by dissolving 20 mg of DPPH radical in 400 mL absolute ethanol. 10 µL of each extracts ranging from 0.1 to 3.3 mg/mL were mixed with 140 µL DPPH solution in 96 well plate. The DPPH radical scavenging capacity of the crude extract and fractions were determined by comparing with standard compound. The reduction of DPPH in the reaction mixture was monitored after 30 minutes incubation at 517 nm with microplate reader (Figure 2.2). The results were expressed as the radical scavenging activity (RSA) in percent and calculated according to the following equation;

Radical Scavenging Activity (%) = 100x[(Absorbance of Sample with DPPH)-(Absorbance of sample blank)]/[(Absorbance of DPPH)-(Absorbance of Solvent)]

EC_{50} values were decided for the evaluation of the antioxidant capacity. EC_{50} values are defined as the concentration of substance which is enough to reduce the 50% DPPH activity. EC_{50} values were calculated by constructing the graph of percent RSA versus the extract concentration.

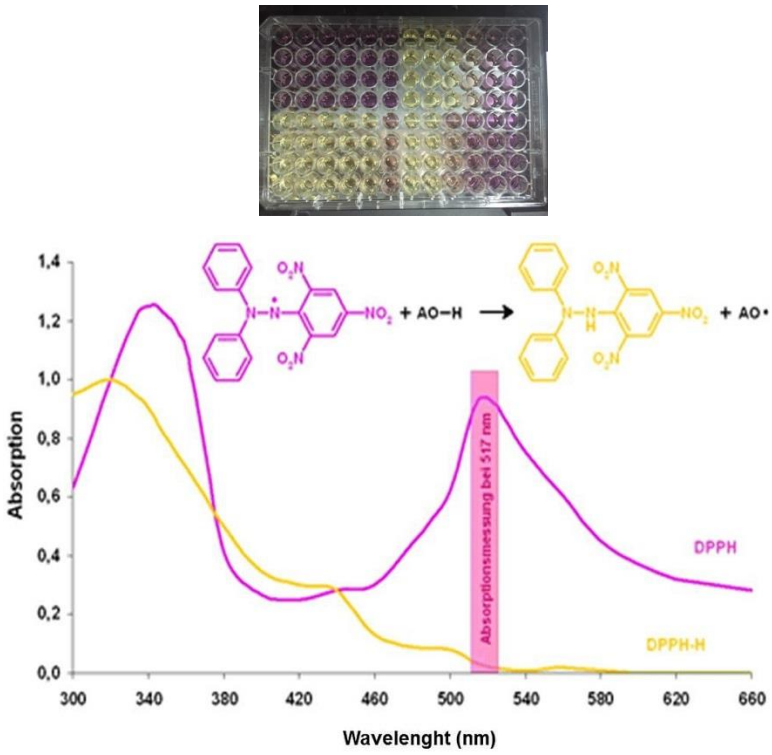

Figure 2.2: Diagrammatic representation of chemical reaction of the reduction of DPPH in the presence of an electron donating antioxidant. (Perez, 2013)

2.3.2. Free radical scavenging capacity by ABTS method

ABTS method was used for the determination of antioxidant capacity. Basically method depends on observing the ability of substances to scavenge a radical cation as equivalent of a standard (Rice-Evans, 1997). Trolox, a vitamin E analogue was used as standard.

The generation of ABTS radical, induces the production of a green $ABTS^{\bullet+}$ chromospheres because of the reaction between ABTS and potassium persulfate ($K_2S_2O_8$) as illustrated in Figure 2.3. Addition of antioxidant substances reduces the ABTS radical and the decrease of the absorbance at 734 nm (0.7±0.02 a.u) is measured. After 6 minutes the reaction stops and the absorbance value is stabilized.

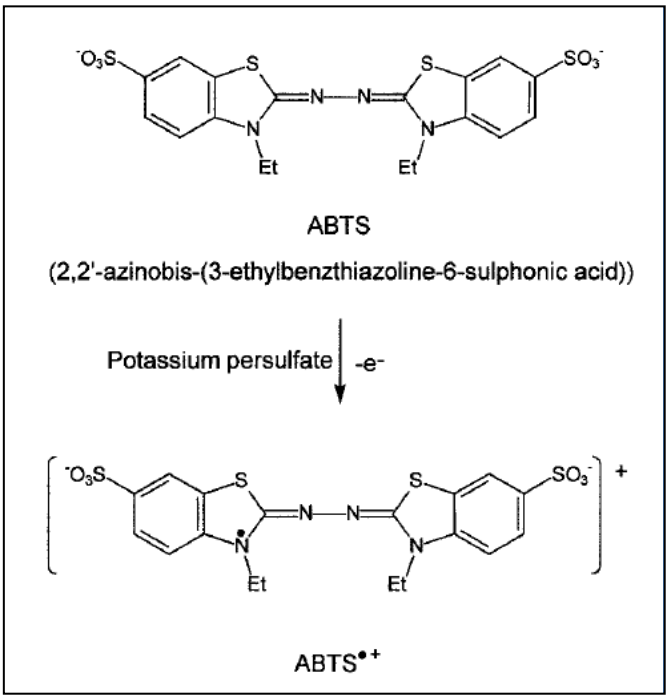

Figure 2.3: Formation of ABTS radical cation (Pannala, 2001)

The ABTS radical cation ($ABTS^{\bullet+}$) was produced by preparing a solution containing 7 mM ABTS and 2.45 mM potassium persulfate. Then this mixture was allowed to stand in dark at room temperature for 16 h. Prior to use, the mixture was diluted by the addition of 100 mL 96% ethanol and the absorbance of solution was

adjusted to 0.7 ± 0.02 a.u. After addition of plant samples and trolox absorbance decrease was measured at 734 nm. ABTS radical scavenging capacity in percent was calculated according to following equation;

$$RSA\ \% = [(A_0-A_1)/A_0] \times 100$$

A_0: ABTS solution with methanol

A_1: ABTS solution with trolox or extract and fractions at various concentrations

After calculation of radical scavenging activity for trolox and plant extracts RSA % versus concentrations were plotted and trolox equivalent antioxidant capacity (TEAC) values were determined. TEAC values were calculated by dividing the slope of extract concentration versus RSA % graph to that of trolox calibration curve. All the experiments were carried out in quadruplets and repeated three times.

2.3.3. Determination of total phenolic content

Total phenolic content of the crude extract and all fractions were evaluated according to the Folin-Ciocaltaeu method proposed previously by Singleton and Rossi (1965) with some modifications. 96 well-plates are used instead of test cuvvettes. Varying concentrations of gallic acid which was used as a standard, crude extracts and all fractions were dissolved in methanol. 10 µL of 1:4 Folin-Ciocaltaeu reagent dissolved in distilled water was mixed with 10 µL of extracts using multi-channel pipette. After 10 min 200 µL of 2% Na_2CO_3 was added and 96-well plates were incubated in dark, at room temperature, for 30 minutes. At the end of the reaction time, absorbance values were measured at 750 nm by Multiskan™

GO Microplate Spectrophotometer (ThermoFisher Scientific, USA) against blank, which constitutes only 10 µL of methanol. The calibration curve of final gallic acid concentration (prepared as 0.023, 0.0114, 0.006, 0.003, and 0.001 mg/mL) versus absorbance was used to calculate the amount of phenolic content in terms of gallic acid equivalent (GAE). All experiments were carried out with 6 various concentrations in triplicates and repeated for two different experiments.

2.3.4. Determination of total flavonoid content

Total flavonoid contents in the extract and fractions were determined according to Zhishen et al. (1999) with slight modifications based on the colorimetric reaction of sodium nitrite and aluminum chloride in the presence of flavonoids. 15 µL of various concentrations of samples and catechin standard, were mixed with 5 µL of 5% sodium nitrite ($NaNO_2$) in 96 well plates. After incubating for 5 minutes at room temperature, 10 µL of 5% aluminium chloride ($AlCl_3$) was added. After 6 minutes 35 µL of 1 M sodium hydroxide (NaOH) was added and then reaction mixtures were diluted to the total volume of 200 µL with 135 µL of distilled water. The absorbance was measured against blank solution at 510 nm by using Multiskan™ GO Microplate Spectrophotometer (ThermoFisher Scientific, USA). Calibration curves of the standard and extract were plotted as absorbance at 510 nm against final concentration. Total flavonoid content was calculated as catechin equivalent (CAE) by using catechin calibration curve equation. All experiments were carried out with 6 various concentrations in triplicates and repeated for two times.

2.4. Cell Culture

2.4.1. Cell lines and Growth Conditions

WM-115 human melanoma cells were grown in Eagle's Minimum Essential Medium with 10 % heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic solution. Primary epidermal melanocytes were grown in Dermal Basal Medium with Adult Melanocyte Growth Kit and 1% antibiotic-antimycotic solution. Adult Melanocyte Growth Kit contains at the final concentration of 5 µg/mL recombinant human insulin, 50 µg/mL ascorbic acid, 6mM L-glutamin, 1 µM epinephrine, 1.5 mM calcium chloride, peptide growth factor and M8 supplement as given by ATCC.Both cell lines incubated at 37 ^{0} C in a 95 % humidified atmosphere of 5 % CO_2.

2.4.2. Cell Passaging

The cells were passaged when the culture had reached approximately 80% to 90 % confluence. If the cells were passaged later, the cells would die and detach. Trypsin-EDTA solution was warmed to room temperature. The growth medium was warmed to 37°C prior to use with the cells. The spent media was aspirated carefully without disturbing the monolayer. Pre-warmed trypsin-EDTA solution was added to each flask. The cells were observed under the microscope. When the cells appeared to detached, quickly equal volume of fetal bovine serum was added to each flask. Dissociated cells were transferred to a sterile centrifuge tube and centrifuged at 150 x g for 3 to 5 minutes. The supernatant was aspirated and the cell pellet was resuspended in 2 to 8 mL complete growth medium to seed new culture flasks. The flasks were placed in a 37°C, 5% CO2 incubator for at least 24 hours before the further processing of the cells.

2.4.3. Freezing and Thawing

In order to freeze the cells, freezing medium was prepared as 5% DMSO in complete growth medium. After trypsinization and centrifugation cell pellet was resuspended in 1.5 mL of freezing medium. The resuspended cells placed into the cryovials. The cryovials were stored at -20 °C for 2 hours and than -80 °C for 24 hours. Finally, cryovials were transferred into tank containing liquid nitrogen.

In order to thaw the cells, cryovials were taken from the liquid nitrogen tank and thawed by gentle agitation (approximately 2 minutes) in 37 °C water bath. When the medium completely dissolved, the vial was decontaminated by spraying with 70% ethanol. The content of vial transferred to a 15 mL conical tube which containing complete growth medium. After centrifugation at 125 xg for 5 minutes cell pellet was resuspended in complete growth medium and seeded into flasks. The flasks were incubated in a 37°C, 5% CO2 incubator for at least 24 hours.

2.4.4. Cell viability (Trypan blue dye exclusion assay)

Trypan blue dye exclusion method was applied to determine cell membrane integrity and to count alive and dead cells. This dye can be actively transported to the outside of the membrane by living cells, but dead cells can not transport the dye. Because of that dead cells are observed blue under the microscope.

Primary epidermal melanocytes and WM-115 human melanoma cells were detached by trypsin/EDTA. After detaching the cells and obtaining cell suspension, 10 µl of trypan blue solution added on 10 µl of Primary epidermal melanocyte and WM-115 human melanoma cell suspensions in a 96 well plate. The cell suspension in trypan blue solution was mixed gently by pipetting and 10 µl of the mixture was loaded to each chambers of a counting slide. Lastly, the numbers of viable and dead cells were monitored under JULI-BR cell imaging system.

2.4.5. Determination of growth curve

Primary epidermal melanocytes and WM-115 human melanoma cell lines were seeded in 6-well plates, at a density of 100000 cells/well. Two wells from each cell line were trypsinized and counted in every 24 hours. The cells were stained with 0.25 % (w/v) trypan blue (Biological Industries, Israel) and counted with JULI. The cells were counted for 7 and 14 days for WM-115 and Primary Melanocytes, respectively. Growth curve was constructed as number of cells ($x10^5$) / mL versus time (day).

2.4.6. Cytotoxicity

2.4.6.1 Preparation of crude extract, ethyl acetate and chloroform fraction stock solution

Crude extract, ethyl acetate and chloroform fraction, were used as melanogenic agents in Primary Epidermal Melanocytes and WM-115. 40 mg dry crude extract and fractions were dissolved in DMSO for cell culture experiments.

Preparation of treatment media: Before the treatment the highest treatment concentratin (40 μg/mL) was prepared by adding 1 μL of stock soliton to each 1 mL of complete growth media. The other concentrations (20/10/5/1 μg/mL) were prepared by diluting this highest concentration with complete growth media. 1 μL DMSO was added for per mL additional complete growth media to keep DMSO concentration constant at 0.1%.

2.4.6.2. XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay

The effect of crude extract, ethyl acetate and chloroform fraction on cell viability of Primary epidermal melanocytes and WM-115 human melanoma cells were investigated by using Cell Proliferation XTT Kit (Biological Industries, Israel). The test is based on quantitative measurement of extracellular reduction of XTT to water soluble orange coloured formazan derivative by metabolically active cells.

This reduction is mediated by mitochondrial dehydrogenase enzyme. Reduction of XTT is greatly enhanced by electron coupling reagent. Electron coupling reagent mediates reduction of XTT by transferring electron from cell surface to extracellular XTT. The tetrazolium salt is reducable to orange colored formazan by metabolically active cells.

Primary epidermal melanocytes and WM-115 human melanoma cells were seeded into 96 well plates (about 60000 cells/well for the primary epidermal melanocytes and about 30000 cells/well for WM-115) and left for 48 hours in order to let them attach and grow. After 48 hours, cells were treated with crude extract and fractions at final concentrations of 0, 5, 10, 20, 40 μg/mL for 72 hours. The DMSO concentration was kept 0.1% in each well. The control wells prepared as containing only growth medium (100 μl) and containing 0.1% DMSO in growth medium. Also the control wells with no cell were used for only growth medium, medium with vehicle (0.1% DMSO) and all treatment concetrations.

The XTT solution and activation solution (PMS (N-methyl dibenzopyrazine methyl sulfate)) were defrosted immediately prior to use in a 37°C bath. 100 μL activation solution was added to 5mL XTT reagent. When the treatment time was completed, 50μl of the reaction solution was added to each well and the plate was incubated in a CO_2 incubator for 2 hours. Following the reaction with XTT reagent mixture for 2 hours in CO_2 incubator at 37 ^{0}C, the absorbance was read at 475 nm with ELISA reader. The percent viability of the primary epidermal melanocytes and WM-115 human melanoma cells after treatment with crude extract, ethyl acetate and chloroform fractions of *G. tournefortii* was calculated by the following equation:

$$\text{Cell viability (\%)} = \frac{\text{(average OD treated cells)- (average OD of sample w/o cells)}}{\text{(average OD of untreated cells)- (average OD of control w/o cells)}} \times 100$$

OD: Optical density

2.4.7. Determination of Secreted Melanin

The secreted melanin assay was performed as previously described (Bhatnagar, 1998). Primary epidermal melanocytes and WM-115 human melanoma cells were treated with the the extract and fractions for 72 hours. After treatment the spent culture medium was collected and centrifuged at 10.000 rpm for 10 minutes. The absorbance of medium was measured at 405 nm with ELISA reader. The secreted melanin was calculated as follows;

$$\text{Secreted Melanin (\%)} = [(C-A)/B] \times 100$$

A: Absorbance of the medium supplemented with each sample before the incubation

B: Absorbance of the untreated medium collected after 72 hours treatment

C: Absorbance of the medium supplemented with each sample and collected after 72 hours treatment

2.4.8. Determination of Intracellular Melanin Content

Determination of intracellular melanin content was performed as previously described by Stasz et al. with slight modifications (Stanisz, 2013). $3x10^5$ cells for WM-115 cells and $6x10^5$ cells for the primary melanocytes were plated in 60mm dishes. After 48 hours cells were treated with various concentrations of crude extract and fractions for 72 hours. Untreated cells were used as control. After 72 hours, cells were washed twice and lysed in phosphate-buffered saline with 1% Triton X-100 at 4°C. The lysate was than centrifuged at 12,000 *rpm* for 45minutes at 4°C. The remaining pellet was dissolved in 230µl 1N NaOH with 10% DMSO at 85°C for 60minutes. To determine the melanin content, absorbance was measured at 405nm. Absolute melanin content was calculated by a melanin calibration curve which is constructed with synthetic melanin. Melanin concentration was normalized to total protein amount.

2.4.9. Determination of Tyrosinase Activity

Tyrosinase enzyme catalyzes the rate-limiting step of melanogenesis (Park, 2004), which hydroxylates tyrosine into 3,4-dihydroxyphenylalanine (DOPA) and converts DOPA into dopaquinone by oxidation (Hearing, 1999). Tyrosinase enzyme activity was determined as previousy described (Stanisz, 2013), with slight modifications. L-DOPA was used as substrate. Melanocytes were pretreated similarly as mentioned determination of melanin content part. Cells were lysed in 200µl phosphate-buffered saline with 1% Triton X-100 at 4°C and centrifuged at 12000 *rpm* for 45 minutes at 4°C. Protein concentrations of supernatants were determined with BCA assay kit using bovine serum albumin as standard. Then, 90 µl of the supernatant containing 40 µg total proteins was mixed with 10 µl 10 mM of L-DOPA. After incubation for 1 hour at 37 ^oC, the absorbance was monitored at 475 nm. The samples without L-DOPA were used as blank.

2.4.10. Determination of mRNA Expression

2.4.10.1 Total RNA Extraction

$3x10^5$ cells and $6x10^5$ cells were plated in 60 mm dishes for WM-115 cells and primary melanocytes, respectively. After 48 hours cells were treated with various concentrations of crude extract and fractions for 72 hours.

For the extraction of total RNA Life Science innuPREP RNA mini kit was used. At the end of treatment spent media was removed by aspiration and the cells were washed twice with PBS. The cells were lysed with 350 µL of lysis solution and the lysates were transferred to D-spin column to remove DNA content of the cells.

After 2 min centrifugation at 12000 rpm remaining eluent was mixed with equal amount of 70% ethanol by gently pipetting and it was transferred into R-spin column and centrifuged for 2 min at 12000 rpm.

After discarding remaining eluent, the column washed with HS and LS washing buffers respectively. The column was allowed to dry for 3 min by centrifugation at12000 rpm. Finally, 30 µL elution buffer was added to the column and the column was centrifuged for 1 min at 8000 rpm.

Total RNA content and purity was determined with AlphaSpec Nanodrop (Alpha Innotech) by the assessment of 260/280 nm ratio. The ratio of OD260/OD280 must range from 1.8 to 2.2. While the values below 1.8 are considered as the DNA contamination, higher than 2.2 considered as the protein contamination.

2.4.10.2 cDNA synthesis

cDNA was synthesized according to the Transcriptor First Strand cDNA synthesis kit version 8 (Roche, Germany). All the reagents were thawed and kept on ice.

Table 2.2. cDNA synthesis reaction mixture

Component	Volume	Final concentration
Template RNA	variable	100 ng
Anchored-oligo $(dT)_{18}$ primer, 50 pmol/µL (vial 5)	1 µL	2.5 µM
Water (PCR-grade)	variable	to make total volume=13 µL
Total volume	**13 µL**	
Transcriptor Reverse Transcriptase Reaction Buffer, 5x conc. (vial 2)	4 µL	1 x (8 Mm $MgCl_2$)
Protector Rnase Inhibitor 40 U/ µL (vial 3)	0.5 µL	20 U
Deoxynucleotide Mix, 10 Mm each (vial 4)	2 µL	1 Mm each
Transcriptor Reverse Transcriptase 20 U/ µL (vial 1)	0.5 µL	10 U
Final volume	**20 µL**	

For cDNA synthesis reaction mixture was prepared as seen in the **Table 2.3**. Mixture was incubated at 55 ^{o}C, for 30 min. Reverse transcriptase was inactivated

for 5 min, at 85 ºC in a conventional thermal cycler, GeneAmp PCR System 9700 (Applied Biosystems). The resulting cDNA was kept at -20 ºC.

2.4.10.3 Quantitative Real-time PCR Analysis

Prior to use 180 µL of RNAse/DNAse free water was added to the synthesized cDNA (1:10 dilution) as described in product manual (Light cycler 480 SYBR Green I Master, Version 12, Roche).

100 µM stock primers were diluted as 1:100 with RNAse/DNAse free water.

Table 2.3: Reaction mixture in a well.

Component	**Volume**
Water, PCR-grade	3 µL
PCR primer	2 µL
Master Mix, 2x concentration	10 µL
Total Volume	**15 µL**

The reaction mixture was prepared as seen in table. Then 5 µL of cDNA template was added to each well and 96 well-plate was sealed. Reactions were run using Roche 480 light cycler with reaction profile as follows; pre-incubation for 5 min at 95°C; PCR amplification for 45 cycles with 10 s at 95°C, 10 s at 60°C and 10 s at 72°C. This was followed by a melt curve analysis to determine the reaction specificity. Expression level of each targeted gene was normalized to GAPDH.

Table 2.4: Primers

Gene name	Forward Primer 5'->3'	Reverse Primer 5'->3'
GAPDH	TCCCTGAGCTGAACGGGAAG	GGAGGAGTGGGTGTCGCTGT
TYR	GATGAGTACATGGGAGGTCAGC	GTACTCCTCCAATCGGCTACAG
TYRP1	GCTCCAGACAACCTGGGATA	TCAGTGAGGAGAGGCTGGTT
TYRP2	AGATTGCCTGTCTCTCCAGAAG	CTTGAGAATCCAGAGTCCCATC
MITF	CTCACAGCGTGTATTTTTCCCA	ACTTTCGGATATAGTCCACGGAT

2.5. Statistical analysis

All values are stated as mean ± standard error of the mean by Microsoft Excel. The student's *t*-test was used for statistical analysis of all data. A statistically significant difference was considered to be at $P < 0.05$.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Extraction of *G. tournefortii*

G. tournefortii dry ground samples (200 g) were extracted with 1200 mL methanol (1:6) by using oval-rotating incubator at 25 °C and 180 rpm overnight and extract was dried completely using a rotary evaporator. After weighing the percent yield was calculated for the extract. According to calculation 200 grams of methanol extract yielded 7.55 %.

3.2. Fractionation of crude extract

A 12.1 g of crude extract was dissolved in methanol-water mixture of total 500 mL (70:30, v/v). Methanol-water mixture was transferred into a separatory-funnel and the same volume of hexane was added to separatory-funnel. When aqueous and organic phase separation obtained by fractionation, separated organic phase was collected and evaporated. Same procedure was carried out with other specified organic solvents in the order of increasing polarity; chloroform and ethyl acetate after the hexane fractionation. After the last fractionation step, the remaining

aqueous fraction was seperated and evaporated. These separation processes were repeated one by one and three times. The subsequent fractionations and their yields are summarised in Figure 3.1.

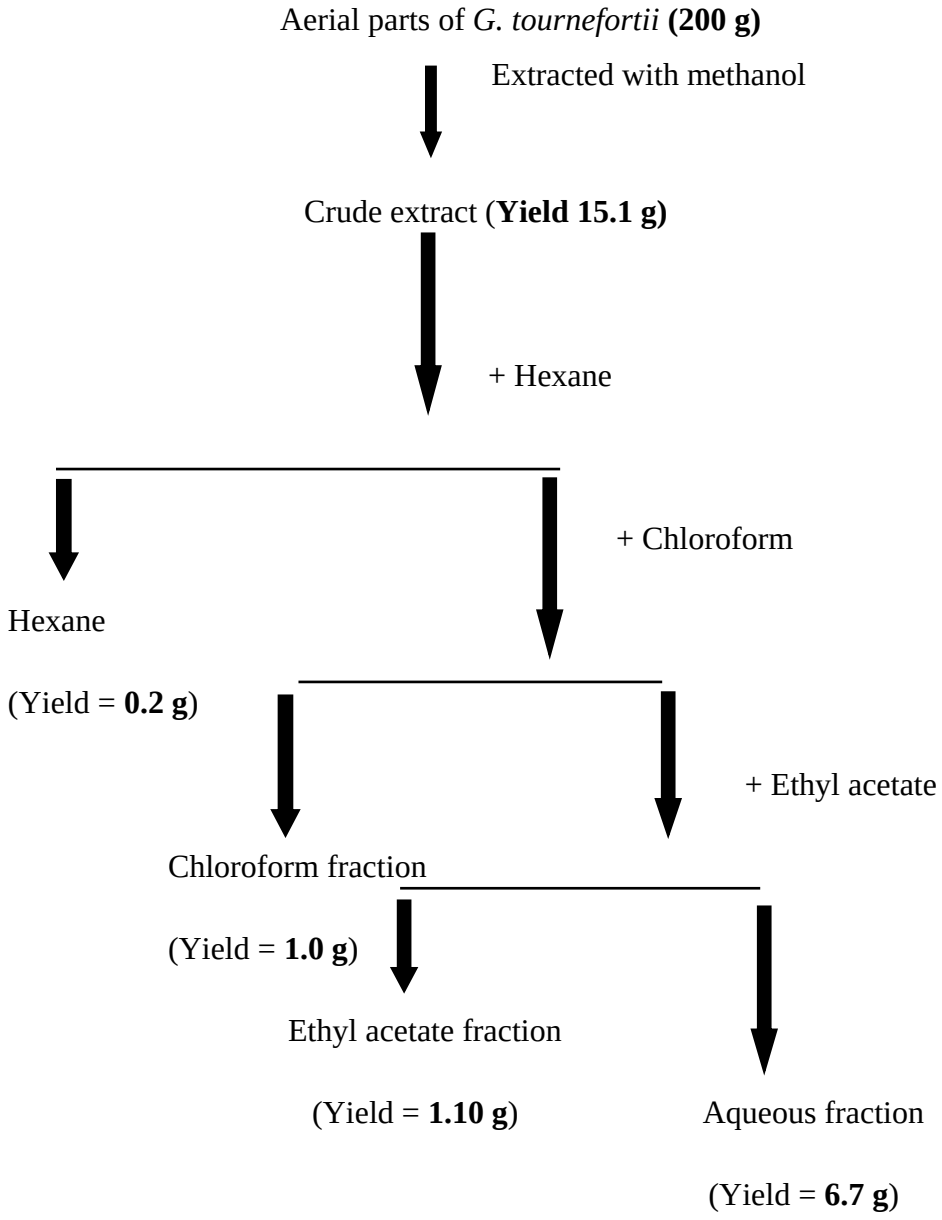

Figure 3.1: Solvent-solvent fractionation scheme of *G. tournefortii* crude methanol extract using different solvents of increasing polarity sequentially.

Because of the efficiency and easy application, one of the widely used procedures for the preparation of extracts from plant samples is solvent extractions. The time spent and the solvents used for extraction, chemical properties of the sample, the ratio of sample to solvent, pH and temperature are important factors which determines the yield of extraction procedure (Pinelo, 2004).

For the extraction of phenolic contents of plant materials different solvents, such as methanol, ethanol, acetone, ethyl acetate and combinations of these solvents have been used. The amount of polyphenols extracted, and rate of extraction depend on correct order and selection of solvents.

The use of methanol which is commonly used as the main extraction solvent gave the highest yield of extract when compared with other solvents (Farhoosh, 2004). In present study, methanol/water mixture was used at the ratio of 70:30 to extract phenolic compounds since the phenolics are generally extracted in higher amounts in more polar solvents when compared with absolute methanol (Shiddhuraju, 2003).

The phenolic contents of the crude extracts may be lower than appropriate fractions because of their high carbohydrate and lipid contituents. There are several widely used procedures for obtaining phenolic rich fractions such as liquid-liquid partitioning, solid phase and sequential extraction.

In this study, among various fractionation methods, each one with different efficiency and complexity, the liquid–liquid or solvent-solvent fractionation processes was preferred for its simplicity and convenience.

The percent yields of the extraction and fractionation of *G. tournefortii* samples obtained using solvents with the increasing polarities, such as hexane, chloroform, ethyl acetate, methanol-water (aqueous) are given in **Table 3.1**

Table 3.1: Yield of fractions of extract of *G. tournefortii*

Extract/Fractions	Yield (%)
Crude extract	7.55
Hexane	1.32
Chloroform fraction	6.62
Ethyl acetate fraction	7.28
Aqueous fraction	44.37

3.3. Evaluation of Antioxidant Capacity

Plant extracts are consisted of many antioxidant compounds. Antioxidant capacities are generally analyzed by *in vitro* and *in vivo* methods. For this study two *in vitro* methods DPPH and ABTS radical scavenging capacity assays were chosen.

3.3.1. Free radical scavenging capacity by DPPH

The DPPH method is routinely practiced to determine antioxidant properties of various compounds including plant extracts (Koleva, 2002; Mishra, 2012). It is simple, quick and economic method to estimate free radical scavenging capacity of samples.

Free radical scavenging capacities of the extract and fractions were calculated as the 50 percent effective concentration (EC_{50}). The antioxidant capacity and EC_{50} value increase indirectly. EC_{50} values were calculated by using the graph of RSA (%) versus concentration (mg/mL) of the crude extract, fraction and standard, which were illustrated in Figure 3.2.

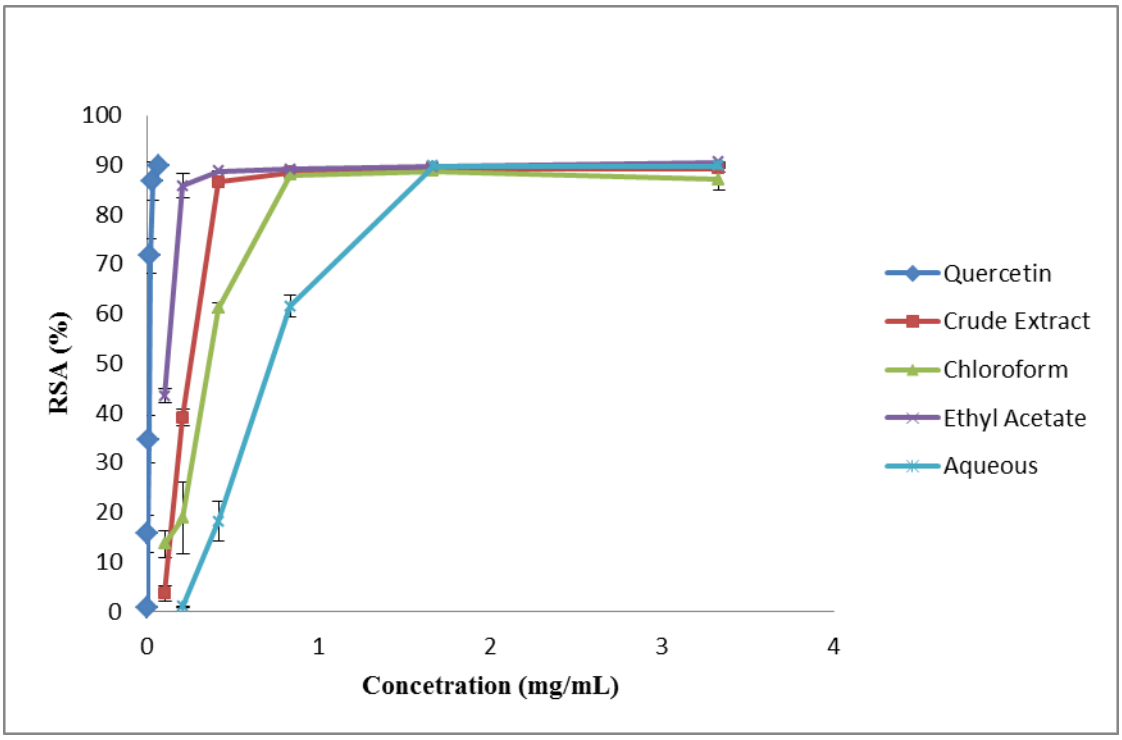

Figure 3.2: Dose dependent DPPH radical scavenging activities (RSA%) versus the concentration of crude extract and fractions (mg/mL) at 517 nm. Each point is the mean of triple measurements from three different sets of experiments (n=3).

In this study, the free radical scavenging capacity of crude extract and fractions of *G. tournefortii* was calculated by DPPH assay as shown in **Table 3.2**. All tested fractions showed a significant antioxidant capacity against the DPPH radical. Among the samples of crude extract and fractions, the lowest EC_{50} value, which means the highest radical scavenging activity, was observed for ethyl acetate fraction with an EC_{50} value of 0.016 ± 0.031 mg/mL. It indicates that this fraction was a good scavenger of DPPH radical. In the literature, DPPH activity of *G. tournefortii* leaves extract in methanol was reported as 11.85±0.50 mM Trolox equalent/g (Ozkan, 2011). The DPPH radical scavenging activity of aerial parts and seeds of *G. tournefortii* was determined by another research group using quercetin as a standart. EC_{50} values were found as 0.442 and 0.073 for aerial parts and seeds,

respectively (Coruh, 2007). On the other hand, the hexane fraction diaplayed the lowest radical scavenging capacity with an EC_{50} value of 1.434 ±0.533 mg/mL. In addition, the EC_{50} values of the ethyl acetate fraction, crude extract, chloroform and aqueous fraction were found as 0.016 > 0.0778> 0.086 > 0.126 mg/ml, respectively.

Quercetin, which is a well-known phenolic standard, with an EC_{50} value of 0.0022 ±0.001 mg/mL, is mostly used as a positive control for the DPPH assay.

Table3.2. Comparison of DPPH EC_{50} (mg/mL) results for extract and fractions.

Crude/Fractions/Standard	**DPPH** **EC_{50} (mg/mL) ± SD***
Quercetin	0.002 ± 0.001
Crude Extract	0.078 ± 0.012
Hexane	1.434 ± 0.533
Chloroform	0.086 ± 0.057
Ethyl Acetate	0.016 ± 0.031
Aqueous	0.126 ± 0.018

DPPH EC_{50}: Effective concentration of crude extract and fractions enough for scavenging 50% of DPPH radical. The reduction of DPPH was monitored at 517 nm with Multiskan™ GO Microplate Spectrophotometer. Each point is the mean of triple measurements from three different sets of experiments (n=3).

In the present study, ethyl acetate fraction exhibited high antioxidant capacity which was determined by DPPH method. This was an expected result, because of

its high polarity most of the phenolic compounds were expected to be dissolved in ethyl acetate fraction.

3.3.2. Free radical scavenging capacity by ABTS

ABTS assay is prevalently used for determination of antioxidant capacity. It is advantageous, easy and reliable to observe total antioxidant capacity in plant and other extracts. Additionally, the principle of ABTS assay is very comparable to DPPH radical scavenging assay.

Antioxidant capacities of the crude extracts and the fractions of the *G. tournefortii* were determined according to their radical scavenging capacity with ABTS method.

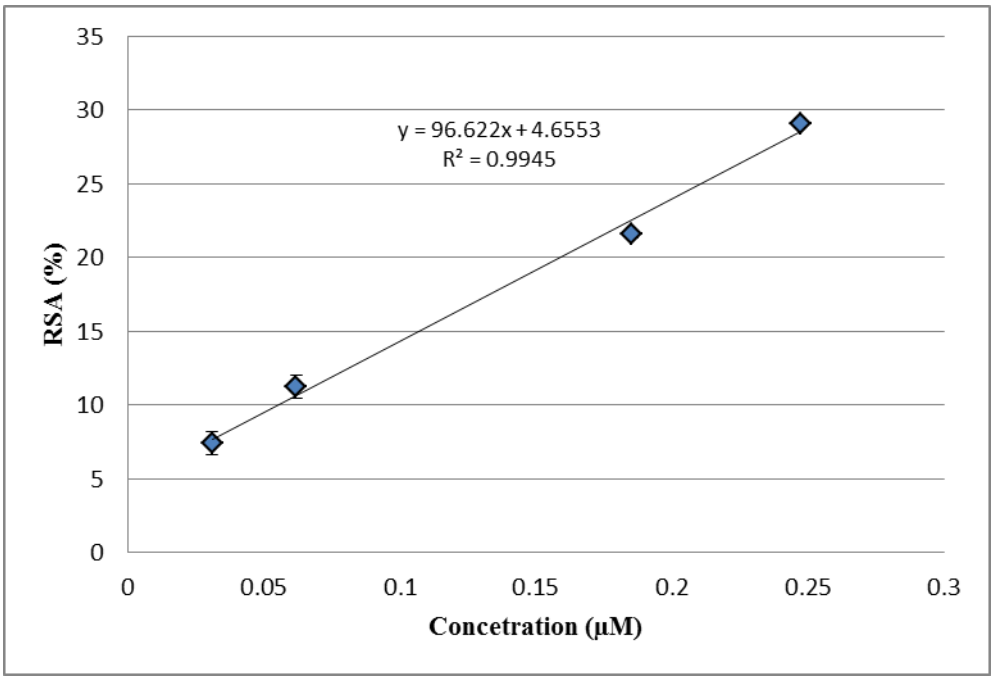

Figure 3.3: Radical scavenging activity in percent versus Trolox concentration.

The concentration of trolox standard versus RSA (%) graph was shown in Figure 3.3. The linear regression equation driven from this curve was further used to calculate the trolox equivalent antioxidant concentration (TEAC) values for the samples. For this, the slopes of regression models of samples were divided to the slope of trolox standard in order to calculate the TEAC values in terms of μM Trolox equivalent/mg extract. Results were illustrated in **Table 3.3.**

Table 3.3: Trolox equivalent antioxidant capacities (TEAC) of crude extract and fractions.

extract/fractions/standard	**TEAC value (μM Trolox ± sd*)**
Crude Extract	3.336 ± 0.030
Hexane fraction	0.444 ± 0.010
Chloroform Fraction	2.724 ± 0.042
Ethyl Acetate Fraction	4.910 ± 0.081
Aqueous Fraction	0.835 ± 0.061

The absorbances were measured at 734 nm with microplate reader. Each point is the mean of quadruplet measurements from three different sets of experiments.

TEAC values calculated for crude extract and for each fraction of hexane, chloroform, ethyl acetate and aqueous phase were found as 3.336 ± 0.030, 0.444 ± 0.010, 2.724 ± 0.042, 4.910 ± 0.081 and 0.835 ± 0.061 μM, respectively. Among the investigated fractions, ethyl acetate displayed the highest TEAC value which means ethyl acetate fraction has the highest radical scavenging capacity. These results were compatible with DPPH method.

There is no study for comparing the antioxidant capacity of crude extract and different fractions obtained from *G. tournefortii*. However, antioxidant capacities of aqueous and methanolic extracts of plant were stated as 57.3 ± 2.7 μmol TE/g, 63.9 ± 2.0 μmol TE/g for aqueous extract and methanolic extract, respectively (Tawaha, 2007). Also, the antioxidant capacity of methanol extract was reported as 5.79 ± 1.36 mmol TE/g in another study (Ozkan, 2011).

3.3.3. Determination of total phenolic content

Total phenolic contents of *G. tournefortii* crude extract and fractions were determined by the method of Singleton and Rossi (1965), the Folin-Ciocalteu reagent assay with some modifications. The Folin-Ciocalteu assay is widely used, because of its simplicity, speed and sensitivity. The phenolic groups are oxidized by Folin reagent and the reaction results with the formation of green-blue product at 750 nm.

The results were expressed as μg phenolic equivalents of gallic acid (GAE) in mg of extract. The graph of gallic acid concentration against absorbance value at 750 nm was drawn. The equation driven from that calibration curve was used for the calculation of samples' phenolic content in terms of gallic acid equivalent value (GAE). The calibration curve was demonstrated in Figure 3.4. The linear regression curve equation is $y= 31.305x - 0.0421$ with $R^2 = 0.997$.

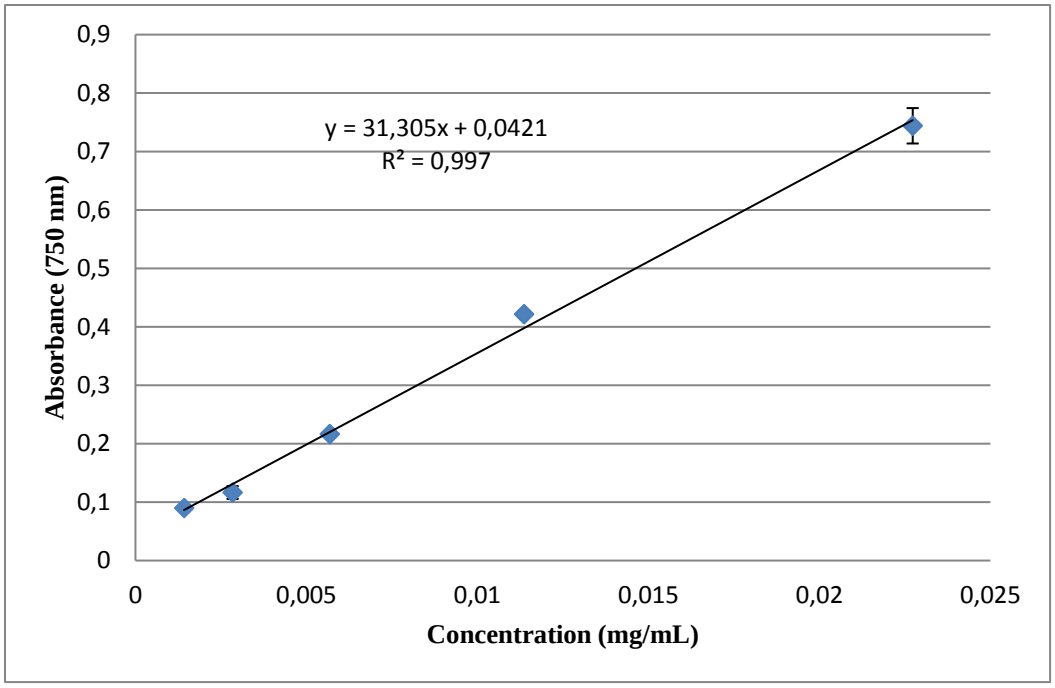

Figure 3.4: Gallic acid calibration curve. Experiments were performed three times in triplicates. The absorbances were measured at 750 nm with microplate reader. Experiments were performed three times in triplicates.

Total phenolic content of extracts were given in Table 3.4. Ethyl acetate fraction, with a value of 62.018 GAE µg/mg extract, displayed the highest total phenolic content. However, the phenolic content of hexane fraction was the lowest with a value of 7.413 GAE µg/mg extract. The highest phenolic content (62.018 GAE µg/mg) value indicates that the ethyl acetate fraction is a rich source of natural phenolic compounds.

Table 3.4: Total phenol contents in crude extract and different fractions of *G. tournefortii* as expressed in gallic acid equivalents (GAE).

extract/fractions/standard	**Total Phenol GAE (µg/mg) ± sd***
Crude Extract	36.178 ± 0.021
Hexane Fraction	7.413 ± 0.003
Chloroform Fraction	37.897 ± 0.024
Ethyl Acetate Fraction	62.018 ± 0.050
aqueous fraction	18.966 ± 0.008

The absorbances were measured at 750 nm with microplate reader. Experiments were performed three times in triplicates.
Total Phenol GAE: Total phenolic contents µg equivalents of gallic acid/mg of plant extract.

The phenolic content of *G. tournefortii* was previously reported as 76.3 µg/mg, 128.4 µg/mg, 103.8 µg/mg for the seeds, leaves at the flowering stage and seeding stage, respectively. In this study chlorogenic acid was used as standard (Haghi, 2011). Another research group reported phenolic content of *G. tournefortii* leaves as gallic acid equivalent. The result was found as 324.13 ± 5.15mg/g (Ozkan, 2011). Also, total phenol contents of aerial parts and seeds were stated as 64.4 ± 4.8 µg GAE/mg, 105.1 ± 8.7 µg GAE/mg (Coruh, 2007).

In addition, total phenolic content of aqueous and methanolic extracts of plant were previously reported as 13.2 ± 5.0 mg GAE/g, 14.7 ± 0.2 mg GAE/g for aqueous and methanolic extract, respectively (Tawaha, 2007).

3.3.4. Determination of total flavonoid content

Flavonoids are the class of important phenolic compounds that found in plants. Many studies have indicated that consumption of flavonoids and other phenolics in food plants would reduce cancer and cardiovascular disease risk (Kahkonen, 1999; Hertog, 1993; Wattenburg, 1990). Total flavonoid contents of any sample can be determined by reaction with sodium nitrite, which causes flavonoid aluminum complex formation in alkaline condition. The resulting colored product is detectable spectrophotometrically at 510 nm (Abu Bakar, 2009).

The equation of catechin calibration was y = 7.9937x + 0.0473 with R^2 = 0.9894 which is used for the calculation of the total flavonoid content as catechin (CAE) equivalents in microgram per milligram crude extract and fractions **(Figure 3.5**).

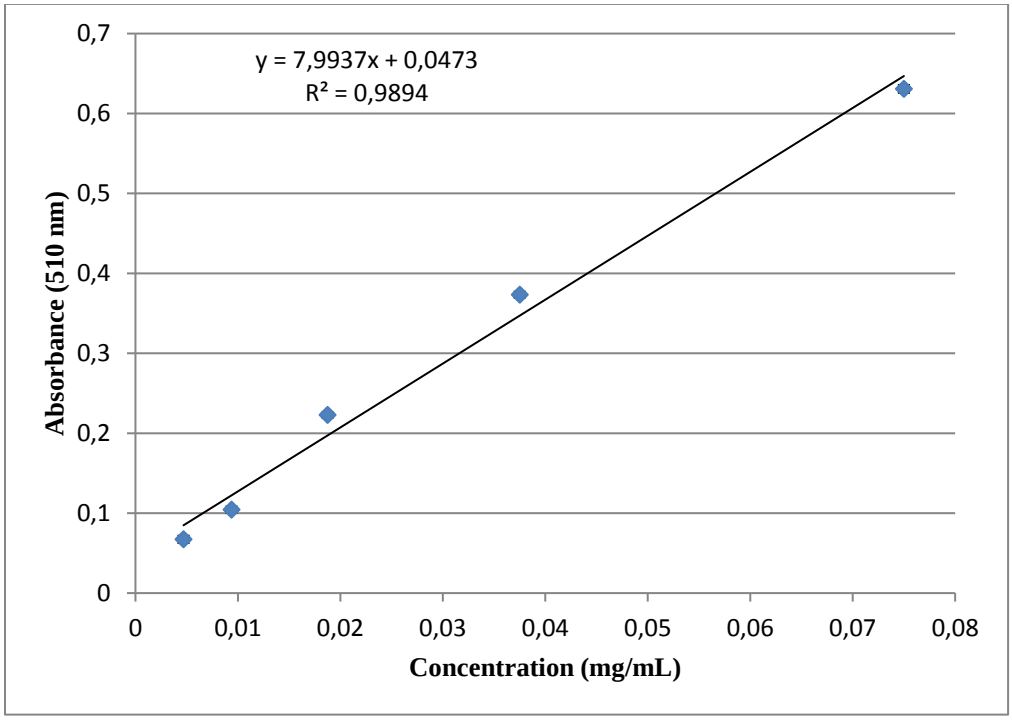

Figure 3.5: Catechin calibration curve. The absorbances were measured at 510 nm with multi-well plate reader. Experiments were performed three times in triplicates.

Total flavonoid content of crude extract and its fractions were given in **Table 3.5**. The highest total flavonoid contents were found in the order of ethyl acetate fraction > crude extract > chloroform fraction > hexane fraction > aqueous fraction. The highest total flavonoid content was found in ethyl acetate fraction with a value of 323.285 ± 0.024 µg CAE/mg of extract. The lowest content of the total flavonoids was detected in the aqueous fraction (14.349 ± 0.008 µg CAE/mg of extract).

Table 3.5: Total flavonoid content of crude extract and fractions.

Extract/fractions	**Total flavonoid CAE (µg/g)**
Crude Extract	137.811 ± 0.075
Hexane Fraction	22.628 ± 0.004
Chloroform Fraction	98.962 ± 0.058
Ethyl Acetate Fraction	323.285 ± 0.024
Aqueous Fraction	14.349 ± 0.008

The absorbances were measured at 510 nm with multi-well plate reader. Experiments were performed three times in triplicates.
TF CAE: Total flavonoid contents µg equivalents of catechin/mg of plant extract

There is no study in the literature related to the total flavonoid content of the fractions of *G. tournefortii*, but methanolic extract of leaves was reported as 54.80 ± 1.21 mg quercetin equivalent/g by Ozkan, et. al. (Ozkan, 2011).

The antioxidant capacity of *G. tournefortii* assayed with ABTS and DPPH method was compatible with each other and as well as with the total phenol and flavonoid content of the plant extract and fractions. This would be the indicator of free radical scavenging activity relation with the phenolic content of *G. tournefortii*. On the other hand the capacity of extract and fractions can be relevant with secondary metabolites like vitamins, oils and carotenoids (Javanmardi, 2003). The seeds of *G. tournefortii* were reported to contain fatty acids, tocopherols and sterols, as well (Matthaus, 2011).

Summary of the antioxidant capacity of crude extract and fractions from *G. tournefortii*, as well as total phenol and flavonoid contents was displayed in the **Table 3.6**.

Table 3.6: Summary of antioxidant capacity results DPPH EC_{50} (mg/mL) and $TEAC_{ABTS}$ (µM Trolox). Total phenol and total flavonoid content results for all fractions and crude extract.

Fraction/Standard	**DPPH EC_{50} (mg/mL)**	**$TEAC_{ABTS}$ (µM Trolox)**	**Total Phenol GAE (µg/mg)**	**Total Flavonoid CAE (µg/mg)**
Crude Extract	0.077 ± 0.012	3.336 ± 0.030	36.178 ± 0.021	137.811 ± 0.075
Hexane Fraction	1.434 ± 0.533	0.444 ± 0.010	7.413 ± 0.003	22.628 ± 0.004
Chloroform Fraction	0.086 ± 0.057	2.724 ± 0.042	37.897 ± 0.024	98.962 ± 0.058
Ethyl Acetate Fraction	0.016 ± 0.031	4.910 ± 0.081	62.018 ± 0.050	323.285 ± 0.024
Aqueous	0.126 ± 0.018	0.835 ± 0.061	18.966 ± 0.008	14.349 ± 0.008
Quercetin	0.002 ± 0.001	NA	NA	NA

NA: not applicable,

EC_{50}: Effective concentration of extract/fraction and standard

Total phenolic content as µg gallic acid equivalents permiligram of extract (GAE).

Micromolar trolox per mg of extracts.

Total flavonoid content is expressed as catechin equivalents (CAE; μg catechin/mg of extract)

As a result, crude extract, ethyl acetate and chloroform fractions of *G. tournefortii* are natural sources of antioxidants and can be used in functional foods and nutraceuticals. Because of that in this study these fractions of *G. tournefortii* were chosen for the following experimental steps.

3.4. Cell culture

3.4.1. Viability of Primary epidermal melanocytes and WM-115 human melanoma cells

The cell viability should be determined to have an idea about the compatibility of medium constituents with the cell line and the effect of environmental conditions. All cultured cells are vulnerable to their environmental, physical and chemical conditions even cancer cell although they are immortal. In order to maintain cell growth physical conditions and nutrient availability must be under control for the cultured cells. Because of this reason cell robustness and survival was under control and determined every 24 hours with JULI-BR cell analyzer.

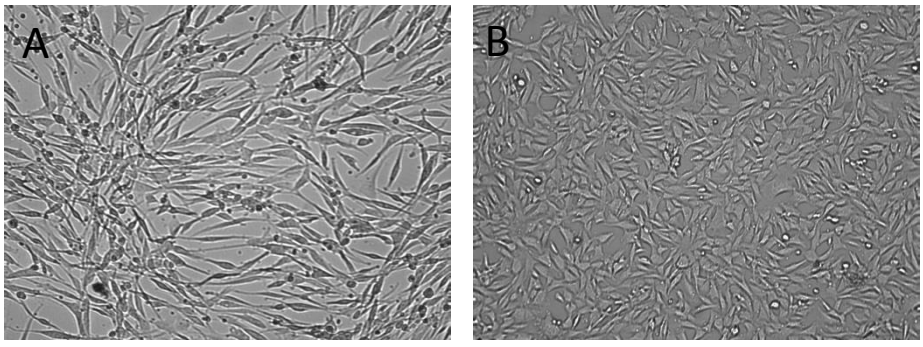

Figure 3.6: The images of A)Primary epidermal melanocytes and B)WM-115 human melanoma cells with JULI-BR

During 14 days of incubation the cell count was repeated every 24 hours. The cell count was determined with the help of the help of trypan blue mixing 10 µL cell suspension with 10 µL trypan blue under aseptic conditions and observing with JULI analyzer system. Because the healthy cells can actively transport the dye out of their membrane they are detected as clear and round. Although the dead cells are filled with trypan blue dye due to absence of active transport and they are observed as blue colored.

The chemical and physical conditions were optimized for the primary epidermal melanocytes and WM-115 melanoma cells to achieve the maximum cell number and normal growth.

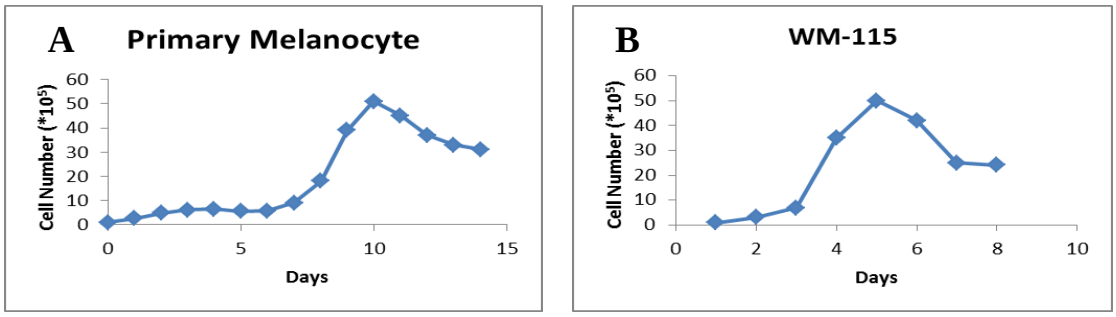

Figure 3.7: The growth curve of A)Primary epidermal melanocytes and B)WM-115 human melanoma cells in 14 days and 7 days, respectively.

Primary epidermal melanocytes and WM-115 human melanoma cells were seeded at a density of 100.000 cells/well as duplicates and incubated in a CO_2 incubator at 37˚C. The cells were harvested from the wells and counted every day. The Growth curves were plotted as growth duration versus number of cells.

3.4.2. XTT cell viability method

Cell viability of the primary epidermal melanocytes and WM-115 human melanoma cells, were determined by the help of (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) (XTT).

XTT is a tetrazolium salt which is slightly yellowish compound when it is in the oxidized form. XTT is reduced by mitochondrial enzymes of metabolically active and alive cells. The final product formazon is orange colored and it is measurable spectrophotometrically by using ELISA. Plant samples were dissolved in DMSO which is used as a vehicle since it allows the entrance of phenolic constituents into the cells easily. The final concentration of DMSO was kept constant as 0.1% for all cell culture applications.

Samples treated with 0.1 % DMSO were used as control and cell viability measurements are expressed as percentage cell viability. Because plant extracts may have ability to reduce tetrazolium salts although presence of dead cells cytotoxicity tests based on this principle might give doubtable results. Also it is reported that serum one of the growth medium constituents may cause formazon production (Talorete, 2006). In order to eliminate these effects control wells without cells and as well as without plant samples were used throughout the cell viability experiments.

Cell viability was measured at 72 h after treatment with crude extract, ethyl acetate and chloroform fraction. The effect on the growth of the primary epidermal melanocytes and WM-115 human melanoma cells at various concentrations of 0-40 µg/mL was determined.

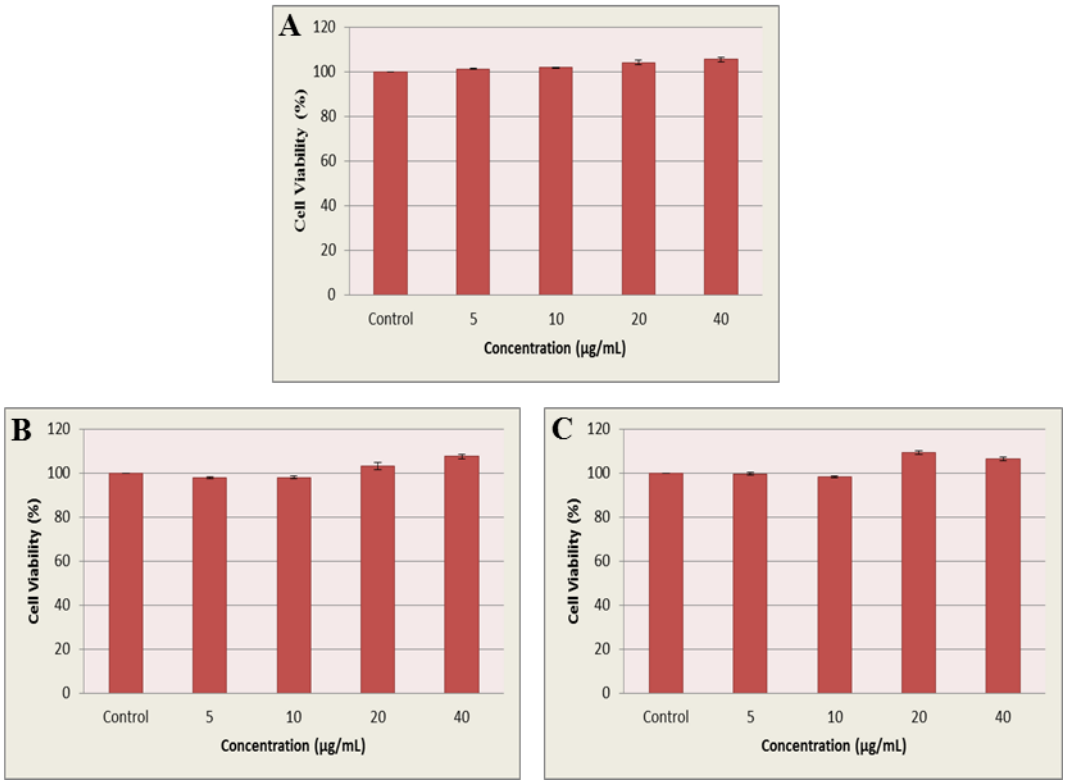

Figure 3.8: Analysis of cell viability for the primary epidermal melanocytes after treatment of various concentration (0, 5, 10, 20 and 40 µg/mL) of A) crude extract and B) ethyl acetate fraction C) chloroform fraction for 72h. The absorbances were measured at 475 nm with ELISA reader. Experiments were performed three times in triplicates.

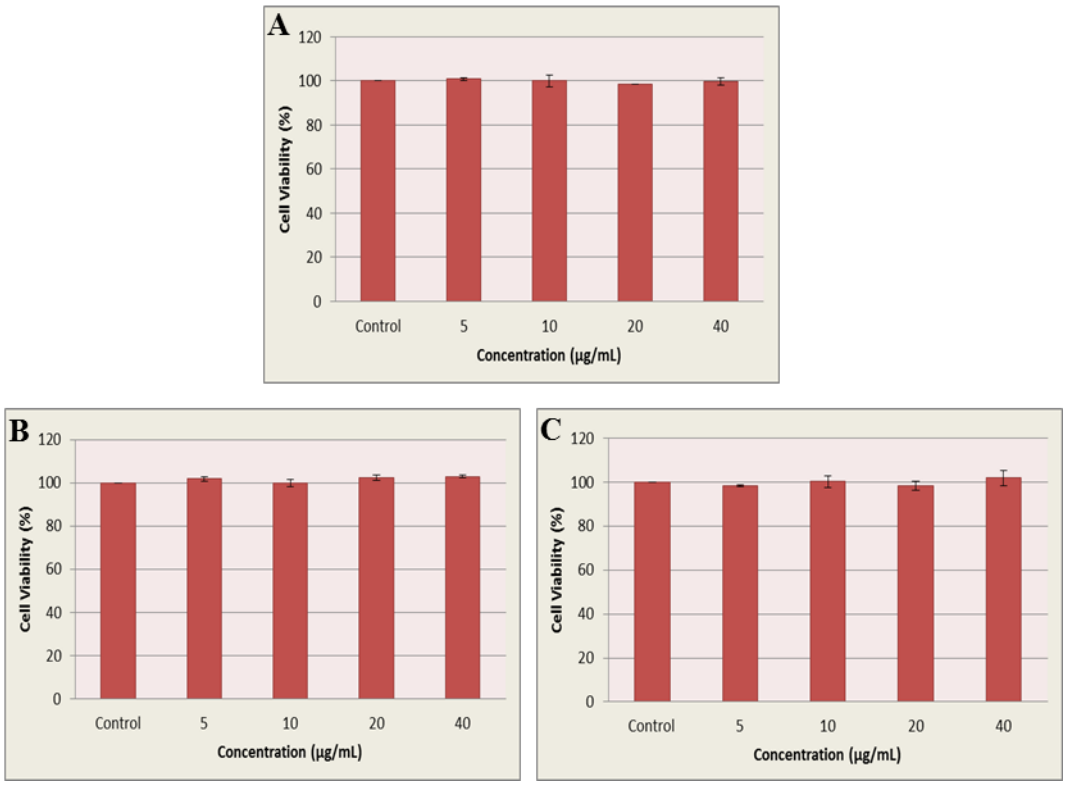

Figure 3.9: Analysis of cell viability for WM-115 melanoma cells after treatment of various concentrations (0, 5, 10, 20 and 40 µg/mL) of A) crude extract and B) ethyl acetate fraction C) chloroform fraction fraction for 72h. The absorbances were measured at 475 nm with ELISA reader. Experiments were performed three times in triplicates.

Between the three samples studied here, crude extract, ethyl acetate and chloroform fraction, no cytotoxic effect was detected for the primary epidermal melanocytes (Figure 3.8) and WM-115 melanoma cells (Figure 3.9). On the contrary, at the concentration of higher than 20µg/mL very slight proliferative effect was detected for the primary melanocytes.

3.4.3. Determination of Secreted Melanin

In order to observe whether the crude extract and fractions of *G. tournefortii* promotes melanogenesis or not, the amount of secreted melanin into the medium was determined as described in the experimental part (Section 2.4.7.). The secreted melanin amount after treatment with various concentrations ranging from 5µg/mL to 40µg/mL for extract and fractions was calculated.

In these experiments, the highest amount of secreted melanin, in the primary epidermal melanocytes, was realized by extract concentrations of 20 µg/mL, 5 µg/mL, 10 µg/mL for crude extract, ethyl acetate and chloroform fraction, respectively (Figure 3.10).

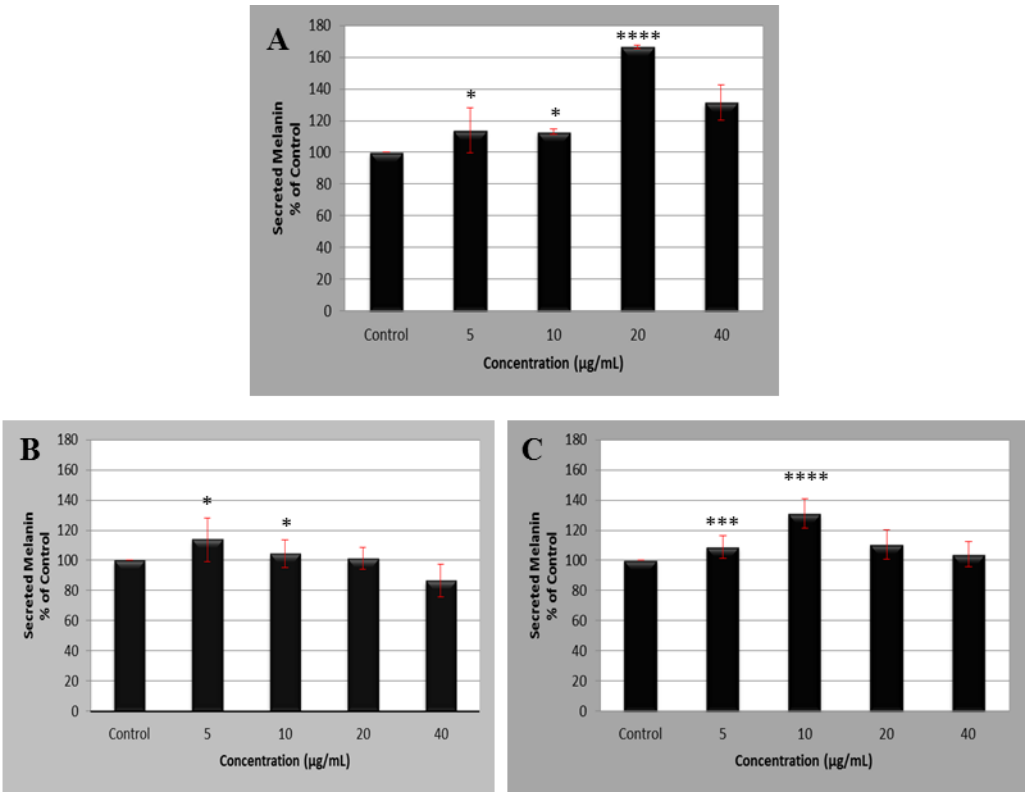

Figure 3.10: After 72 hours treatment, effect of A) crude extract and B) ethyl acetate fraction C) chloroform fraction on the secreted melanin amount in the primary melanocytes. The absorbance of medium was measured at 405 nm with ELISA reader. Each percentage value in the treated cells was calculated with respect to that in the control cells. The results were expressed as ± SD of two different experiments each carried out in triplicate. *Significantly different from the control (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

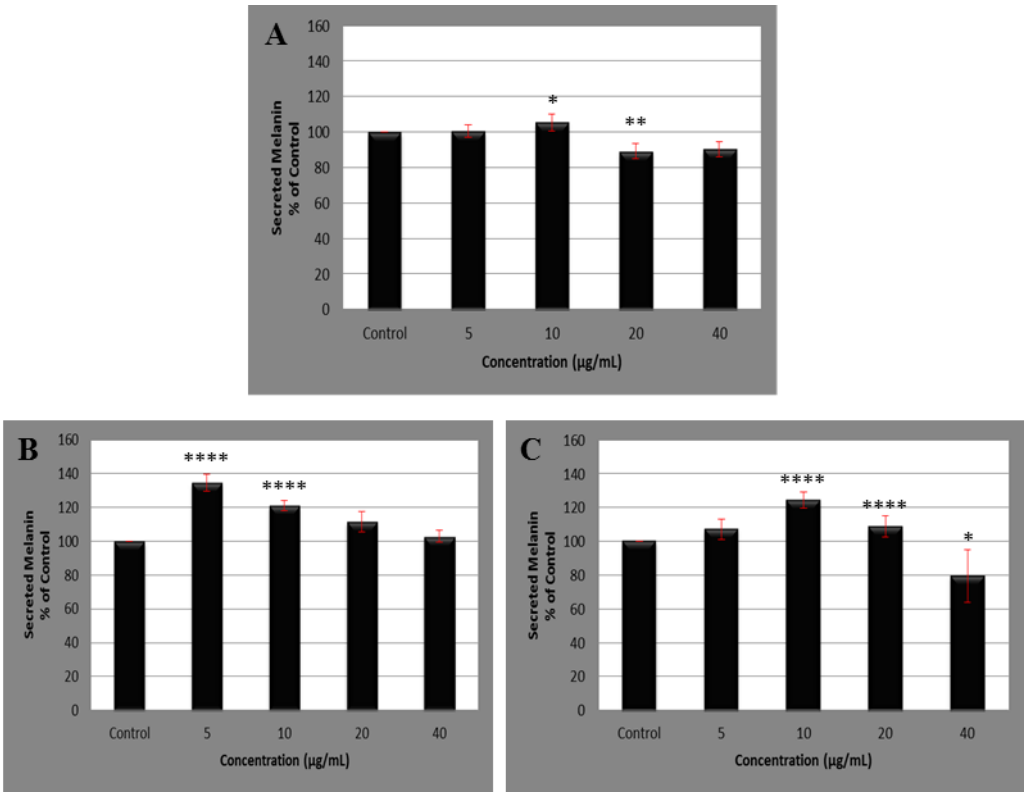

Figure 3.11: After 72 hours treatment, effect of A) crude extract and B) ethyl acetate fraction C) chloroform fraction on the secreted melanin amount for WM-115 melanoma cells. The absorbance of medium was measured at 405 nm with ELISA reader. Each percentage value in the treated cells was calculated with respect to that in the control cells. The results were expressed as ± SD of two different experiments each carried out triplicate. *Significantly different from the control (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

On the other hand, in WM-115 melanoma cells the highest secreted melanin amount was observed at lower concentrations. The melanin secretion increased after treatment with various concentrations of the extract and fractions. High amount of secretion was detected at the concentrations of 10 µg/mL, 5 µg/mL, 10 µg/mL for crude extract, ethyl acetate and chloroform fractions, respectively (Figure 3.11).

In litreture, there were no reports up to date regarding of G. *tournefortii*'s promoting effect on the melanin secreted from melanocytes. Yet, there have been a few studies which determined the promoting effect of other extracts and compounds. Mouse melanoma cells (B16/F10) were reported to secrete more melanin into the medium in presence of citrate (Bhatnagar, 1998). Additionally, aqueous extract of Garcinia *mangostana* L. was proved to increase the secreted melanin amount from B16/F10 melanoma cells (Abdul Hamid, 2012).

3.4.4. Determination of Melanin Content

In order to clarify the effect of *G. tournefortii* crude extract and fractions on melanogenesis the amount of intracellular melanin content was determined in human melanocytes and WM-115 melanoma cells. After 3 days treatment with various concentrations of crude extract and fractions the results revealed that the melanin content was increased in a dose-dependent manner in the primary melanocytes. The most effective concetrations was observed as 20 µg/mL, 5 µg/mL, 10 µg/mL for crude extract, ethyl acetate and chloroform fraction, respectively (Figure 3.12). There was a decrease in the melanin content of cells after the treatment with concentration excess of 20µg/mL of crude extract and 10µg/mL of the fractions.

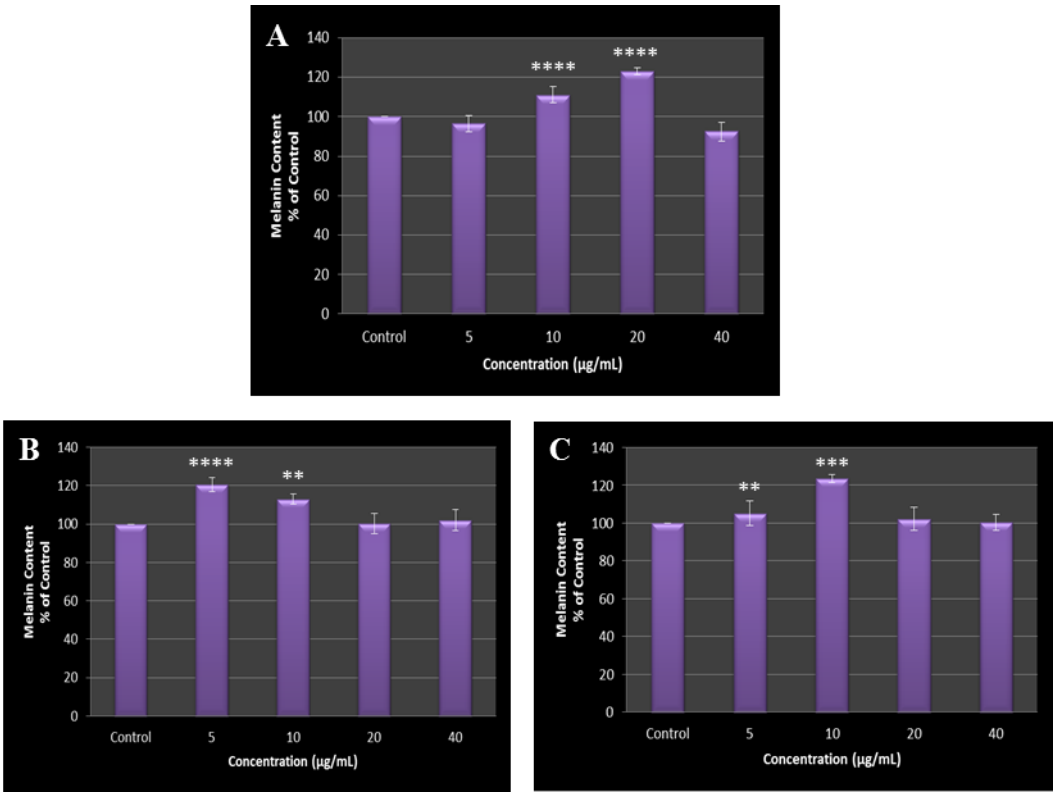

Figure 3.12: Effect of 72 hours treatment with A) crude extract and B) ethyl acetate fraction and C) chloroform fraction on the intracellular melanin content of the primary epidermal melanocytes. The absorbances were measured at 405 nm. The results were expressed as ± SD of three different experiments each carried out duplicate. *Significantly different from the control cells (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

As shown in Figure 3.13. treatment with 5 µg/mL crude extract, ethyl acetate and chloroform fraction resulted in ~2-2.5 fold increase in the melanin content of WM-115 melanoma cells compared to the control cells.

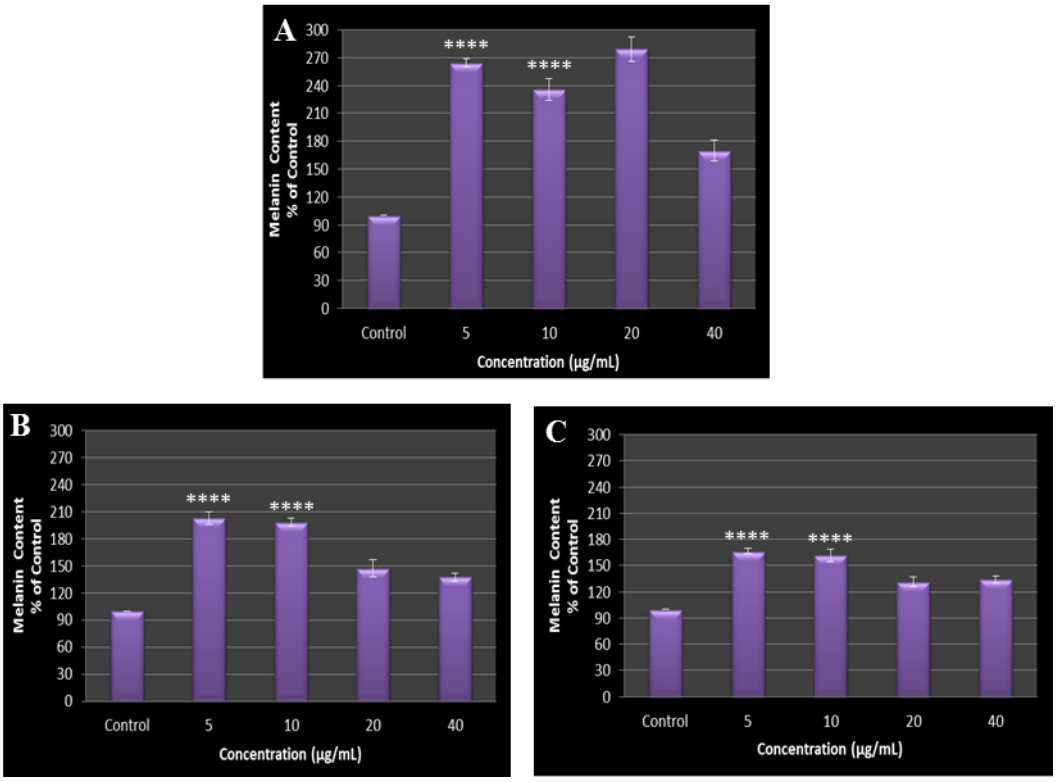

Figure 3.13: Effect of 72 hours treatment with A) crude extract and B) ethyl acetate fraction and C) chloroform fraction on intracellular melanin content of WM-115. The absorbances were measured at 405 nm. The results were expressed as ± SD of three different experiments each carried out duplicate. *Significantly different from the control cells (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

The melanogenic effect of *G. tournefortii* was not determined previously but there are few studies about hyperpigmentation activity of plant extracts. The ethanolic extracts from leaves and flowers of *Pyrostegia venusta* were reported to increase melanin content in B16F10 melanoma cell line (Moreira, 2012). Also, *Vernonia anthelmintica* L. extract was shown to have promoting effect on the intracellular

melanin content (Jia, 2012). Another research group reported Mangosteen leaf extract to increase melanin content in melanoma cells (Abdul Hamid, 2011).

3.5.5. Determination of Tyrosinase Activity

The essential enzyme of melanin synthesis is tyrosinase which catalyses the rate limiting step of pathway and it is responsible for hydroxylation of tyrosine into3,4-dihydroxyphenylalanine (DOPA) and further oxidation into dopaquinone (Hearing, 1999; Park, 2004).

Because increased tyrosinase activity related with the melanin content of melanocytes, in this study the effects of *G. tournefortii* on tyrosinase activity in the primary melanocytes and melanoma cells was examined. To clarify the mechanism of melanogenesis induced by *G. tournefortii*, the cells were treated with the crude extract and fractions at the same concetrations in melanin content assay.

In these experiments, tyrosinase activity of the primary epidermal melanocytes was maximized at ethyl acetate concentration of 5 µg/mL, chloroform concentration of 10 µg/mL and crude extract concentration of 20 µg/mL. (Figure 3.14).

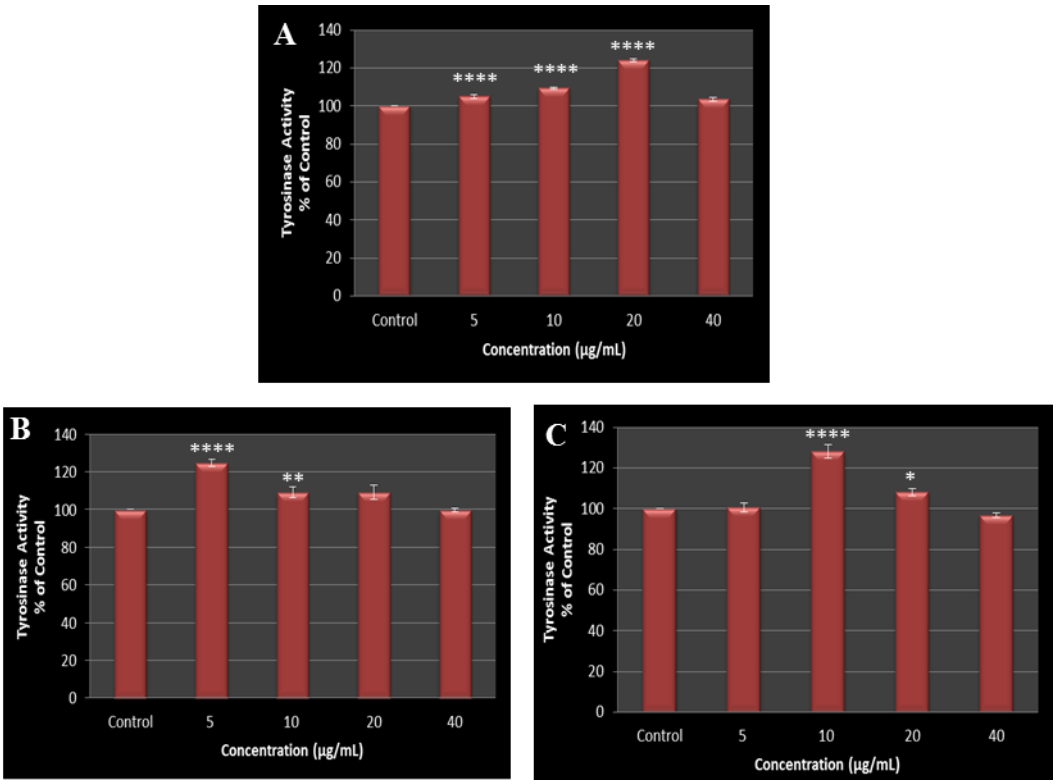

Figure 3.14: Effect of 72 hours treatment with A) crude extract and B) ethyl acetate fraction and C) chloroform fraction on the tyrosinase activity of the primary melanocytes. The absorbance was measured at 475 nm with ELISA reader.The results were expressed as ± SD of three different experiments each carried out triplicate. *Significantly different from the control cells (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

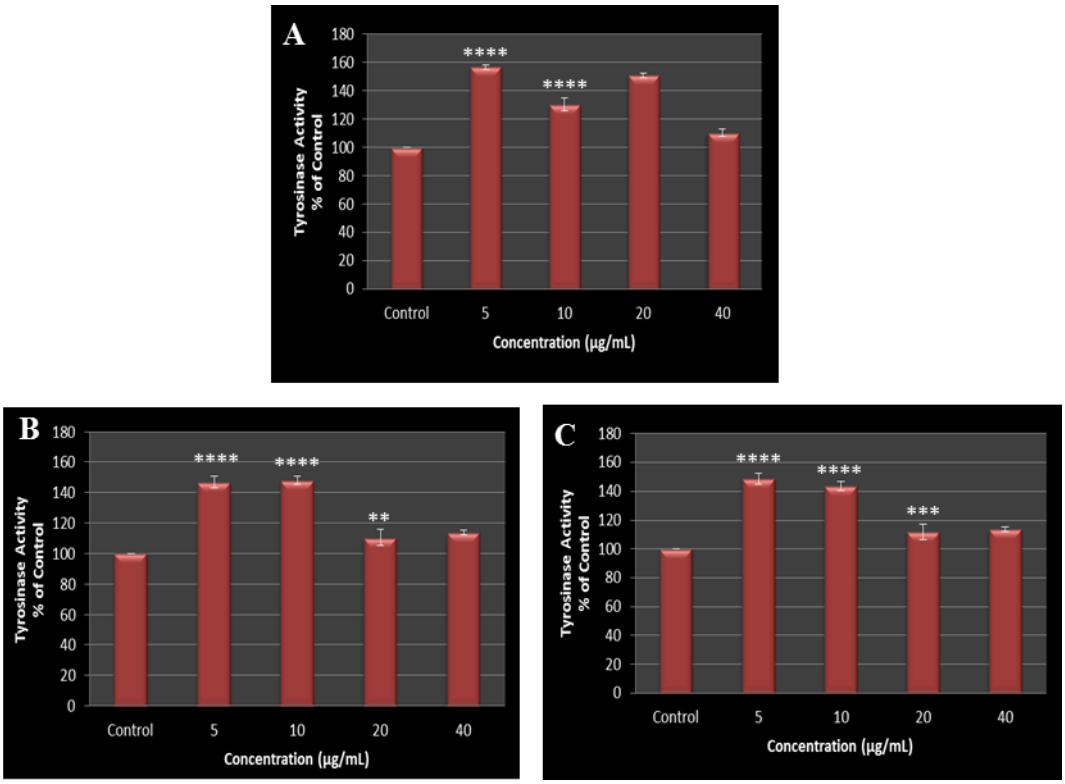

Figure 3.15: Effect of 72 hours treatment with A) crude extract and B) ethyl acetate fraction and C) chloroform fraction on the tyrosinase activity of WM-115. The absorbance was measured at 475 nm with ELISA reader. The results were expressed as ± SD of three different experiments each carried out triplicate. *Significantly different from the control cells (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

The treatments with 5 µg/mL concentrations of crude extract, ethyl acetate and chloroform fractions on Wm-115 melanoma cells resulted in almost 1.5 fold increase in the tyrosinase activity in comparison to the untreated control cells as shown in Figure 3.15.

After 3 days treatment with the various concentrations of crude and fractionated extracts, displayed a change in the tyrosinase activity which was compatible with

the intracellular melanin content of melanocytes (Figure 3.12 and 3.14) and melanoma (Figures 3.13 and 3.15) cells. On the contrary, there was a report regarding the stimulatory effect of crude extract of *Pyrostegia venusta* on melanin content; however any influence on the activity of tyrosinase was indicated in melanoma cells (Moreira, 2012). Nevertheless, in parallel to our results there are other research groups stated matching results for melanin content and tyrosinase activity (Jia, 2012; Abdul Hamid, 2012; Noriko, 2007).

3.5.6. Gene Expression Analysis

Although tyrosinase is the key enzyme of melanin synthesis pathway, there are two distinct enzymes, contribute to regulation of melanogenesis, which are called as tyrosinase-related protein-1 (TRP-1), and tyrosinase related protein-2 (TRP-2). Tyrosinase, hydroxylates tyrosine into 3,4-dihydroxyphenylalanine (DOPA), and converts DOPA into dopaquinone by oxidation. While TRP1 is responsible for the oxidation of indole 5,6-quinone-2-carboxylic acid (DHICA), TYRP2 converts dopachrome into DHICA. Because these three enzymes are transcriptionally regulated by MITF, this specific transcription factor considered as powerful regulator of the melanogenesis (Simon, 2009; Widlund, 2003).

In this study, whether the induction of melanin synthesis the *G. tournefortii* was promoted via mRNA expression of melanogenesis related enzymes (TYR, TYRP1 and TYRP2) and transcription factor (MITF) or not was investigated with RT-PCR.

Primary epidermal melanocytes and WM-115 cells were treated with the effective concentrations of crude extract and fractions which were determined with the enzyme activity and melanin assays. After 3 days of treatment with extracts, alterations in the mRNA expressions were observed with comparative CT method ($2^{-\Delta\Delta Ct}$). Comparison of gene expression of two different samples was calculated according to the following formula;

$\Delta\Delta Ct = [(Ct\ target\ gene - Ct\ internal\ control)_{treated} - (Ct\ target\ gene - Ct\ internal\ control)_{untreated\ control}]$

$Fold\ change = 2^{-\Delta\Delta Ct}$

The primary epidermal melanocytes were treated with maximum effective concentrations of crude extract (20 µg/mL), ethyl acetate fraction (5 µg/mL), and chloroform fraction(10 µg/mL), for 72 hours. Expression levels of four genes increased after the treatments. Especially, mRNA expression of rate limiting enzyme tyrosinase and related transcription factor MITF increased dramatically after treatment with ethyl acetate fraction, altohough the minimum concentration (5 µg/mL), was applied for this fraction (Figure 3.16). The results were comparible with the melanin assays. The ethyl acetate fraction of *G. tournefortii* L. has the most promoting effect on melanogenesis at the level of mRNA expression.

In the light of evidences gathered from our results, it could be possible that the promoting effect of *G. tournefortii* on melanogenesis is attributable to up-regulation of MITF and tyrosinase mRNA expression and subsequently increased the tyrosinase activity which in return for the increase in melanin synthesis.

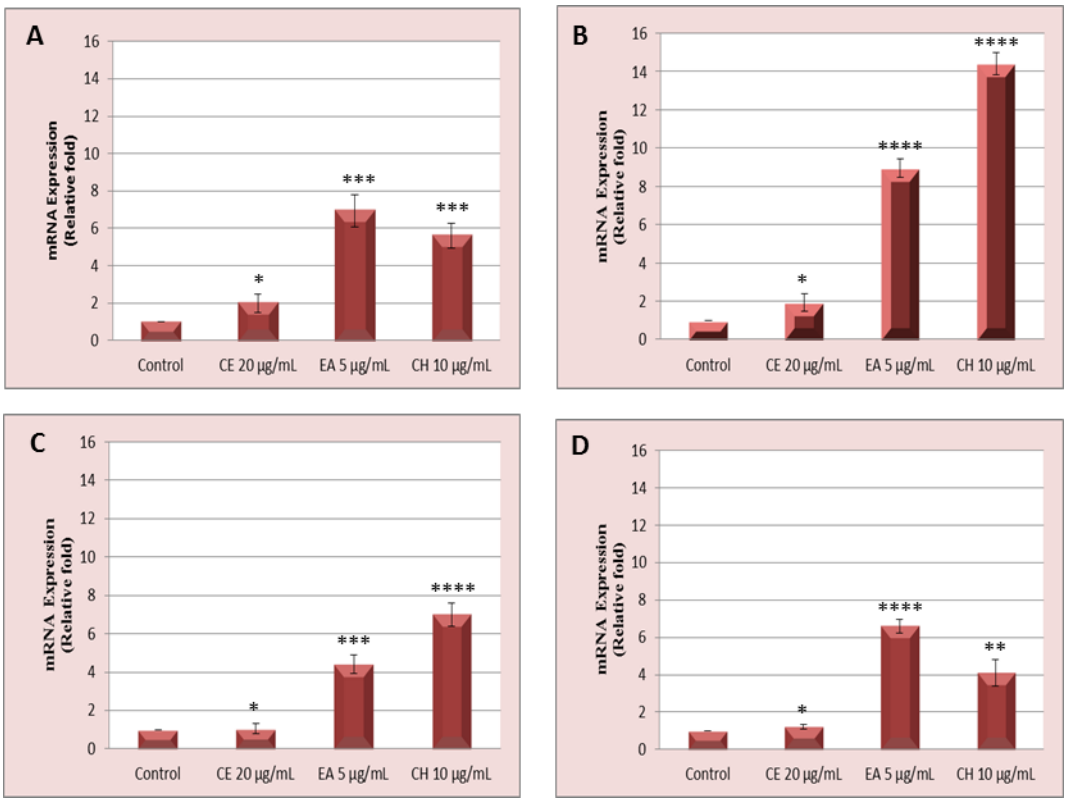

Figure 3.16: Effect of 72 hours treatment with 20 µg/mL crude extract, 5 µg/mL ethyl acetate and 10 µg/mL chloroform fractions on A) TYR B) TYRP1 C) TYRP2 D) MITF mRNA expression in the primary epidermal melanocytes. The quantifications are expressed as ± SD of the relative fold expression. *Significantly different from the control cells (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

WM-115 melanoma cells were treated with 5 µg/mL crude extract, ethyl acetate and chloroform fractions for 72 hours. Expression levels of four genes did not alter after all treatments importantly, except for the ethyl acetate fraction. The mRNA expression of TYRP1 and related transcription factor MITF increased dramatically after treatment with ethyl acetate fraction (Figure 3.17). The results supported that only ethyl acetate fraction has an effect on the mRNA expression and increased amount of melanin in WM-115 cells can be attributed to upregulated expression of MITF and TYRP1 genes. Since, the possible roles of TYRP1 in the stabilization of tyrosinase enzyme (Kobayashi, 1998) and formation of a heterodimer with

tyrosinase *in vivo*, also important functions in intracellular trafficking of these enzymes were reported in some studies (Luo, 1994; Kobayashi, 2007). Other studies demonstrated that decrease in TYRP1 can result in with decreasing effects on tyrosinase activity (Manga, 2002). Furthermore, the stimulation of tyrosinase activity was demonstrated after the re-expression of the TYRP1 protein in human melanoma cells (Zhao, 1996).

Additionally in the present study, melanogenesis promoting effect of crude extract and chloroform fraction of G. *tournefortii* can be considered to be a reflection of increased tyrosinase activity in melanoma cells. Consequently, the ethyl acetate fraction exhibited the highest promoting effect among the compared extracts of G. *tournefortii* on melanogenesis at the level of mRNA expression in WM-115 melanoma cells (Figure 3. 17).

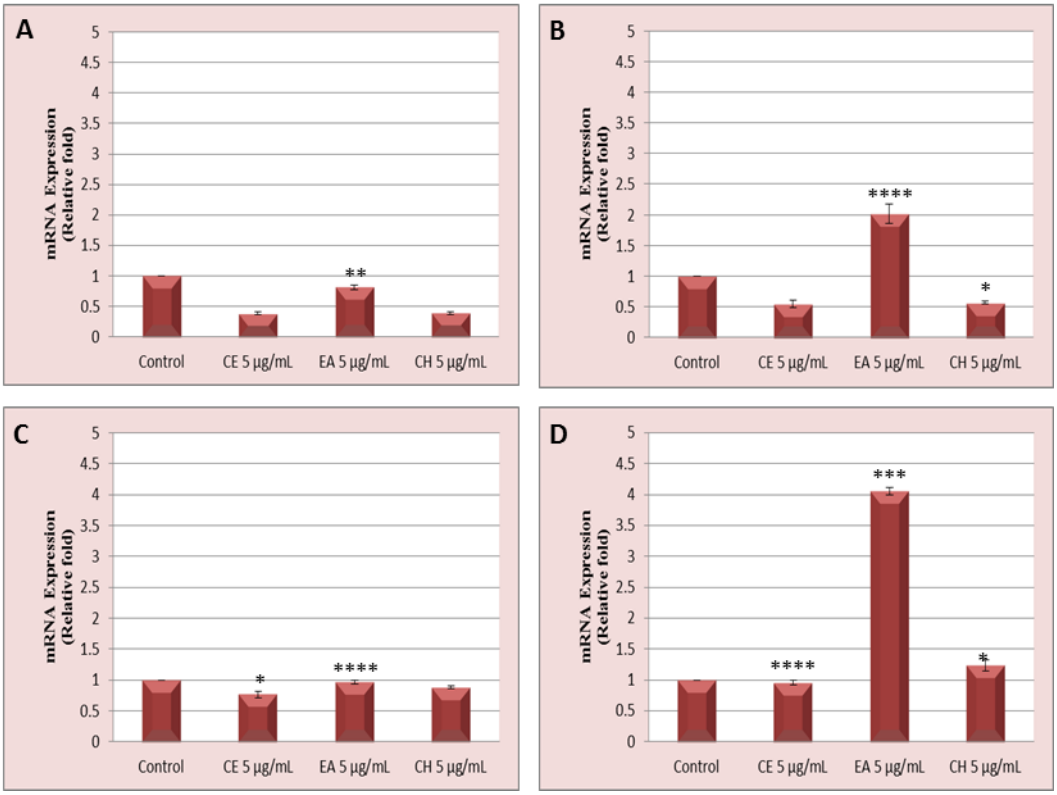

Figure 3.17: Effect of 72 hours treatment with 20 µg/mL crude extract, 5 µg/mL ethyl acetate and 10 µg/mL chloroform fractions on A) TYR B) TYRP1 C) TYRP2 D) MITF mRNA expression in WM-115 melanoma cells. The quantifications are expressed as ± SD of the relative fold expression. *Significantly different from the control cells (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

There are several research groups dealt with melanin synthesis and related gene regulation observed similar relation between enzyme activity and mRNA expression of tyrosinase, TYRP1, TYRP2 and MITF (Jin, 2012; Jang, 2012). Another research group studied melanin synthesis stimulating effect of cubein had proven the increased melanin content and tyrosinase activity was found to be related with the up-regulation of tyrosinase mRNA expression (Noriko, 2007).

Makpol and co-workers found comparable results among melanin content, tyrosinase activity and TYR, TYRP1,TYRP2 mRNA expression (Makpol, 2009). Additionally, Abdul Hamid et. al. observed up-regulation of tyrosinase and MITF expression which well correlated with intracellular tyrosinase activity (Abdul Hamid, 2012). On the contrary, Nakajima et. al. observed an increased amount of melanin in melanocytes although they did not recognize any differences in the level of tyrosinase mRNA expression and activity after treatment with arbutin (Nakajima, 1998). Up to now, the present work is the first investigation in literature in regard to the effect of *G. tournefortii* on modulation of melanin synthesis and its mechanism.

CHAPTER 4

CONCLUSION

An abnormal pigmentation of skin is caused by undesired modulation of melanin production. Hence, the regulation of melanin synthesis is imperative for the recovery of skin color. In folk medicine, *Gundelia tourneforti* was used for the treatment of skin diseases, characterized by skin discoloration. Because of this reason the present study was desined in order to determine potential of *G. tournefortii* for the induction of melanin synthesis.

Initially, methanolic extraction of *G. Tournefortii* was performed and subsequently, plant crude extract was obtained and fractionation was carried out with solvents in sequential polarity from non-polar to polar. Followed by the determination of antioxidant capacity and phenolic contents of *G. tournefortii* crude extract and fractions, since phenolic compounds in plants are responsible for the most of their bioactivities. Among the fractions of *G. tournefortii* ethyl acetate fraction displayed the highest radical scavenging capacity in both DPPH and ABTS antioxidant assays because of it's high phenolic content. In addition to ethyl acetate fraction, crude extract and chloroform fraction were revealed high amounts of phenolics and flavonoids. Thus, these fractions of *G. tournefortii* were chosen for the following experimental steps. Those extracts were tested on melanocytes and WM-115 melanoma cells for the comparison of healthy and cancer conditions. In order to clarify, whether there would be any cytotoxic effect existing due to application of the plant extracts was determined by XTT cell viability assay on both of the cell lines. Hereby, nontoxic concentrations were defined and continued to the next step.

For the investigation of the melanogenic effect on intracellular and secreted melanin amounts were determined in the primary epidermal melanocytes and WM-115 melanoma cells. Melanin content of primary epidermal melanocytes was very high at the concentrations of 20 μg/mL, 5 μg/mL, 10 μg/mL for crude extract, ethyl acetate and chloroform fractions, respectively. On the other hand, for the same fractions, the increased melanin secretion was observed even at lower concentrations (5μg/mL) in WM-115 melanoma cells.

The activity of tyrosinase responsible for the melanogenesis was also examined to determine whether the increased amount of melanin synthesis was related with the activity of this enzyme. The alteration in the tyrosinase activity was found comparable with the intracellular melanin content of melanocytes and melanoma cells after three days of treatment in various concentrations of crude extract and fractions.

The mechanism of melanogenesis induced by *G. tournefortii* was investigated by examining the mRNA expression level of melanogenesis related enzymes (TYR, TYRP1 and TYRP2) and their transcription factor (MITF). The results supported the promoting effect of *G. tournefortii* extracts on melanogenesis which might be related with the up-regulated expression of MITF and tyrosinase and as well as increased tyrosinase activity in the primary melanocytes. However, the expression levels of melanogenesis related genes in WM-115 melanoma cells did not increase upon the extract treatments, except for the ethyl acetate fraction. The mRNA expression of TYRP1 and related transcription factor MITF increased dramatically in melanoma cells after treatment with ethyl acetate fraction. In melanoma cells the increase of melanin synthesis can be considred as a consequence of upregulated TYRP1 and MITF mRNA expression as well as increased tyrosinase activity.

When all the results are taken into consideration; the present research is a pioneering study in regard to the potential effect of the phytochemical components of *G. tournefortii* on melanin synthesis induction in melanocytes.

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APPENDIX

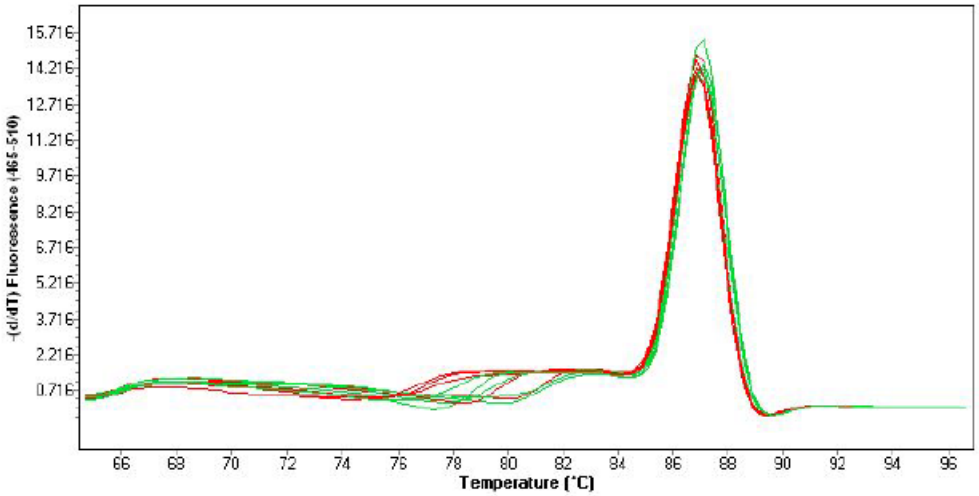

Figure A.1: GAPDH Melting curve the fluorescence emission change versus temperature. Detection of single peak means single PCR product.

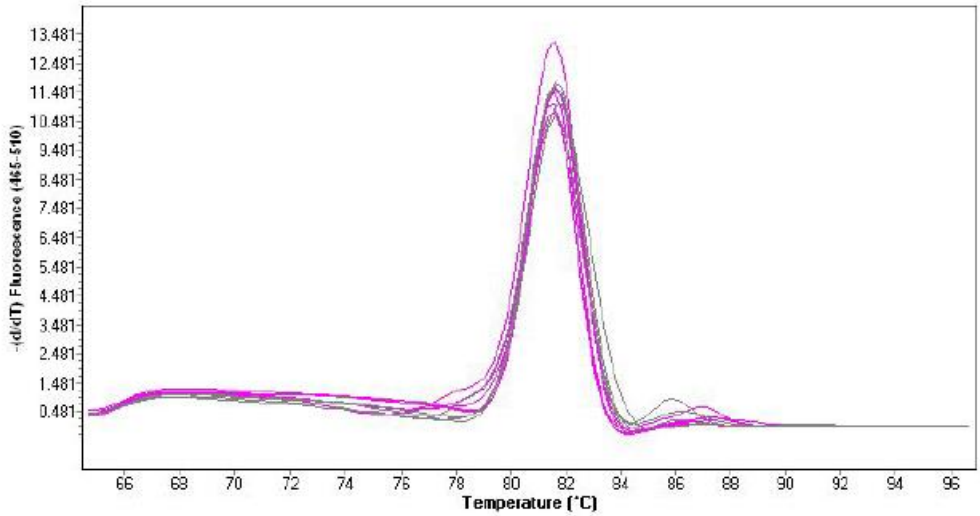

Figure A.2: TYR Melting curve the fluorescence emission change versus temperature. Detection of single peak means single PCR product.

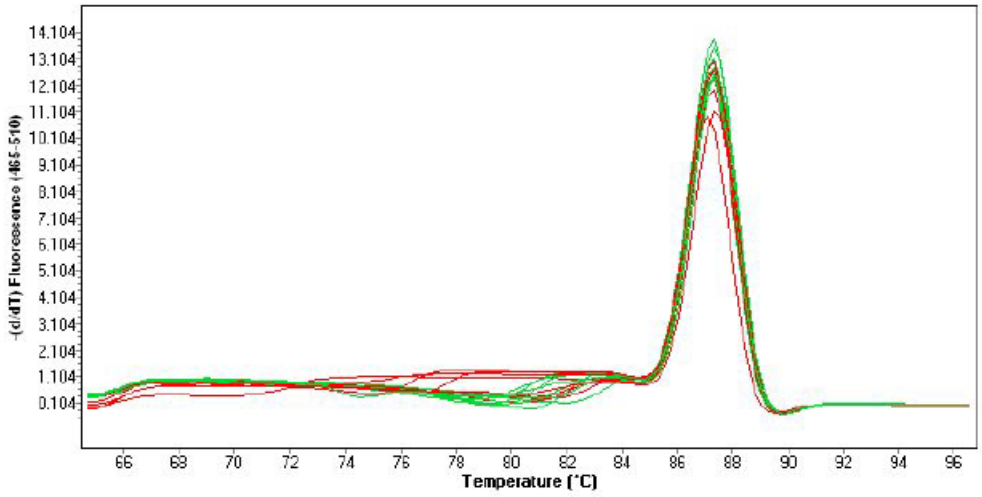

Figure A.3: TYRP1 Melting curve the fluorescence emission change versus temperature. Detection of single peak means single PCR product.

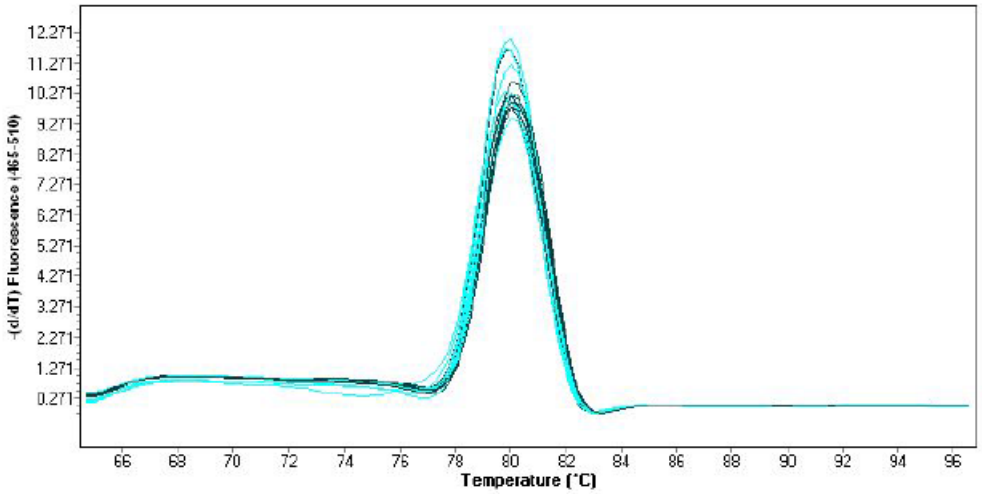

Figure A.4: TYRP2 Melting curve the fluorescence emission change versus temperature. Detection of single peak means single PCR product.

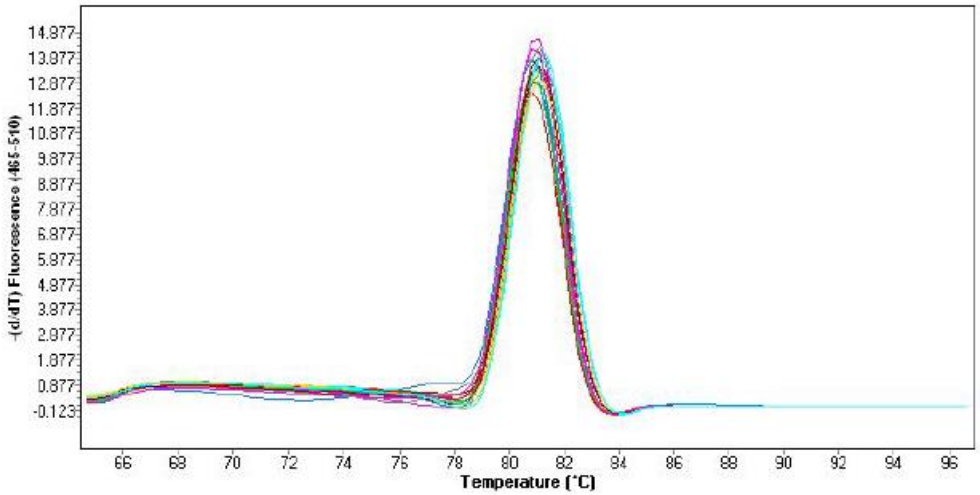

Figure A.5: MITF Melting curve the fluorescence emission change versus temperature. Detection of single peak means single PCR product.

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Poster Presentations in National and International Congresses:

- **S. Sahin**, N. Coruh, F. Ozgokce. (2015). Anti-oxidant properties of the seeds of Gundelia tournefortii L. FEBS Journal, 282 (1), 126.
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- N. Ozdogan, **S. Sahin**, N. Coruh, F. Ozgokce. "Antioxidant Properties of Flowers Extract Of Gundelia Tournefortii L." International workshop on Plant Products Chemistry and Symposium on Medicinal and Aromatic Plant, Izmir, TURKEY.
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Paticipation in National and International Congress:

- 40th FEBS Congress, Biochemical Basis of Life. Berlin, Germany, 2015

- 38th FEBS Congress, Mechanisms in Biology. St. Petersburg, Russia, 2013
- 25th National Biochemistry Congress, Izmir, Turkey, 2013.
- 36th FEBS Congress, Biochemistry for tomorrow's medicine. Torino, Italy, 2011
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- Primary Cell Culture Course, 23-25 September 2010, Ege University Department of Bioengineering, İzmir, Turkey.