MOLECULAR INVESTIGATION OF THERAPEUTICAL EFFECTS OF
Ɣ-TOCOTRIENOL CONJUGATION THERAPY WITH DOXORUBICIN
ON HEPATOCELLULAR CARCINOMA CELL LINE HEPG2

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ABSTRACT

**MOLECULAR INVESTIGATION OF THERAPEUTICAL EFFECTS
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Hepatocellular carcinoma (HCC) is the fifth leading tumor of the liver tissue in terms of frequency of occurrence and third in terms of mortality. Even though, the most widely used chemotherapeutic agent doxorubicin in treatment of HCC had a great impact for development of therapeutic strategies preventing the progression of tumor formation and elucidating the physiological characteristics of tumor tissue. Moreover, use of doxorubicin minimizes the side effects of the drug in the individual and prevents the resistance developed by the HCC cells. However, vitamin E derivatives, especially γ-tocotrienol rich fractions have an anti-proliferative and inhibitory effect on cancer cells by several means, including evading the gained resistance of HCC cells to chemotherapic agents and inducing apoptosis. Furthermore, when cells were co-treated with γ-tocotrienol and chemotherapy drugs, synergistic effect was observed. In this study It has been shown that the combination of doxorubicin chemotherapeutic agent with γ-tocotrienol, which belongs to Vitamin E family of molecules, causes changes in the molecular mechanism of liver cancer cells and lipid metabolism.

In this study, we have shown that these changes are caused not only by reducing the side effects caused by doxorubicin, but also by reversing the resistance of HCC cells for the actions of chemotherapeutic agents.

ÖZ

KARACİĞER KANSERİ HÜCRE HATTINDA, γ-TOKOTRİENOL AJANININ DOXORUBİCİN İLE KONJUGE TEDAVİSİNİN TERAPÖTİK ETKİLERİNİN MOLEKÜLER İNCELENMESİ

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Hepatosellüler karsinom (HCC) bir karaciğer öncü tümörüdür ve dünyada en çok görülen kanser türleri arasında 5. sırada, mortalite açısından ise 3. sırada yer almaktadır. Bunula birlikte, HCC tedavisinde kullanılan temel kemoterapötik ajan doxorubicin, tümör oluşumunun ilerlemesi ve tümör dokusunun fizyolojik özellikleri açısından terapötik stratejilerin geliştirilmesi için büyük önem taşımaktadır. Dahası, doxorubicin kullanımı ilacın kişide görülen yan etkilerini ve HCC hücreleri tarafından geliştirilen direncin amaçlanan etkisini göstermemektedir. Bunula birlikte, vitamin E türevleri, özellikle γ-tokotrienol açısından zengin fraksiyonlar, apoptoza neden olduğundan kanser hücreleri üzerinde anti-proliferetif ve inhibe edici etkiye sahiptir. Hücreler, γ-tokotrienol ve kemoterapi ilaçlarıyla birlikte işlem gördüklerinde sinerjik etki gözlenmiştir. Bu çalışmada, doxorubicin kemoterapötik ajan ile bir E vitamini türevi olan γ-tokotrienol kombinasyonunun karaciğer kanseri hücrelerinin moleküler mekanizması ve lipit metabolizmasında değişikliğe neden olduğunu gösterilmiştir.

Çalışmalarımız sonucunda sözü edilen değişikliklerin, yalnızca doxorubicin kaynaklı yan etkilerin azaltılmasından değil, aynı zamanda HCC hücrelerinin kemoterapötik ajanlara daha duyarlı hale gelmesinden de kaynaklandığı tespit edilmiştir.

Anahtar Kelimeler: E vitamini, γ-tocotrienol, doxorubicin, karaciğer kanseri, lipid metabolizması

Dedicated to my mother,

Alev Bartın

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

ATR   Attenuated Total Reflectance

DOX   Doxorubicin

GTT   Ɣ-tocotrienol

HCA   Hierarchical cluster analysis

HCC   Hepatocellular carcinoma

MDR   Multidrug resistance

OD   Optical Density

PCA   Principal component analysis

STAT3   Signal Transducer and Activator of Transcription 3

TBARS   Thiobarbituric Acid Reactive Substances

CHAPTER 1

INTRODUCTION

1.1 Cancer

Healthy body cells, with the exception of muscle and some nerve cells, have the ability to divide. They use these abilities to replace dead cells and repair injured tissues. But their abilities are limited. Throughout its life, every cell has the capacity to divide a finite number of times. A normal cell has an accurate spatial programming and timing of the cell division. On the other hand, cancer cells, begin and multiply in an uncontrolled fashion. Cancer cells accumulate to form tumors, which can apply pressure on normal tissues, or can infiltrate into healthy tissues and destroy them. If cancer cells are detached from the tumor, in the blood stream they can go to other parts of the body through the lymphatic circulation. Landing on tissues where they grow further, they form new tumor colonies. The spread of cancer to other parts of the body is termed metastasis (National Cancer Institute, 2015). Cancers are usually categorized based on their morphology. Different types of cancer grow at different rates, show different metastatic patterns and respond to different treatments. For this reason, in the treatment of cancer patients, a number of treatments are available based on the cancer type. Risk factors for cancer vary depending on people lifestyle, age, sex, and family history (Murray and Lopez, 1996).

1.2 Liver Cancer

Liver cancers are malignant tumors that originate from the tissue of the liver. The most common type is called hepatocellular carcinoma and it accounts for about 90% of liver cancers. The remainder of the tumors is called cholangiocarcinomas, mostly originating from the bile duct cells of liver (Fong *et al*., 2015).

1.3 Cancer Chemotherapy and Drug Metabolism

1.3.1 Chemotherapy for Liver Cancer

Chemotherapy is a type of treatment that includes a medication or combination of medications to target cancer cells by slowing or stopping their growth (National Cancer Institute, 2015). Systemic chemotherapy uses anti-cancer drugs that can enter the bloodstream and reach all of the body. Liver cancer has been shown to be resistant to several anti-cancer drugs (American Cancer Society, 2016). Most effective drugs are doxorubicin (Adriamycin), 5-fluorouracil, and cisplatin (Forner *et al*., 2012). Now it is standing their significant success in chemotherapeutic success, these chemotherapeutic agents are able to cause a minimal decrease in tumor size.

1.3.2 Side Effects of Chemotherapy

Chemotherapeutic drugs show their anti-cancer effects by attacking cells that undergo cell rapid cell division. It is important to note that other cells in the body, such as bone marrow cells, those lining of the mouth and intestines, and the hair follicles, also divide quickly. These cells, therefore, are also likely to be affected by chemotherapeutic drugs, which can lead to various other unwanted side effects (American Cancer Society, 2016).

Side effects of chemotherapeutic drugs depend on several factors including the type and the dose of drugs applied and the duration of their application. Common side effects include hair loss, mouth sores, loss of appetite, nausea and vomiting, diarrhea, increased chance of infections, easy bruising or bleeding, fatigue, etc. (American Cancer Society, 2017; Lencioni *et al*., 2010).

1.3.3 Anticancer Drugs

Most anticancer drugs target cancer cells by arresting their growth in their cell cycle. They target cancer cells by aiming for cells that undergo rapid cell division, and might inadvertently also affect normal cells with high growth rates. This is true especially in tissues with high cell turnover such as bone marrow, skin, gastrointestinal tract, hair roots, nails, etc. Consequently, side effects are commonly observed with chemotherapies (OMICS International, 2017).

1.3.4 Anticancer Drug Resistance

In order to provide effective treatment, chemotherapy resistance is still one of the major problems. Resistance can occur in two ways: prior to drug treatment or develop over time following exposure. In some patients, prolonged exposure to a single chemotherapeutic agent may lead to the development of resistance to multiple other structurally unrelated compounds. This process is known as cross resistance or multidrug resistance (MDR) (Akhdar *et al.*, 2012). In primary resistance, MDR can occur without prior exposure to chemotherapy. There are several mechanisms that can be responsible for anticancer drug treatment such as alterations in drug metabolism, modification of drug target expression, changes in apoptotic signaling pathways or expression of proteins which directly affect cellular drug transport.
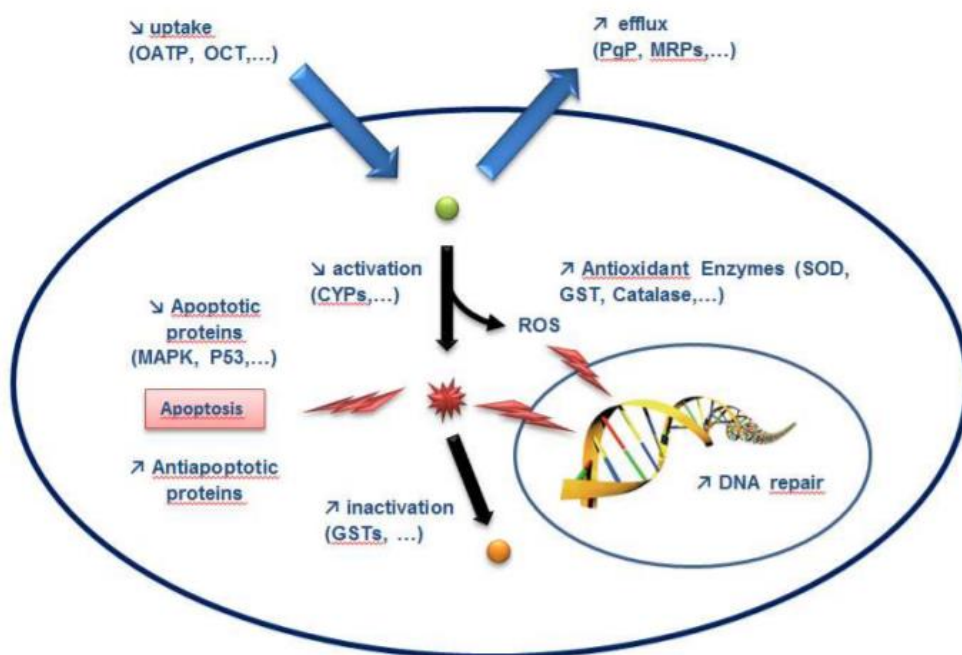
Figure 1 Representation of different mechanisms involved in anticancer drug resistance. OATP: organic anion-transporting polypeptide; OCT: organic cation transporter; Pgp: P-glycoprotein; MRP: multidrug resistance associated proteins; CYP: cytochrome P-450; SOD: superoxide dismutase; GST: glutathione transferase; MAPK: mitogen activated protein kinase (Adapted from Akhdar *et al.*, 2012).

1.3.5 Doxorubicin as an Anticancer Drug

Doxorubicin is a kind of chemotherapy drug which can slow or stop the growth of cancer cells. Two mechanisms are proposed by which doxorubicin acts in the cancer cell (i) intercalation into DNA and disruption of topoisomerase-II-mediated DNA repair and (ii) generation of free radicals and their damage to cellular membranes, DNA and proteins (shown in Fig. 2) (Gewirtz, 1999). Doxorubicin is oxidized to semiquinone which is converted back to doxorubicin. During this process, reactive oxygen species are released and they can lead to lipid peroxidation, membrane damage, DNA damage, oxidative stress, and apoptotic pathways of cell death can be triggered.
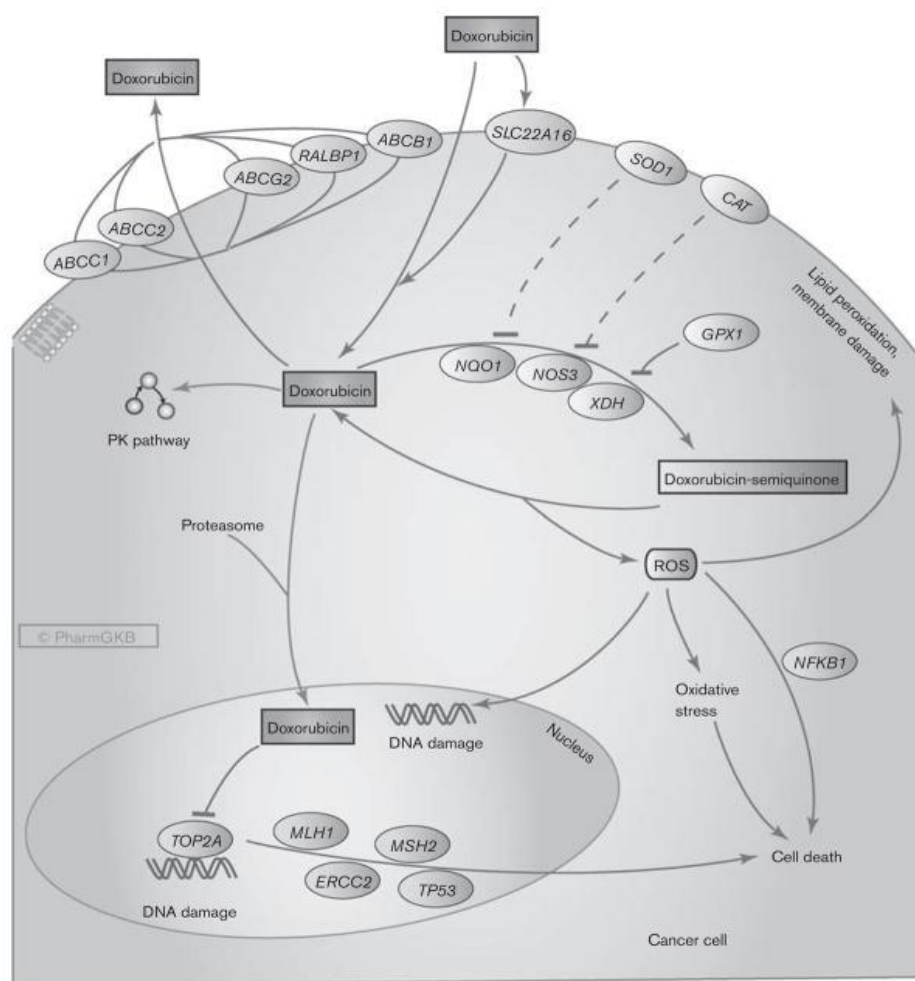
Figure 2 Graphical representation of the candidate genes involved in the pharmacodynamics of doxorubicin in a stylized cancer cell (Adapted from Thorn *et al.*, 2011).

1.3.6 Role of Vitamin E in Cancer Research

1.3.6.1 Structure of Vitamin E

Vitamin E which is a generic term that represents derivatives of tocopherol and tocotrienol. There are four tocopherols and four tocotrienols that can exhibit antioxidant properties. Alpha- and beta-tocotrienols have been found to be the least active forms overall, while delta- and γ-tocotrienols are the most active (Jiang, 2014).

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The structural difference between both groups of isoforms is that, tocopherols have a saturated phytyl chain while tocotrienols have the unsaturated chain (Figure 3).

Vitamin E is found in vegetable oil, nuts, sunflower, wheat, green leafy vegetables, and fish. It is a fat-soluble vitamin that acts as an antioxidant. Storage of the vitamin occurs in adipose tissue, the liver, and muscle. Since it is a fat soluble molecule, it can accumulate in the tissues (Subliov, 2015). In a 2000 report, the Food and Nutrition Board of the National Academy of Sciences specified the tolerable upper intake levels of any form of Vitamin E as 1000 mg/day (Rosenbloom, 2016).
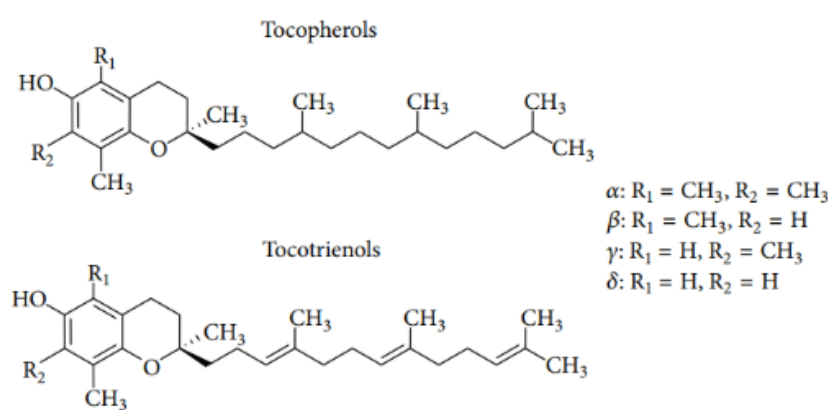
Figure 3 Structure of the natural isoforms of Vitamin E (Adapted from (Neophytou & Constantinou, 2015).

1.3.6.2 Therapeutic use of Vitamin E in Cancer

Antioxidants's inhibitory effect on development of cardiovascular diseases and cancer is proposed to be through neutralizing the damage caused by free radical (Meydani, 1995). Therefore, prevalence of these two diseases can be reduced by consumption of Vitamin E–enriched foods. The most drastic cure for cancer is the surgical resection of the primary tumor. Nowadays, it is shown that tocotrienol rich fraction has an antiproliferative and inhibitory effect on cancer cells since it can induce apoptosis by generating free radicals. Moreover, γ-tocotrienol treatment leads to suppression of mesenchymal markers and the restoration of E-cadherin and γ-catenin expression, which was associated with suppression of cell invasion capability (Chang, *et al.*, 2009).

The effect of γ-tocotrienol on normal cell growth should be considered as well. Cell culture studies have shown that treatment with natural forms and synthetic derivatives of Vitamin E significantly inhibits growth and initiates apoptosis in neoplastic cells using treatment doses that have little or no effect on normal cell growth or viability (McIntyre *et al.*, 2000a; Neuzil *et al.*, 2001a; Weber *et al.*, 2002). These experiments have also demonstrated that the highly malignant cells were the most sensitive whereas normal cells were the least sensitive to the apoptotic effects of these individual Vitamin E forms (McIntyre *et al*., 2000a; McIntyre *et al*., 2000b).

Activation of signal transducer and activator of transcription 3 (STAT3) play a critical role in the survival, proliferation, angiogenesis, and chemoresistance of tumor cells. Therefore, agents that suppress STAT3 phosphorylation have potential as cancer therapies. The apoptotic, antiproliferative and chemosensitizing effects of γ-tocotrienol are associated with its ability to suppress STAT3 activation in hepatocellular carcinoma (Rajendran *et al.*, 2010).
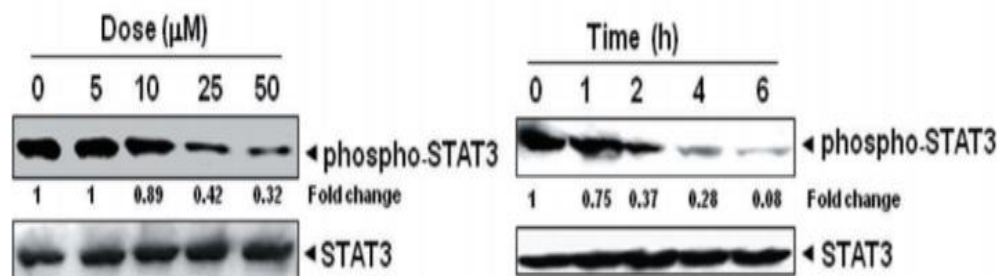
Figure 4 γ-tocotrienol inhibits constitutively active signal transducer and activator of transcription 3 (STAT3) in HepG2 cells (Adapted from Rajendran *et al.*, 2010).

According to Rajendran's study, γ-tocotrienol suppresses phospho-STAT3 levels in a dose-dependent manner (Figure 4). HepG2 cells were treated with the indicated concentrations of γ-tocotrienol for 4 h, after which whole-cell extracts were prepared, and 30 mg of protein was resolved on 7.5% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3.

The graph in Figure 5 also shows that γ-tocotrienol suppresses phospho-STAT3 levels in a time-dependent manner. HepG2 cells were treated with the 50 mM γ-tocotrienol for the indicated times, after which Western Blot analysis was performed.
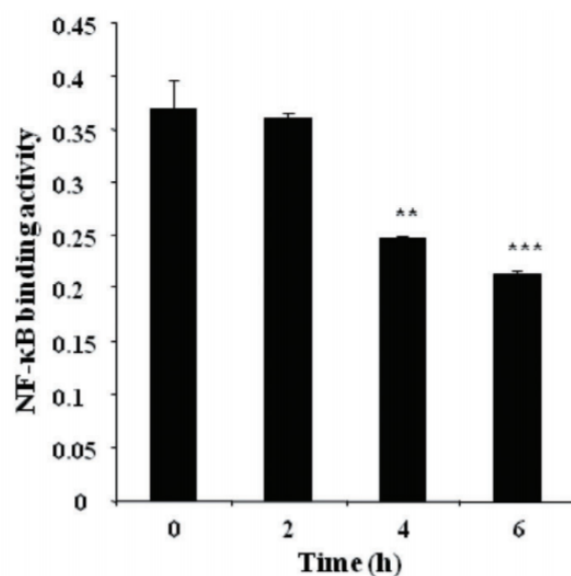
Figure 5 γ-tocotrienol suppresses NF-kB activation in HepG2 cells (Adapted from Rajendran *et al.*, 2010).

Investigations have also shown that combined treatment of Vitamin E with other chemotherapeutic agents resulted in a significant decrease in viable tumor cell number as compared to either treatment alone. Thus findings strongly suggest that Vitamin E can be used effectively to enhance therapeutic efficacy and reduce toxicity of other anticancer agents (Anderson *et al.*, 2004a; Guthrie *et al.*, 1997; Kline *et al.*, 2004; Zhang *et al.*, 2004; Zu & Ip, 2003). Taken the experimental evidence obtained from cell culture studies, Vitamin E derivatives appear to be strong candidates for use as cancer chemotherapeutic agents. This suggestion is even more attractive when taking into account the low toxicity displayed by tocotrienol derivatives against normal cells and when cells were co-treated with γ-tocotrienol and chemotherapy drugs, a synergistic effect may be observed.

1.4 Use of Infrared Spectroscopy for Drug Research

As a new perspective, infrared spectrum of cells treated by anti-cancer drugs could present an opportunity to obtain fingerprint of all molecules that forms to cells, and offer to observe metabolic changes induced by drug exposure with high sensitivity. Middle IR (MID-IR) spectroscopy measurements based on the absorption of MID-IR light in the electromagnetic spectrum by vibrational transitions in covalent bonds. While the wavenumbers in the spectrum relate to the nature of these covalent bonds including their structure and the molecular environment, the intensities / areas under the curve, give quantitative information (Severcan & Haris, 2012). Infrared spectrum of a cell is a sophisticated molecular representation with sum of the contributions coming from proteins, lipids, nucleic acids and other chemical species present in the cells. These spectra could be used as signatures to monitor global effects of drug on cell constituents (Severcan & Haris, 2012).

In this study, it has been suggested that the cells exposed to potential anti-cancer drug will have metabolic modifications which are possibly correlated with drug's cellular mode of action. The wealth of information present in IR spectra related to drug-cell interaction was demonstrated in previous studies (Gasper *et al*., 2009; Inan Genc *et al*., 2016).

1.5 Aim of the Study

In the proposed project, primary goals are;

- To determine the therapeutic effect of γ-tocotrienol in hepatocellular carcinoma cell line HepG2,
- To examine its mechanism of action in relation to lipid metabolism,
- To characterize resistance to chemotherapeutic agents when combined therapy with γ-tocotrienol is applied on HCC cells, and
- To show that whether side effects of chemotherapeutic agents can be reduced with combined drug therapy.

Biochemical, spectroscopic, chemometric measurements and molecular biological techniques were used to achieve these goals.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 HepG2 Cell Line

HepG2 cell line is a hepatocellular carcinoma cell line, which was derived from a 15 years old Caucasian male, was purchased from ATCC (American Type Culture Collection, USA).

2.1.2 Chemicals and Kits

All laboratory equipment which used in cell culture experiments were sterilized. In this study we used T-25/T-75 Cell culture flasks and 6 well/ 96-well cell culture plates (Sarstedt, Germany), Cryovials (Grenier-Bio, Germany), Micro pipettes; 1000, 100, 10 µL (Eppendorf, Germany), Sterile Filtered Pipette Tips; 10, 100, 200 and 1000 µL (Grenier-Bio, Germany), Serological pipettes; 10, 5 mL (Sarstedt, Germany), centrifuge tubes; 50 mL, 15 mL (Sarstedt, Germany) and Hemacytometer (Marienfeld, Germany). In this study, we used DMEM medium with L-Glutamine, Na-pyruvate, Non-essential amino acids, and PBS (Bio-west, United States). Fetal Bovine Serum (FBS), Trypsin-EDTA 1X (Biochrom, Germany). Glacial acetic acid, Dimethyl Sulfoxide (Sigma-Aldrich, USA). In this study, commercially available kits were used for Cell Proliferation kit XTT Based (Roche, Switzerland), MDA lipid peroxidation kit (Abcam, USA).

2.1.3 Instruments

The instruments used in these experiments were Laminar flow cabinet (NUVE, Turkey) CO_2 incubator (NUVE, Turkey), Inverted phase contrast microscope (BEL Engineering, Italy), Eppendorf Centrifuge 5810 R (Eppendorf, Germany), WiseMix Vortex, -80 °C freezer , microplate reader (Multiskan™ GO- Thermo Scientific), WiseBath Water Bath (Wisd Laboratory, Czech Republic), Fourier Transform Infrared Spectroscopy equipped with Attenuated Total Reflectance (Pelkin Elmer, USA).

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Cell Culture Conditions and Medium

HepG2 cells were grown in Dulbecco's Modified Eagle's medium (DMEM), which formulation is shown in the figure below, supplemented with 1% L- Glutamine, 10% fetal bovine serum (FBS). Medium was changed every 2 days and cells were incubated at 37°C atmosphere with 5% CO_2.

Table 1 Formulation of DMEM Medium

Substance	Concentration (mg/l)	Substance	Concentration (mg/l)
NaCl	6400	L-methionine	30
KCl	400	L-phenylalanine	66
$CaCl_2$	200	L-threonine	95
$MgSO_4 \cdot 7H_2O$	200	L-tryptophane	16
NaH_2PO_4	124	L-tyrosine	72
D-glucose	1000	L-valine	94
$Fe(NO_3)_3 \cdot 9H_2O$	0.1	Glycine	30
Na-pyruvate	110	L-serine	42
Phenol red	15	Cholin chloride	4
$NaHCO_3$	3700	Folic acid	4
L-arginine·HCl	84	Myo-inositol	7.2
L-cystine	48	Nicotinamide	4
L-glutamine	580	D-Ca-pantothenate	4
L-histidine·HCl·H_2O	42	Pyridoxal·HCl	4
L-isoleucine	105	Riboflavin	0.4
L-leucine	105	Thiamine·HCl	4
L-lysine·HCl	146		

2.2.1.2 Cell Thawing

10 mL of total volume was used for T-75 cultures as 9 mL medium and 1 mL of cells. First, growth medium was warmed to 37°C in a water bath and followed by procedure took place into the laminar flow sterilized using 70% ethanol. 9 mL of pre-warmed medium at 37°C was transferred into Falcon tube. After that, cryovials were warmed in hand until only residual ice crystals remain and one mL of semi-thawed cell suspension were added into falcon tube containing growth medium.

Then it was slowly mixed with gentle shaking/pipetting. Tube was centrifuged at 1,000 x g for 5 min. The supernatant was discarded and cell pellets were transferred into a T-75 flask containing 10 mL of complete growth medium. T-75 flasks were incubated in a CO_2 incubator at 37 °C. Following a 24 hours' incubation, culture media was removed, adherent cells were washed with PBS and fresh medium added to vials containing the cells.

2.2.1.3 Cell Growth

The cells were transferred into another culture flask when they reached to at least a confluence of 70% (shown in the figure below).
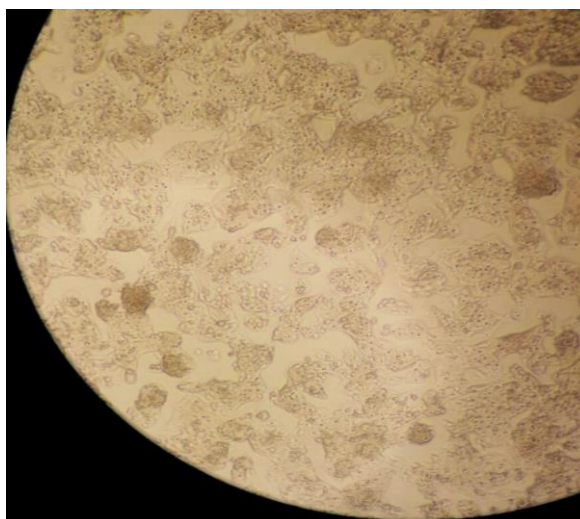
Figure 6 Microscope view of 80% confluent HepG2 cells which grown in the T-75 flask.

- T-75 Growth Procedure:

1. Medium is discarded

2. Cells grown in a monolayer formation is washed with 2 mL PBS

3. 2 mL 0.05% Trypsin-EDTA solution is added for 15 minutes

4. 6 mL culture medium (DMEM + 10% FBS + Antibiotics Penicillin & Streptomycin) is added and pipetted properly

5. 8 mL of solution is transferred to falcon tube and contents were mixed by gentle using vortex for 5 minutes

6. 4 mL of solution is added to T-75 by syringe and medium is completed to 10 ml

2.2.1.4 Cell Freezing

Similar to the passaging procedure, supernatant is removed, cells monolayer is washed with 2 mL PBS, 2 mL 0.05% Trypsin-EDTA solution is added for 10 minutes, 6 mL culture medium (DMEM + 10% FBS + antibiotics) is added and pipetted properly and 8 mL of solution is transferred to falcon tube and vortexed in 5 minutes. After that cell suspension was centrifuged at 1000 x g for 5 min and the supernatant was removed while cell pellet was re-suspended in freezing medium. Freezing medium for HepG2 cells were consisted of 90% (v/v) FBS with 10% (v/v) dimethyl sulfoxide (DMSO). Cells were dissolved in freezing medium and *immediately* put into in cryovials. Cryovials were placed into Mr. Frosty™ Freezing Container (Sigma-Aldrich, USA) first and then transferred to -80°C in Mr. Frosty. Cryovials were then transferred into liquid nitrogen for long term storage.

2.2.1.5 Cell Counting

Amount of viable cells was determined with a hemocytometer and trypan blue staining. After HepG2 cells were harvested, cell pellet is re-suspended in medium and 1 volume of cell solution was added to 1 volume of Trypan Blue Solution (0.25 M) which was used to analyze cell viability. 100 µL of Trypan Blue-treated cell suspension was taken by using a pipette and gently applied to the hemocytometer and both chambers were filled underneath the coverslip, allowing the cell suspension to diffuse out by capillary action. Viable cells are easily detected under a light microscope with a 10X objective since they don't contain blue color whereas dead cells do.

Using a hand tally counter, live ones were counted when they are set within a square or on the right-hand or bottom boundary line. The hemocytometer contains squares with volume the of $0,0001 \text{ mm}^2$ so the average cell count from each of the sets of 16 corner squares was taken and multiplied by 10,000 (10^4). The formula is shown below:

Total Cells/mL= (Total Cell Counted x Dilution Factor x 10^4) x #of Squares

A

B

C
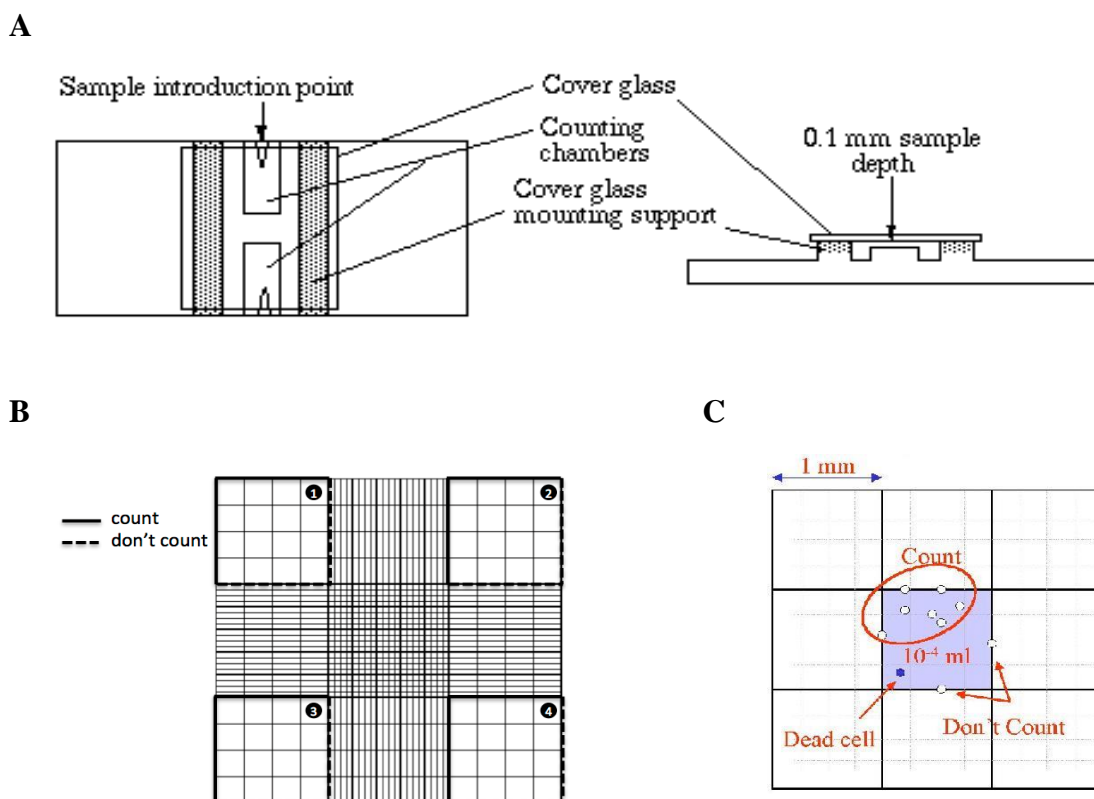
Figure 7 Hemocytometer chamber(A), Counting technique (B and C).

2.2.2 XTT Cell Proliferation Assay

The principle of this assay is based on the metabolism of the yellow tetrazolium salt XTT to form an orange formazan dye (shown in Figure 6). This conversion can only occur in viable cells. Since the formazan dye formed is soluble in aqueous solutions it can directly be quantified using an ELISA reader.
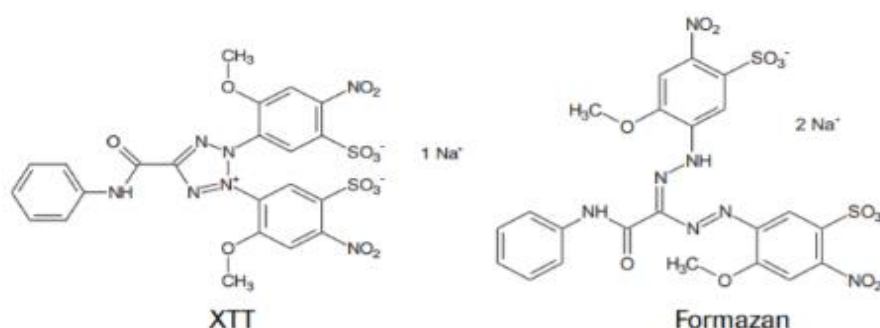
Figure 8 Metabolism of XTT to a water-soluble formazan salt by viable cells.

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Cells were grown in T-75 flasks and then transferred to 96 well plates for XTT assay. The cells were cultured in a 96 well plate (100.000 cells/mL) with 100 µL/well. Medium is discarded from 96 well plate and cells were washed with PBS and treated with medium containing doxorubicin and γ-tocotrienol at different concentrations for 100µL/well. After 24 hours of treatment, XTT solutions were warmed to 37 °C in a water bath until a clear solution is obtained. *Immediately before use*, the XTT reagent was prepared. 0.1 ml of electron coupling reagent and 5 ml of XTT labeling reagent were mixed and added onto cells as 50 µl per well, including the blanks. The plates were incubated for 4 and 8 hours at 37 °C, 5% CO_2 and OD was read at 450nm with a reference wavelength at 690 nm. Roche Cell Proliferation Kit was used and kit contents are presented in Table 2.

Table 2 Cell proliferation kit contents

Vial	Label	Contents
1	XTT Labeling Reagent	• 5× 25 ml XTT (sodium 3′-[1-(phenylaminocarbonyl)- 3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) labeling reagent, in RPMI 1640, without phenol red • 1 mg/ml, filtered through 0.2 µm pore size membrane
2	Electron coupling reagent	• 5× 0.5 ml PMS (N-methyl dibenzopyrazine methyl sulfate) • 0.383 mg/ml (1.25 mM), in phosphate buffered saline (PBS), filtered through 0.2 µm pore size membrane

2.2.3 Wound Healing Assay

Wound healing assay is simple, inexpensive, and one of the earliest developed methods to studying cell migration in vitro. By applying this method, cell migration during wound healing in vivo can be mimicked by creating a "wound" in a cell monolayer, capturing the images at the beginning and at regular intervals. The migration rate of the cells can be quantified by comparing the images (Rodriguez *et al.*, 2010).

1. 6 well plate was inoculated with 750 µl cell suspension/well

2. After it is grown to confluence of 80%, wounds were created by using 100 ml pipette tips

3. Medium was discarded and wells were washed with PBS

4. Cells were divided into in 4 groups;

Group 1 – untreated cells

Group 2 – cells treated with 0.5 µM DOX

Group 3 – cells treated with 300 µM GTT

Group 4 – cells treated with 0.5 µM DOX + 300 µM GTT

Group 5 – cells treated with 0.25 µM DOX + 300 µM GTT

Group 6 – medium

5. During the addition of the new medium, photos of cells were taken to detect the wound formation

6. At 24h of treatment, photos are taken again to detect the cell migration.

2.2.4 MDA Lipid Peroxidation Assay (TBARS)

TBARS assay utilizes the chemical formation of TBARS (Thiobarbituric acid reactive substance) as a byproduct of lipid peroxidation. Lipid peroxidation is the degradation of lipids and it occurs as a result of oxidative damage. It is a useful marker for the detection of oxidative stress.

Polyunsaturated lipids are susceptible to an oxidative attack and this attack results in a chain reaction with the production of end product malondialdehyde (MDA). In this assay, lipid peroxidation assay kit (Abcam, USA) was used. Kit contents are tabulated below:

Table 3 Lipid Peroxidation Assay Kit Contents

Content	Amount	Storage Condition Prior to Preperation	Storage Condition Following Preparation
MDA Lysis Buffer	25 mL	-20 °C	-20 °C
Phosphotungstic Acid Solution	12.5 mL	-20 °C	-20 °C
BHT (100X)	1 mL	-20 °C	-20 °C
TBA Solution	4 vials	-20 °C	4 °C
MDA Standard (4.17 M)	100 µL	-20 °C	-20 °C

2.2.4.1 Standard Curve Construction

In order to accurately determine the amount of oxidation, we need to perform TBARS assay standard curve construction protocol:

1. 0.1 M MDA standard was prepared by diluting 10 µL 4.17 M MDA Standard in 407 µL of ddH2O.

2. 2 mM MDA standard was prepared by diluting 10 µL 0.1 M MDA Standard in 490 µL of ddH2O.

3. Using 2 mM MDA standard, standard curve dilutions were prepared following volumes shown in Table 4:

Table 4 Standard curve dilution volumes

Standard #	Volume of MDA Standard (µL)	Volume ddH2O (µL)	Final Volume standard in well (µL)	End Conc MDA in well (nmol/well)
1	0	600	200	0
2	6	594	200	4 (20 µM)
3	12	588	200	8 (40 µM)
4	18	582	200	12 (60 µM)
5	24	576	200	16 (80 µM)
6	30	570	200	20 (100 µM)

Each dilution has enough amount of standard to set up duplicate reading (2 x 200 µL).

2.2.4.2 Sample Preparation

Approximately 2×10^6 cells were harvested for each assay and washed with cold PBS. Lysis Solution was prepared by mixing 300 µL of the MDA Lysis Buffer with 3 µL BHT (100X). Cells were homogenized in 303 µL Lysis Solution (Buffer + BHT) using a Dounce homogenizer (10-50 times) on the ice. Cells were centrifuged at 13,000 x g for 10 minutes to remove pellet and the supernatant was collected.

2.2.4.3 Assay Procedure

600 µL of TBA reagent was added into each vial or well containing 200 µL standard and 200 µL sample, and incubated at 95 °C for 60 minutes. Once cooled to room temperature in an ice bath for 10 minutes and 200 µL supernatant was (containing MDA-TBA adduct) placed into a 96-well microplate for analysis and measured on ELISA reader at OD_{532}.

2.2.5 ATR-FTIR Spectroscopic Studies

2.2.5.1 Sample Preparation

For ATR-FTIR spectroscopic studies, 4 groups of cells (control, doxorubicin (DOX) treated, γ-tocotrienol (GTT) treated and DOX+GTT treated cells) were grown in an independent manner, harvested and the cell pellet was re-suspended in 10 µl phosphate buffer saline solution (PBS). Cells were grown until a confluence of 80% was reached and then treated with trypsin. Approximately 2×10^6 cells from each group was taken and centrifuged at 1000 x g for 5 min. Cell pellet was re-suspended in 10 µl phosphate buffer saline solution (PBS) for ATR-FTIR measurements.

The analyses were performed on vector normalized second derivative spectra. Vector normalization and derivation were both performed using the Unscrambler X 10.3 (Camo Software AS) multivariate analysis (MVA) software. While studying FTIR, examined samples were not being dried as we wanted to preserve the natural environment of cells and reactions taken place on them.

2.2.5.2 Data Acquisition

For the collection of the IR spectra of the cells, the one-bounce ATR mode in a Spectrum 100 FTIR spectrometer (Perkin-Elmer Inc., Norwalk, CT, USA) equipped with a Universal ATR accessory was used. Cell spectra were obtained in the 4000–650 cm^{-1} region, 100 scans and at a resolution of 8 cm^{-1} at room temperature. Experiments were performed in triplicates.

2.2.5.3 Data Analysis

Four groups of cells were differentiated from each other based on spectral alterations and Principal Component Analysis (PCA) was performed using Unscrambler X 10.3 (Camo Software AS) multivariate analysis (MVA) software. The mean centered absorbance spectra at 4000-650 cm^{-1} spectral region were used for all groups. Discrimination between the groups was shown as score plots.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 XTT Cell Proliferation Assay

After total cell treatment, amount of cell proliferation was assayed with XTT Assay. The absorbance values were measured at 490 nm in ELISA plate reader. As shown in Figure 9, the % cell viability of the control cells is valued at 100%.

In literature, there are different concentrations for doxorubicin agent. According to research done by Capone and his colleagues, doxorubicin has 50% cytotoxic effect on HepG2 cells at concentration 0.87 μM (Capone *et al.*, 2014). Another research shows that doxorubicin reduced cell viability to 50% at concentration of 0.58 μM (Yang, *et al.*, 2010). On the other hand, there are high concentration treatments as well. It was found that doxorubicin has 50% cytotoxic effect on HepG2 cells at concentrations 1.06 μM (Qubaisi *et al*., 2011), 1.25 μM (Fan *et al*., 2010), 5 uM (Manov et al., 2007).

Optimal dose for doxorubicin was determined by applying different doses from low to high concentrations. We tried 0.1 μM, 0.2 μM, 0.3 μM, 0.4 μM, 0.5 μM, 0.8 μM and 1 μM of doxorubicin agent for 24, 36, and 48 hours. After that, different treatment trials with GTT is done with concentrations such as 50 μM, 100 μM, 200 μM, 300 μM and 400 μM for 24, 36 and 48 hours. In literature, GTT is applied in concentrations as 20 μM, 40 μM, 80 μM, 100 μM and its anti-proliferative effects were observed (Yap *et al.*, 2008). After treatment trials, optimal concentrations were calculated as 0.5 μM for doxorubicin hydroxylate and 300 μM for γ- tocotrienol for 24 h in HepG2 cells. Combined-treatment of these two compounds (DOX and GTT) was observed to be more effective to reduce cell proliferation than GTT or DOX alone for 24 h.
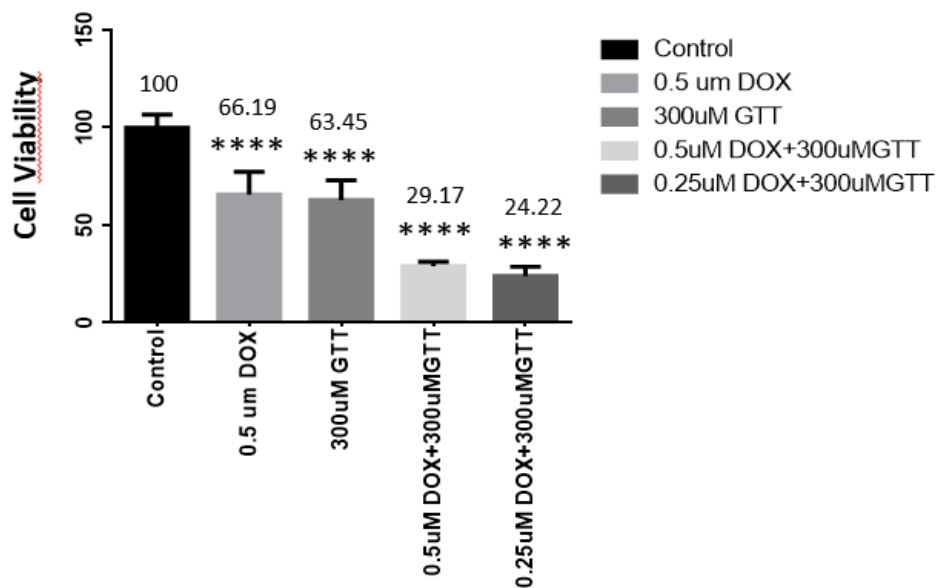
Figure 9 One-way ANOVA with Tukey's multiple comparisons test for XTT results (p values equal to or less than 0.05 were considered as significantly different from the control group. The degree of significance was denoted as: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

As it can be also referred in figure 9, the overall data indicated that when vitamin E derivative GTT applied with chemotherapeutic agent DOX for 24 h, an increase in the intensity of the damage was observed. Choi and his colleagues have shown that treatment with doxorubicin can reduce the viability of the HepG2 cells up to 50% at a concentration of 0.5 µM (Choi *et al.*, 2008). It means that an enhancement of viability repression can be induced by DOX at this concentration. Another study demonstrated that γ-tocotrienol suppressed the expression of several STAT3-regulated genes; including proliferative gene cyclin D1. The down-regulation of cyclin D1 expression by γ-tocotrienol may explain its reported anti-proliferative effects on various tumor cells since cyclin D1 is required for the transition from G1 to S phase of the cell cycle (Rajendran *et al.*, 2011). This study shows that our results are reliable and GTT can be used as anti-proliferation agent on hepatocellular carcinoma cells.

Moreover, the same study shows that γ-tocotrienol significantly enhanced the apoptotic effects of doxorubicin from 14% to 48%. This data supports our results as cell viability in 0.5 µM DOX treated cells was 66% while viability in combined treatment was found as 30%. It shows that there is a reduction in cell viability when combined treatment is applied. Therefore, it appears that GTT can potentiate the effect of chemotherapeutic agent DOX.

According to XTT results, there is a significant decrease in proliferation of treated HepG2 cells compared to the control group. All groups were compared with the control group and only DOX or GTT treated cells showed a significant amount of reduction in cell viability up to 60% and when combined treatment is applied cell viability is reduced down to 30%. After these concentrations were determined, we tried to go one step further and applied a half dose of DOX while GTT concentration remains constant. In literature, the lowest amount of DOX is represented as 0.5 µM. However, we tried to use 0.25 µM DOX instead of 0.5 µM and we observed that reduction in liver cancer cell proliferation was the same. Based on our XTT results, we decided to choose concentrations as 0.5 µM for DOX, 300 µM for GTT, 0.25 µM DOX and 300 µM GTT for combined treatment for our further experiments.

Results obtained from XTT assay shows that half of doxorubicin concentration can be used along with the same amount of GTT and it can be interpreted as side effects of chemotherapy may be reduced.

On the other hand, it should be considered that there are adverse reactions have been recorded in high doses. Especially for people who have conditions such as heart disease and diabetes, vitamin E may be unsafe when taken in very high amounts. The recommended doses by Food and Nutrition Board of the National Academy of Sciences in 2000 report is 1000 mg per day. In our experiments, approximately 123 mg of γ-tocotrienol was used for an application. Even this dose is under the daily uptake level, people who take this dose should be careful about the side effects of excess amount of γ-tocotrienol.

3.2 Wound Healing Assay

The control and the treated HepG2 cells were inoculated in 6 well plates and after wounds were created healing processes were observed and images were analyzed with Image J program.

	0 hour	**24 hours**
A		
B		
C		
D		
E		
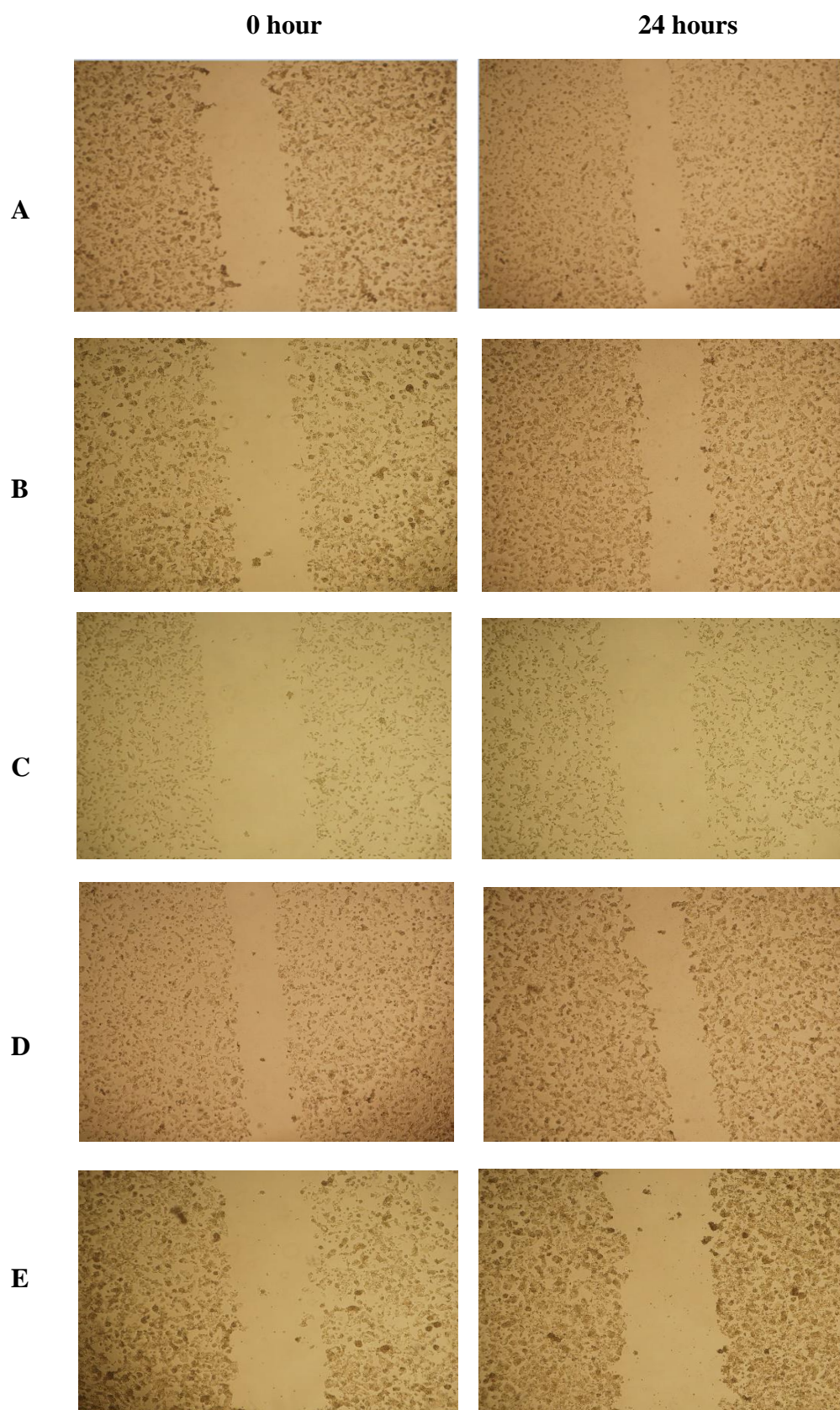
Figure 10 Wounds created by using 100 mL pipette tips. Photos were taken and represented by using Image J when wound was created (0 hour) and after 24 hours (A) The control group (no treatment was applied) (B) Cells treated with 0.5 µM DOX (C) 300 µM GTT treated cells (D) 0.5 µM DOX + 300 µM GTT treated cells (E) 0.25 µM DOX + 300 µM GTT treated cells.
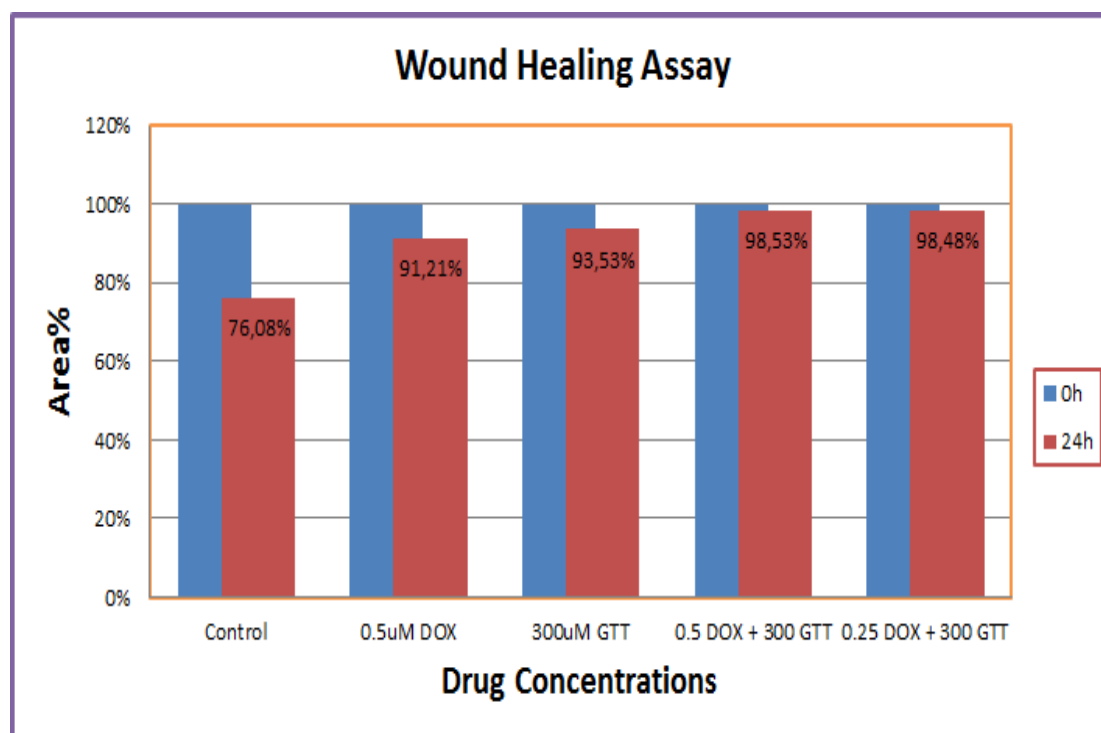
Figure 11 Wound healing assay results.

Percentages of wound area are represented in Figure 11. If we consider that the beginning area is 100%, the decrease in wound area means that there is a cell migration. Since we exposed our cells to toxic agents, we expect to observe cell death, so, there was no migration. For the control group; Wound area is decreased to 76% from 100% in 24 hours. Migration occurred, so cells are more viable. For 0.5 µM DOX; decrease in wound area to 91% from 100% shows that there is less migration and less viability of cells. For 300 µM GTT; Wound area decreased to 93% from 100% and there is less migration compared to the control and 0.5 µM DOX and obviously it was more effective than 0.5 µM on cell migration.

For 0.5 μM DOX + 300 μM GTT; Wound area decreased to 98% from 100%, indicating less migration of cells. This combined concentration was more effective on cell death. For 0.25 μM DOX + 300 μM GTT; Wound area decreased to 98% from 100% and also less migration of cells. This combined concentration has also higher effect on migration capability of cells. Last two combined concentrations show almost the same effect on migration of cells. Although, we dropped the chemotherapeutic agent concentration, migration percentage stayed constant due to the GTT concentration. It means that GTT can be used with lower doses of DOX and the same effect can be shown by decreasing the side effects of DOX. So, wound healing assay results are in agreement with our XTT results.

3.3 MDA Lipid Peroxidation Assay TBARS

Lipid peroxidation refers to the metabolic process in which reactive oxygen species (ROS) result in the oxidative deterioration of lipids (Placer *et al*., 1966). In this process free radicals take electrons from the lipids, resulting in cell damage. Quantification of lipid peroxidation is essential to assess oxidative stress. Lipid peroxidation forms reactive aldehydes such as malondialdehyde (MDA) and measuring the MDA amount is one of the most widely accepted assays for oxidative damage (Niki, 2008). TBARS test determines malondialdehyde that reacts with thiobarbituric acid. Therefore, the test is named as thiobarbituric acid reactive substances (TBARS) test. Butylated hydroxytoluene (BHT) is used to prevent the artificial increase of MDA during the experiment. This method is widely used to determine lipid peroxidation in biological systems (Jain *et al*., 1995; Sevenian *et al*., 1985; Severcan *et al*., 2005). There is no doubt that ROS and lipid peroxidation play a role in cancer development. However, evidence also indicates that cancer cells require certain amounts of ROS for proliferation and survival. This, therefore, suggests strategies aimed at further increasing the levels of ROS and oxidative damage such as lipid peroxidation products may be deleterious to cancer cells and thus beneficial in cancer management.

It is demonstrated that lipid peroxidation products exert antitumor effects and also potentiate the cytotoxicity of anticancer drugs and radiotherapy (Erejuwa *et al.*, 2013).

Lipid peroxidation biomarkers could also serve as a diagnostic tool to predict the chances of cancer recurrence and be used to monitor the progress or effectiveness of therapy in cancer patients (Barrera *et al.*, 2008; Kumar *et al.*, 2008). The findings also reveal insights into the possible molecular mechanisms (via modulation of key cancer-related transcription factors) by which lipid peroxidation products may inhibit or suppress the growth of cancers.

Standard Curve

$y = 0,2597x - 0,2799$
$R^2 = 0,9934$

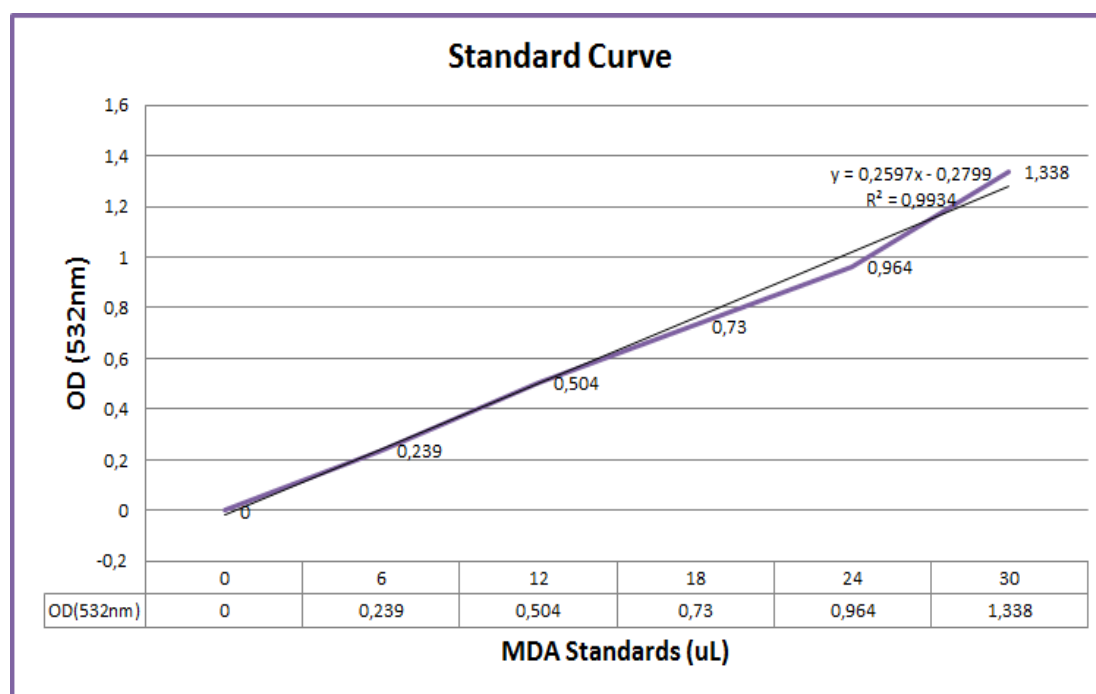|	0	6	12	18	24	30
OD(532nm)	0	0,239	0,504	0,73	0,964	1,338

MDA Standards (uL)

Figure 12 Standard curve graph for TBARS assay

For the accuracy of the TBARS assay, standard curve must be constructed prior to performing the test in order to be used as guidance (shown in Figure 12). R-squared is a statistical measure of how close the data are to the fitted regression line.

It is also known as the "coefficient of determination" and the percentage of the response variable variation that is represented by a linear model, where a 100% coefficient of determination indicates that the model explains all the variability of the response data around its mean. In general, the higher the R-squared, the better the model fits the data. This shows that, our model is in good fit to the data.

Following the construction of standard curve, the 4 groups of cells were examined and MDA concentrations were calculated as presented in Table 5 and Table 6.

Table 5 Representation of average of 4-MDA lipid peroxidation assay results

	CONTROL	0,5 µM DOX	300 µM GTT	0,25 µM DOX + 300 µM GTT
1st TBARS	27,8 µM MDA	28,1 µM MDA	26,9 µM MDA	29,6 µM MDA
2nd TBARS	20 µM MDA	29 µM MDA	33 µM MDA	29 µM MDA
3rd TBARS	16 µM MDA	22 µM MDA	33 µM MDA	35 µM MDA
4th TBARS	22 µM MDA	30 µM MDA	32 µM MDA	33 µM MDA

Table 6 Average MDA concentrations

		Average of 4 TBARS assays			
		CONTROL	0,5 µM DOX	300 µM GTT	0,25 µM DOX + 300 µM GTT
OD (532nm)		0,831	1,133	1,345	1,356
MDA (µM)		21.5 µM MDA	27 µM MDA	31 µM MDA	32 µM MDA

Results were also represented as a bar graph in Figure 13. An increase in MDA concentration was observed and it means that there is an increase in lipid peroxidation and reactive oxygen species (ROS). Lipid peroxidation and ROS are initiators and essential mediators of apoptosis, which leads to the carcinogenesis (Begin *et al*., 1985; Seigal *et al*., 1987).

Therefore, combined treatment with DOX and GTT can force the cancerous cells to apoptosis by triggering lipid peroxidation and increasing oxidative stress. As a result of ROS production, the vital point is to successfully, effectively, and selectively target cancer cells in humans using lipid peroxidation products. This is an area that requires further and detailed research as it may prove to be an important strategy to complement current treatment regimens for cancer patients.
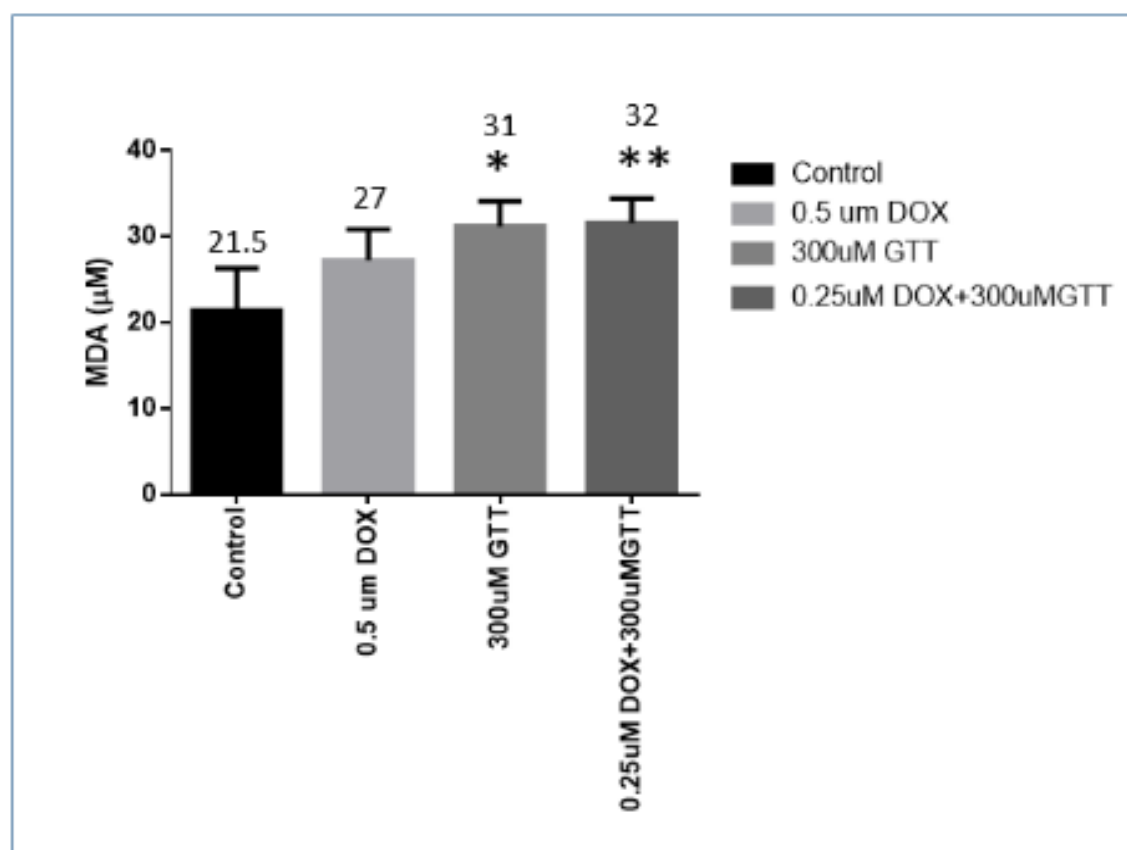
Figure 13 Representation of MDA concentrations from 4 groups of cells

3.4 ATR-FTIR Spectroscopic Studies & PCA Analysis

Figure 14 A and B show representative absorption and second derivative spectra, respectively of the groups of cells in different regions.

A

B
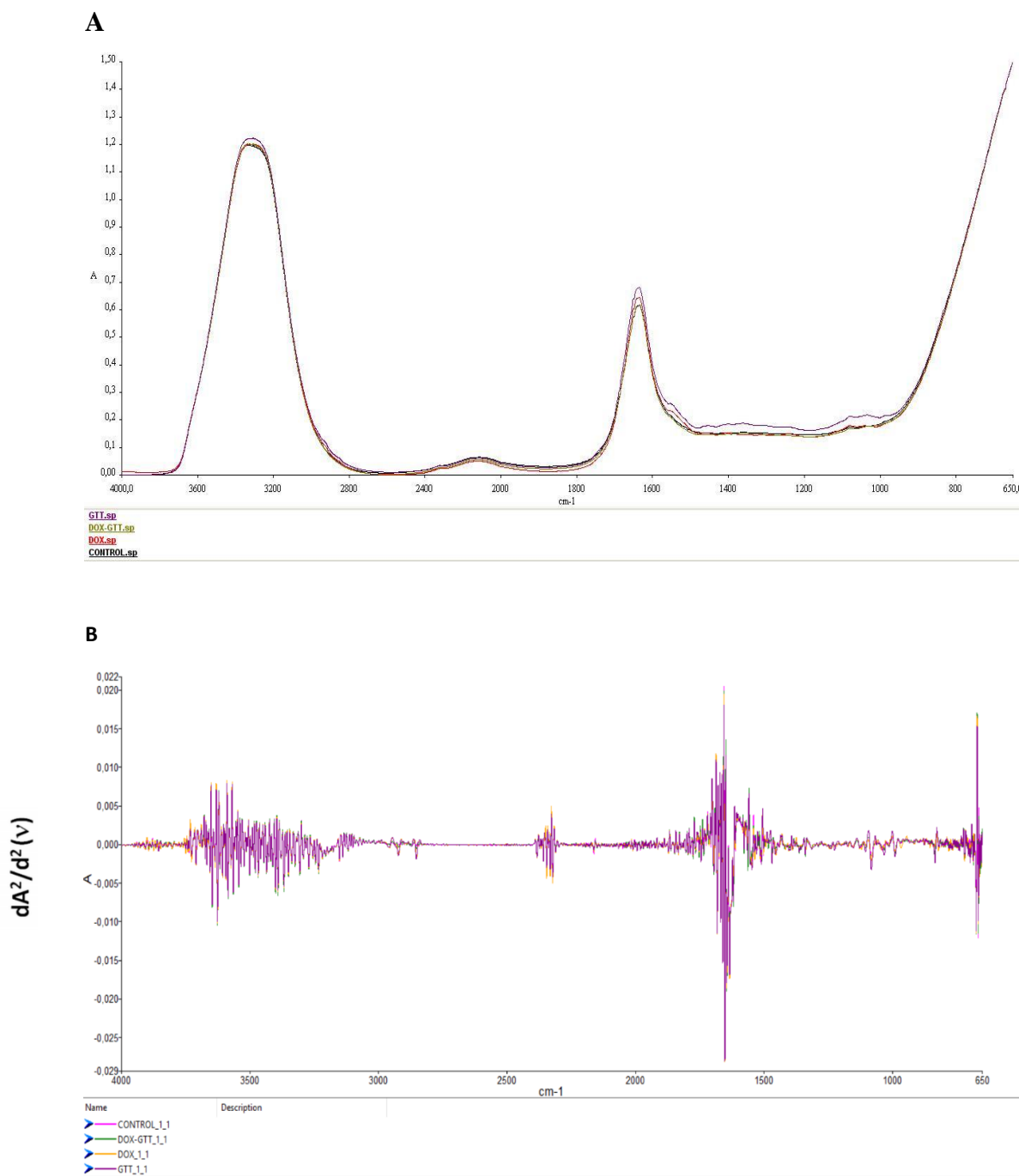
Figure 14 Representative spectra of groups of cells in the 4000- 650 cm^{-1} region (A). Vector normalized second derivative spectra of 4 groups of HepG2 cells in the 4000- 650 cm^{-1} region (B).

ATR-FTIR provides rapid, sensitive and simultaneous monitoring of different functional groups of biomolecules in the biological systems (Garip *et al*., 2007; Severcan *et al*., 2010). As seen from the Figure 14 A, the absorption spectra do not show the main bands of biomolecules such as lipid and protein, which were masked by free water bands of the system. As we mentioned in the materials and method section since we wanted to preserve the natural environment of our biological system, we did not apply drying process and therefore the spectra of free water bands were present. These bands almost mask the bands related to major biomolecules of the system which are partly resolved in the second derivative spectra.

A
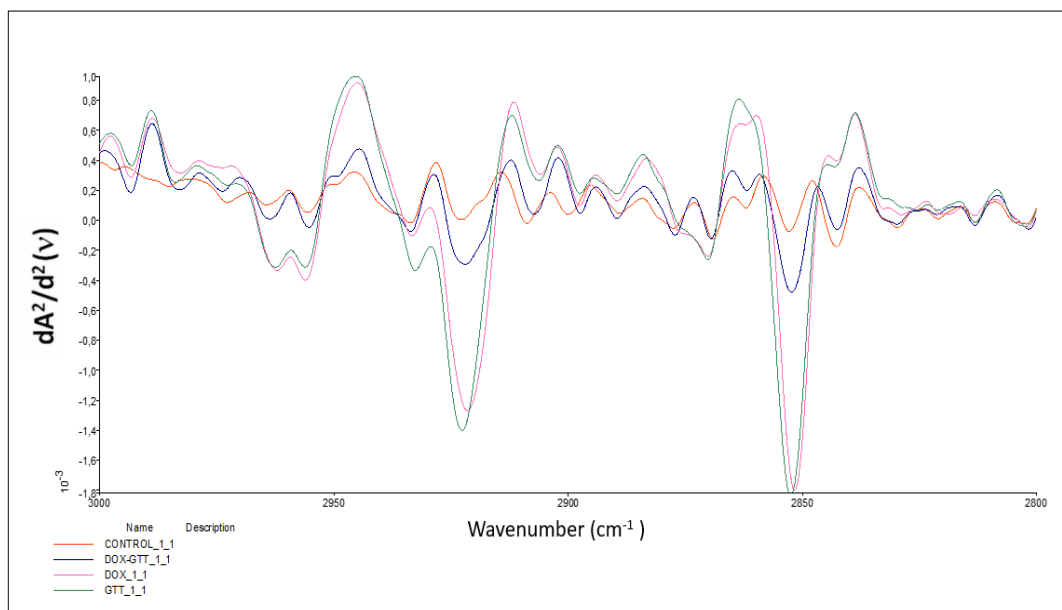
B
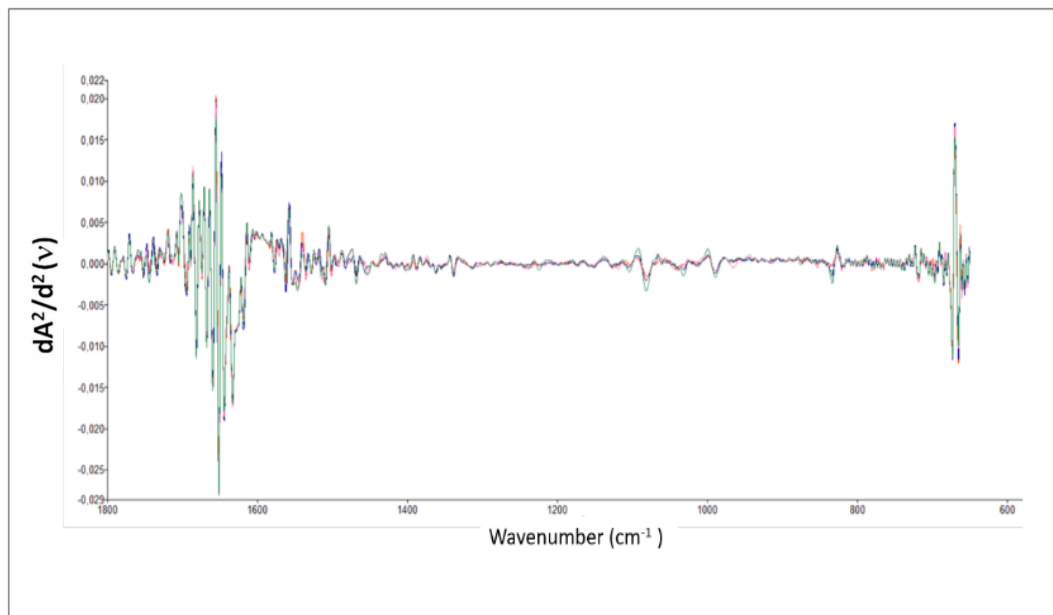
Figure 15 Representative vector normalized second derivative spectra of 4 groups of HepG2 cells in the 3000- 2800 cm^{-1} region (A). In the 1800-650 cm^{-1} fingerprint region (B).

ATR-FTIR spectroscopy gives valuable information about the changes in biochemical components and processes in diseases or drug-induced pathological conditions (Ozek *et al*., 2010; Cakmak *et al*., 2011). As can be seen from these figures changes in the C-H stretching (3000-2800 cm-1) region mainly contains lipid bands and fingerprint (1800-650 cm-1) regions (Figure 15A & 15B) contains the bands arise from the functional groups belonging to biomolecules such as proteins, carbohydrates, and nucleic acids, as well as lipids and small molecules such as primary metabolites, secondary metabolites and natural products. The changes in the intensity of the spectral bands in these regions are clearly seen from the figures. These changes can be due to any bio molecular difference between the groups but these alterations are enough to say that there is a distinction between the studied groups.

A
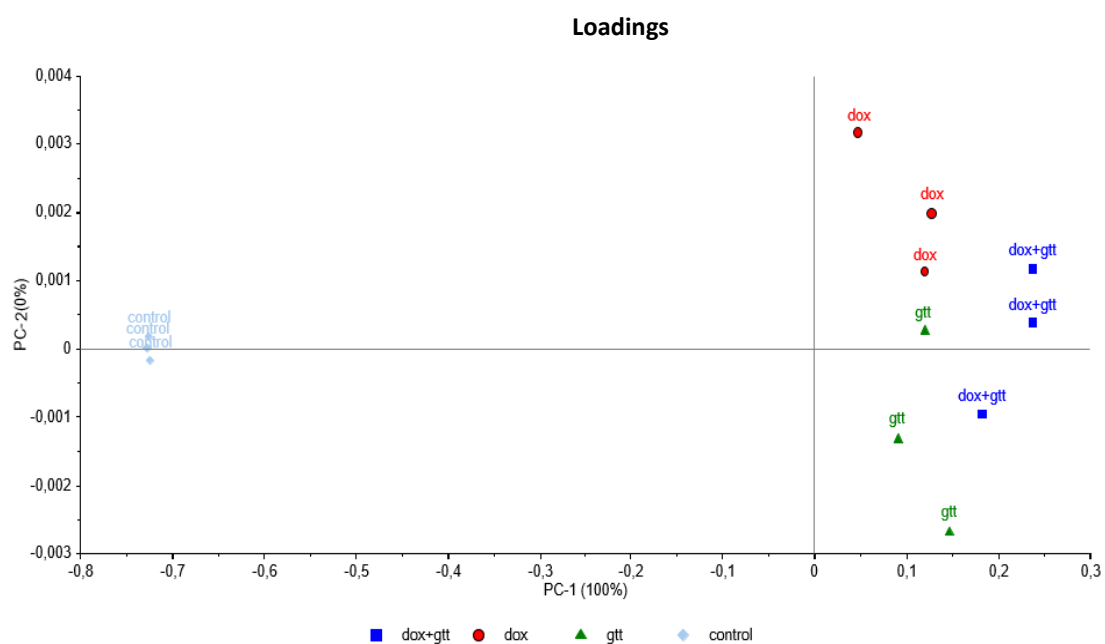
B

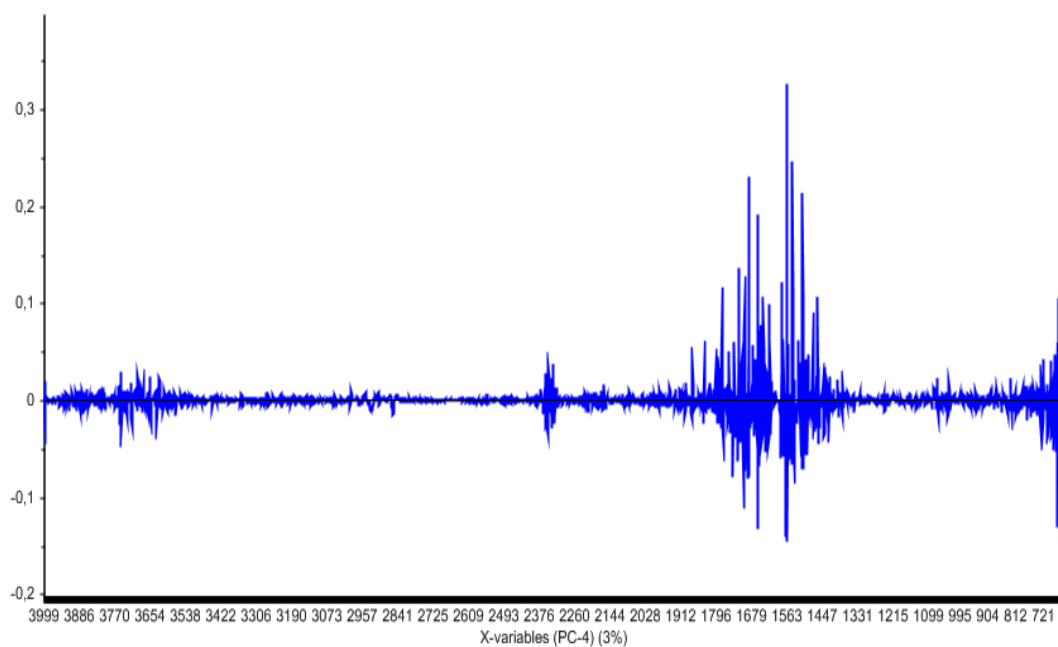**Figure 16** Principle component analysis of vector normalized second derivative spectra of 4 different cell groups as score plot (A). PCA loading plot for vector normalized second derivative spectra of 4 groups of cells in the 4000-650 cm-1 spectral region (B).

Principle Component Analysis (PCA) is used to reduce the dimensions of data into a series of orthogonal eigenvectors, while retaining the information about the variations in data points (Nieuwoudt *et al.*, 2004). For a FTIR spectral input, this corresponds to the reduction of hundreds of absorbance values at corresponding spectral wavenumbers into a single point. The coordinates are the principal components (PC) and the plot obtained is called the scores plot (Nakamura *et al.*, 2010). Each PC describes the spectral variability among samples in decreasing order. Thus, the first principle component (PC1) expresses most of the variance in the data; PC2 expresses the second largest variance in the data and so on (Demir *et al.*, 2015). As a result, we can get the information about the discrimination between groups. The more increase in the separation between the two points in a scores plot, the more increase in the dissimilarity between these two samples. Furthermore, the spectral variability among the samples is described with decreasing order in the principal components (Demir *et al.*, 2015).

PCA has been widely used to differentiate experimental groups in cancer studies (Gok *et al.*, 2016; Ghosh and Barman, 2013; Hsu *et al.*, 2014). For example, in one of those studies bladder cancer group was differentiated from the health subject successfully using bladder wash samples (Gok *et al.*, 2016). Another study demonstrated that PCA analysis allowed correct separation of all serum specimens of ovarian cancer patients from healthy samples (Odunsi *et al.*, 2005).

In our results, the discrimination between the control group, untreated hepatocellular carcinoma cells, and the treated groups can easily be seen in Figure 16A. As expected, all the treated groups were discriminated from untreated cancer cells and combined therapy on HepG2 cells shows more dissimilarity than HepG2 cells treated with only DOX or GTT.

Score plots are used to indicate relationship studied groups while loading plots which is represented in figure 16B are used to indicate the degree of contributions of spectral variations among sample groups. Both loading and score plots are created based on the principal components (PCs). The advantage of PCA lies in the fact that it is an unbiased technique in the sense that no information about the classes, so it is resulting in a representation of the true variability of the data.

In our PCA model, loading plots of PC score values demonstrate the spectral origin of the variations which differentiate the data groupings according to the wavenumbers. This plot which is shown in Figure 16 has a spectral dimension, where positive and negative peaks can be observed. These obvious differences in the loading plot of cell groups indicate the altered profiles of cells. To be able to detect which biomolecule has a difference, detailed characterization studies will be done in future studies.

CHAPTER 4

CONCLUSION

During our investigations, we used 4 groups of cells to prepare from the Hep-G2 cell line represented as Control group Hep-G2 cells, γ-tocotrienol (GTT) applied Hep-G2 cells, only doxorubicin treated cells, and finally HCC cells treated with γ-tocotrienol and combination in order to investigate resistance to chemotherapy agents. Chemotherapeutic agent used in combination with γ-tocotrienols was doxorubicin, which is a commonly used chemotherapeutic agent.

The aim of our investigation was to be able to show the therapeutic effects of combined therapy of γ-tocotrienol (GTT) and doxorubicin, and to be able to show that combined therapy can reduce the side effects of doxorubicin by reducing the drug concentration. To evaluate the impact of combination therapy we used XTT procedure, owing to its ease of implementation and reliability of its results. Briefly, in the XTT assay, the accurate growth rate of HepG2 cells was measured by using the degree of enzymatic degradation that cells cause during this time in the ELISA reader. The percentages of cell viability were measured calorimetrically and the impact of combination therapy on cell growth was investigated. GTT and DOX to be used during our research by starting with the aim of determining the concentrations and the published values in the literature, the XTT experiment was repeated at different GTT and DOX concentrations. As a result, concentrations are found as 0.5 μM for doxorubicin and 300 μM for γ-tocotrienol and these results are consistent with previously published values (Choi *et al.*, 2008; Yang *et al*., 2010). Moreover, the concentration of GTT which is applied on cells was appropriate regarding daily uptake level. Secondly, for wound healing assay the Image J program was used to examine the photographs and the cell growth was described as related to the closure performance of the wound simulator.

After that, TBARS experiments in which we examined lipid peroxidation were carried out by using MDA lipid peroxidation kit. Lastly, the ATR-FTIR study were done with 4 groups of HepG2 cells to investigate structural and contextual changes.

Based on the experiments, when XTT, wound healing and TBARS experiments are evaluated together, the growth characteristics of HepG2 cells used in our experiments was in a normal environment. However, when doxorubicin is added, this chemical agent gives the expected response and finally, it was observed that the addition of GTT increased the effect of doxorubicin. By moving one step further, we observed that cell viability was constant even though doxorubicin concentration was reduced to halved. So, these results show us that side effects of doxorubicin may be reduced with the combined therapy of γ-tocotrienol agent.

For future studies, we are planning to do experiments with lower doses of γ-tocotrienol since decreasing the dose of vitamin E is important considering effects of higher doses on healthy cells. Furthermore, studies are planning to search the effects of combined treatment on resistant liver cancer cell line to be able to show the prevention of resistance developing by HH resistant cells.

REFERENCES

A. Sevanian, P. Hochstein, Mechanisms and consequences of lipid peroxidation in biological systems, Annu. Rev. Nutr. 5 (1985) 365–390.

Akhdar, H., Legendre, C., Aninat, C., & Morel, F. (2012). Anticancer Drug Metabolism: Chemotherapy Resistance and New Therapeutic Approaches. *Topics on Drug Metabolism*, 137–170

Al-Qubaisi, M., Rozita, R., Yeap, S. K., Omar, A. R., Ali, A. M., & Alitheen, N. B. (2011). Selective cytotoxicity of goniothalamin against hepatoblastoma HepG2 cells. *Molecules*, *16*(4), 2944–2959. https://doi.org/10.3390/molecules16042944

Anderson K, Lawson KA, Simmons-Menchaca M, Sun L, Sanders BG, Kline K (2004a) Alpha-TEA plus cisplatin reduces human cisplatin-resistant ovarian cancer cell tumor burden and metastasis. Experimental biology and medicine (Maywood, NJ) 229: 1169-1176

Anderson K, Simmons-Menchaca M, Lawson KA, Atkinson J, Sanders BG, Kline K (2004b) Differential response of human ovarian cancer cells to induction of apoptosis by vitamin E Succinate and vitamin E analogue, alpha-TEA. Cancer research 64: 4263-4269

Barrera G, Pizzimenti S, Dianzani MU. Lipid peroxidation: control of cell proliferation, cell differentiation and cell death. *Molecular Aspects of Medicine*. 2008;29(1-2):1–8.

Begin, M.E., Das, U.N., Ells, G., Horrobin, D.F. Selective killing of human cancer cells by polyunsaturated fatty acids. *Prostaglandins Leukotrienes Med*. 1985; 19:177–186.

Capone, F., Guerriero, E., Sorice, A., Colonna, G., Storti, G., Pagliuca, J., Costantini, S. (2014). Synergistic antitumor effect of doxorubicin and tacrolimus (FK506) on hepatocellular carcinoma cell lines. *The Scientific World Journal*, *2014*.

Demir, P., Onde, S., Severcan, F. (2015). Phylogeny of cultivated and wild wheat species using ATR–FTIR spectroscopy. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 135*, 757-763. https://doi.org/10.1016/j.saa.2014.07.025

F. Severcan, O. Bozkurt, R. Gurbanov and G. Gorgulu, FT-IR spectroscopy in diagnosis of diabetes in rat animal model. J. Biophotonics, 2010, 3, 621–631.

Fagard, R., Metelev, V., Souissi, I., & Baran-Marszak, F. (2013). STAT3 inhibitors for cancer therapy: Have all roads been explored? *Jak-Stat*, *2*(March), e22882. https://doi.org/10.4161/jkst.22882

Fan, L.-L. (2010). Melatonin and Doxorubicin synergistically induce cell apoptosis in human hepatoma cell lines. *World Journal of Gastroenterology*, *16*(12), 1473. https://doi.org/10.3748/wjg.v16.i12.1473

Fong,Y, Dupey,DE,, Feng M, Abou-Alfa G. Cancer of the liver. In: DeVita VT, Lawrence TS, Rosenberg SA, eds. DeVita, Hellman, and Rosenberg's *Cancer: Principles and Practice ofOncology*. 10th ed. Philadelphia, Pa: Lippincott Williams & Wilkins; 2015:696-714.

Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. *Lancet*. 2012;379:1245-1255.

G. Cakmak, F. Zorlu, M. Severcan, F. Severcan, screening of protective effect of amifostine on radiation-induced structural and functional variations in rat liver microsomal membranes by FT-IR spectroscopy, Anal. Chem. 83 (2011) 2438–2444.

Gasper R, Dewelle J, Kiss R, Mijatovic T, Goormaghtigh E (2009) IR spectroscopy as a new tool for evidencing antitumor drug signatures. Biochimica et Biophysica Acta (BBA)-Biomembranes 1788: 1263-1270

GBD 2015 Mortality and Causes of Death, Collaborators. (8 October 2016). "Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: a systematic analysis for the Global Burden of Disease Study 2015.". *Lancet*. 388 (10053): 1459–1544 https://doi.org/10.1155/2014/450390

Gewirtz, D. A. (1999, April 1). A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochemical Pharmacology.*

Ghosh, A., & Barman, S. (2013). Prediction of Prostate Cancer Cells Based on Principal Component Analysis Technique. Procedia Technology, 10, 37–44. https://doi.org/10.1016/j.protcy.2013.12.334

Guthrie N, Gapor A, Chambers AF, Carroll KK (1997) Inhibition of proliferation of estrogen receptor-negative MDA-MB-435 and -positive MCF-7 human breast cancer cells by palm oil tocotrienols and tamoxifen, alone and in combination. The Journal of nutrition 127: 544s-548s

Har CH, Keong CK (2005) Effects of tocotrienols on cell viability and apoptosis in normal murine liver cells (BNL CL.2) and liver cancer cells (BNL 1ME A.7R.1), in vitro. Asia Pac J Clin Nutr 14:374–380

Hsu, Y., Huang, P., & Chen, D. (2014). Sparse principal component analysis in cancer research. Translational Cancer Research, 3(3), 182–190. https://doi.org/10.3978/j.issn.2218-676X.2014.05.06

Inan Genc A, Gok S, Banerjee S, Severcan F (2016) Valdecoxib Recovers the Lipid Composition, Order and Dynamics in Colon Cancer Cell Lines Independent of COX-2 Expression: An ATR-FTIR Spectroscopy Study. Applied spectroscopy

Jiang, Q., 2014. Natural forms of vitamin E: metabolism, antioxidant, and anti-inflammatory activities and their role in disease prevention and therapy. Free Radic Biol Med. 2014; 72:76–90.

Kamal-Eldin A, Appelqvist LA. The chemistry and antioxidant properties of tocopherols and tocotrienols. Lipids. 1996; 31:671–701. doi: 10.1007/BF02522884

Kannappan R, Yadav VR, Aggarwal BB. {Gamma}-tocotrienol but not {gamma}-tocopherol blocks STAT3 cell signaling pathway through induction of protein-tyrosine phosphatase SHP-1 and sensitizes tumor cells to chemotherapeutic agents. J Biol Chem. 2010; 285:33520–33528.

Kannappan, R., Gupta, S. C., Kim, J. H., & Aggarwal, B. B. (2012). Tocotrienols fight cancer by targeting multiple cell signaling pathways. *Genes & Nutrition*, *7*(1), 43–52

Kline K, Yu W, Sanders BG (2004) Vitamin E and breast cancer. The Journal of nutrition 134: 3458s-3462s

Kumar B, Koul S, Khandrika L, Meacham RB, Koul HK. Oxidative stress is inherent in prostate cancer cells and is required for aggressive phenotype. *Cancer Research*. 2008;68(6):1777–1785.

Lencioni R, Chen XP, Dagher L, Venook AP. Treatment of intermediate/advanced hepatocellular carcinoma in the clinic: How can outcomes be improved? *Oncologist*. 2010;15(suppl 4):42–52.

Manov, I., Bashenko, Y., Eliaz-wolkowicz, A., Mizrahi, M., Liran, O., & Iancu, T. C. (2007). High-Dose Acetaminophen Inhibits the Lethal Effect of Doxorubicin in HepG2 Cells : The Role of P-glycoprotein and Mitogen-Activated Protein Kinase p44 / 42 Pathway. *The Journal of Pharmacology and Experimental Therapeutics*, *322*(3), 1013–1022. https://doi.org/10.1124/jpet.107.121772.)

McIntyre BS, Briski KP, Gapor A, Sylvester PW (2000a) Antiproliferative and apoptotic effects of tocopherols and tocotrienols on preneoplastic and neoplastic mouse mammary epithelial cells. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY) 224: 292-301

McIntyre BS, Briski KP, Tirmenstein MA, Fariss MW, Gapor A, Sylvester PW (2000b) Antiproliferative and apoptotic effects of tocopherols and tocotrienols on normal mouse mammary epithelial cells. Lipids 35: 171-180

Meydani M. Vitamin E. Lancet. 1995; 345:170–175. doi: 10.1016/S0140-6736(95)90172-8

Murray C, Lopez A. The global burden of disease. Geneva: World Health Organization; 1996.

N.S. Ozek, Y. Sara, R. Onur, F. Severcan, Low dose simvastatin induces compositional, structural and dynamic changes in rat skeletal extensor digitorum longus muscle tissue, Biosci. Rep. 30 (2010) 41–50.

Nesaretnam, K., & Meganathan, P. (2011). Tocotrienols: Inflammation and cancer. *Annals of the New York Academy of Sciences*, *1229*(1), 18–22

Neuzil J, Kagedal K, Andera L, Weber C, Brunk UT (2002b) Vitamin E analogs: a new class of multiple action agents with anti-neoplastic and anti-atherogenic activity. Apoptosis: an international journal on programmed cell death 7(2):179-187

Neuzil J, Weber T, Schroder A, Lu M, Ostermann G, Gellert N, Mayne GC, Olejnicka B, Negre-Salvayre A, Sticha M, Coffey RJ, Weber C (2001a) Induction of cancer cell apoptosis by alpha-tocopheryl succinate: molecular pathways and structural requirements. FASEB journal: official publication of the Federation of American Societies for Experimental Biology 15: 403-415

Neuzil J, Weber T, Terman A, Weber C, Brunk UT (2001b) Vitamin E analogues as inducers of apoptosis: implications for their potential antineoplastic role. Redox report: communications in free radical research 6: 143-151

Niki, E. (2008). Lipid peroxidation products as oxidative stress biomarkers. *BioFactors*. https://doi.org/10.1002/biof.5520340208

Odunsi, K., Wollman, R. M., Ambrosone, C. B., Hutson, A., McCann, S. E., Tammela, J., … Alderfer, J. L. (2005). Detection of epithelial ovarian cancer using 1H-NMR-based metabonomics. *International Journal of Cancer*, *113*(5), 782–788. https://doi.org/10.1002/ijc.20651

Placer, Z. A., Cushman, L. L., & Johnson, B. C. (1966). Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. *Analytical Biochemistry*, *16*(2), 359–364. https://doi.org/10.1016/0003-2697(66)90167-9

Rajendran, P., Li, F., Manu, K. A., Shanmugam, M. K., Loo, S. Y., Kumar, A. P., & Sethi, G. (2011). γ-Tocotrienol is a novel inhibitor of constitutive and inducible STAT3 signalling pathway in human hepatocellular carcinoma: Potential role as an antiproliferative, pro-apoptotic and chemosensitizing agent. *British Journal of Pharmacology*, *163*(2), 283–298. https://doi.org/10.1111/j.1476-5381.2010.01187.x

Rodriguez, L. G., Wu, X., & Guan, J.-L. (n.d.). Wound-Healing Assay. In *Cell Migration* (pp. 023–030). New Jersey: Humana Press. https://doi.org/10.1385/1-59259-860-9:023

S. Garip, F. Bozoglu and F. Severcan, Differentiation of Mesophilic and Thermophilic Bacteria with Fourier Transform Infrared SpectroscopyAppl. Spectrosc., 2007, 61, 186–192

S.K. Jain, S.N. Levine, Elevated lipid peroxidation and vitamin E– quinone levels in heart ventricles of streptozotocin-treated diabetic rats, Free Radic. Biol. Med. 18 (1995) 337–341

Sabliov CM, Astete CE, Spivak D. Vitamin E conjugates, and their uses as antioxidants and prodrug delivery vehicles. US 20130210702 A1

Sakai M, Okabe M, Tachibana H, Yamada K (2006) Apoptosis induction by gamma-tocotrienol in human hepatoma Hep3B cells. J Nutr Biochem 17:672–676

Sakai M, Okabe M, Yamasaki M, Tachibana H, Yamada K (2004) Induction of apoptosis by tocotrienol in rat hepatoma dRLh-84 cells. Anticancer Res 24:1683–1688

Seigal, I., Lin, T.L., Yaghoubzadch, E., Kaskey, T.S., Greicher, N. Cytotoxic effects of free fatty acids on ascites tumour cells. *J Natl Cancer Inst*. 1987; 78:271–277

Severcan F, Bozkurt O, Gurbanov R, Gorgulu G (2010) FT-IR spectroscopy in diagnosis of diabetes in rat animal model. Journal of biophotonics 3: 621-631

Severcan F, Haris PI (2012) Vibrational Spectroscopy in Diagnosis and Screening-Advances in Biomedical Spectroscopy. Severcan F, Haris PI (eds), 1st edn, pp 386–418. Amsterdam, The Netherlands: IOS Press

Thorn, Caroline; Oshiro, Connie; Marsh, Sharon; Hernandez-Boussard, Tina; McLeod, Howard; Klein, Teri; Altman, R. (2012). Doxorubicin pathways: pharmacodynamics and adverse effects. *Pharmacogenet Genomics*, *21*(7), 440–446

Weber T, Lu M, Andera L, Lahm H, Gellert N, Fariss MW, Korinek V, Sattler W, Ucker DS, Terman A, Schroder A, Erl W, Brunk UT, Coffey RJ, Weber C, Neuzil J (2002) Vitamin E succinate is a potent novel antineoplastic agent with high selectivity and cooperativity with tumor necrosis factor-related apoptosis-inducing ligand (Apo2 ligand) in vivo. Clinical cancer research: an official journal of the American Association for Cancer Research 8: 863-869

Yang, L., Liu, X., Lu, Z., Yuet-Wa Chan, J., Zhou, L. L., Fung, K. P., … Wu, S. (2010). Ursolic acid induces doxorubicin-resistant HepG2 cell death via the release of apoptosis-inducing factor. *Cancer Letters*, *298*(1), 128–138. https://doi.org/10.1016/j.canlet.2010.06.010

Yap, W. N., Chang, P. N., Han, H. Y., Lee, D. T. W., Ling, M. T., Wong, Y. C., & Yap, Y. L. (2008). Gamma-tocotrienol suppresses prostate cancer cell proliferation and invasion through multiple-signalling pathways. *British Journal of Cancer*, *99*(11), 1832–41. https://doi.org/10.1038/sj.bjc.6604763

Zhang S, Lawson KA, Simmons-Menchaca M, Sun L, Sanders BG, Kline K (2004) Vitamin E analog alpha-TEA and celecoxib alone and together reduce human MDA-MB-435-FL-GFP breast cancer burden and metastasis in nude mice. Breast cancer research and treatment 87: 111-121

Zu K, Ip C (2003) Synergy between selenium and vitamin E in apoptosis induction is associated with activation of distinctive initiator caspases in human prostate cancer cells. Cancer research 63: 6988-6995