T.C.
YEDİTEPE UNIVERSITY
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DEPARTMENT OF PHARMACEUTICAL TOXICOLOGY

COMPARATIVE EVALUATION OF *IN VITRO* BIOLOGICAL ACTIVITIES OF CHEMICALLY CHARACTERIZED *TILIA* SPECIES: PROTECTIVE EFFECTS AGAINST INFLAMMATION AND CYTOTOXIC EFFECTS ON PANCREATIC CANCER CELLS WITH 2D AND 3D SPHEROID MODELS

DOCTOR OF PHILOSOPHY THESIS

Gamze YÜKSEL, Pharm.

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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated ..... and numbered .....

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DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

Gamze Yüksel

DEDICATION

This thesis is dedicated to my beloved family

with deepest love and thanks.

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LIST OF SYMBOLS and ABBREVIATIONS

2D: Two-Dimensional

3D: Three-Dimensional

AP-1: Activator Protein-1

cAMP: Cyclic Adenine Monophosphate

cGMP: Cyclic Guanosine Monophosphate

CNS: Central Nervous System

COX-2: Cyclooxygenase-2

CRP: C-Reactive Protein

CUPRAC: Cupric Reducing Antioxidant Capacity

DAD: Diode Array Detector

DMEM: Dulbecco’s Modified Eagle's Medium

DNA: Deoxyribonucleic Acid

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EC_{50}: Median Effective Concentration

eNOS: Endothelial Nitric Oxide Synthase

FRAP: Ferric Ion Reducing Antioxidant Power

GABA: Gamma Aminobutyric Acid

GAE: Gallic Acid Equivalent

GSH: Glutathione

HPLC-DAD: High-Performance Liquid Chromatography-Diode Array Detector

HPLC-UV: High-Performance Liquid Chromatography-Ultraviolet

HPLC: High-Performance Liquid Chromatography

HPTLC: High-Performance Thin Layer Chromatography

IC_{50}: The Half Maximal Inhibitory Concentration

IFN: Interferon

IL: Interleukin

iNOS: Inducible Nitric Oxide Synthase

JAK: Janus Kinase

LOD: Limit of Detection

LOQ: Limit of Quantification

LPS: Lipopolysaccharide

MAPK: Mitogen-Activated Protein Kinase

MeOH: Methanol

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NF-κB: Nuclear Factor Kappa-B

nNOS: Neuronal Nitric Oxide Synthase

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

Nrf-2: Nuclear Factor Like 2

NSAID: Non-Steroidal Anti-inflammatory Drugs

PCA: Protocatechuic Acid

PEG 400: Polyethylene Glycol 400

$PGE_{2:}$ Prostaglandin E_2

PRR: Pattern Recognition Receptors

QE: Quercetin Equivalent

$R_{F:}$ Retention Factor

$t_{R:}$ Retention Time

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

RSD: Relative Standard Deviation

SOD: Superoxide Dismutase

STAT: Signal Transducer and Activator of Transcription

TCB: *Tilia cordata* bracts

TCF: *Tilia cordata* flower

TCI: *Tilia cordata* inflorescences

TEAC: Trolox Equivalent Antioxidant Capacity

TFC: Total Flavonoid Content

TGF: Transforming Growth Factor

TLC: Thin-Layer Chromatography

TLR: Toll-Like Receptor

TNF-α: Tumor Necrosis Factor-α

TPB: *Tilia platyphyllos* Bracts

TPC: Total Phenolic Content

TPF: *Tilia platyphyllos* Flowers

TPI: *Tilia platyphyllos* Inflorescences

TPTZ: 2,4,6-Tris(2-Pyridyl)-s-Triazine

TRB: *Tilia rubra* Bracts

TRF: *Tilia rubra* Flowers

TRI: *Tilia rubra* Inflorescences

TTB: *Tilia tomentosa* Bracts

TTF: *Tilia tomentosa* Flowers

TTI: *Tilia tomentosa* Inflorescences

ABSTRACT

Yüksel G, (2022), Comparative Evaluation of *in vitro* Biological Activities of Chemically Characterized *Tilia* Species: Protective Effects Against Inflammation and Cytotoxic Effects on Pancreatic Cancer Cells with 2D and 3D Spheroid Models Yeditepe University, Institute of Health Sciences, Department of Pharmaceutical Toxicology, Ph.D. Thesis, İstanbul.

Cancer is the second leading cause of death worldwide. The most common methods for cancer treatment are radiation therapy and chemotherapy; however, these methods adversely affect the healthy cells. Medicinal plants could be safer alternatives with rich secondary metabolites having anti-inflammatory and anticancer properties for decreasing the risk of oxidative stress-related diseases including inflammatory conditions and cancer. This study aims to comparatively assess the hydroalcoholic extracts of chemically characterized *Tilia* species in terms of their antioxidant activities, protective effects against inflammation, and anticancer potentials. For the antioxidant activities DPPH, CUPRAC, and FRAP assays were performed. The protective role against inflammation was evaluated by *in vitro* nitrite assay in LPS-induced RAW 264.7 murine macrophage cells and IL-6 releasing and PGE_2 production inhibition assays. Anticancer activities of the extracts were assessed with a 2D cytotoxicity assay and 3D spheroid formation/growth assay towards MIA PaCa-2 pancreatic cancer cell line. The highest phenolic and flavonoid contents were determined in *T. rubra* flowers and bracts which showed the highest antioxidant activities in FRAP, CUPRAC and DPPH assays. All extracts inhibited LPS-induced nitrite production by more than 50 % at 0.125–1 mg/mL concentrations range. *T. tomentosa* bracts exerted the best anti-inflammatory activity with 90% NO inhibition. In addition, all *Tilia* species exerted potent reducing capacity on IL-6 releasing and PGE_2 production. Bracts, flowers, and inflorescences extracts of *T. rubra* exerted the highest cytotoxic activity with IC_{50} values of 0.16 ± 0.01, 0.21 ± 0.01, and 0.24 ± 0.05 mg/mL, respectively in 2D cytotoxicity assay. Similarly, bracts of *T. rubra* showed the strongest spheroid size change with 50 % reduction with the 3D spheroid assay. In conclusion, our results suggest that all investigated *Tilia* species showed potent protective activity against inflammation and especially *T. rubra* flowers and bracts have shown promising anticancer activity against MIA PaCa-3 pancreatic cancer cell line.

Keywords: Antioxidant activity, anti-inflammatory activity, anticancer activity, *Tilia* species, 3D spheroid model

ÖZET

Yüksel G, (2022). Kimyasal Olarak Karakterize *Tilia* Türlerinin *in vitro* Biyolojik Aktivitelerinin Karşılaştırmalı Değerlendirilmesi: Enflamasyona Karşı Koruyucu Etki ve Pankreatik Kanser Hücre Hattında 2D ve 3D Sferoid Modeliyle Sitotoksik Etki. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmasötik Toksikoloji ABD, Doktora Tezi, İstanbul.

Kanser, dünyada ikinci en sık ölüm nedenidir. Kanser tedavisinde en sık kullanılan yöntemlerden radyoterapi ve kemoterapi sağlıklı hücreleri de olumsuz etkiler. Tıbbi bitkiler zengin biyoaktif sekonder metabolitleri ile kanser ve enflamatuvar hastalıklar dahil olmak üzere ROT ile ilgili hastalıkların oluşum riskini azaltmak için daha güvenli alternatiflerdir. Bu çalışmanın amacı, kimyasal olarak karakterize edilen farklı *Tilia* türlerinin hidroalkolik ekstrelerinin antioksidan aktiviteleri, enflamasyona karşı koruyucu etkileri ve antikanser potansiyelleri açısından karşılaştırmalı olarak değerlendirmektir. Antioksidan aktiviteler için DPPH, CUPRAC ve FRAP deneyleri yapıldı. Enflamasyona karşı koruyucu etki, LPS ile indüklenen RAW 264.7 mürin makrofaj hücrelerinde *in vitro* nitrit düzeyi belirlenmesi ve IL-6 salıverme ve PGE_2 üretimi inhibisyonu deneyi ile gerçekleştirildi. Ekstrelerin antikanser aktiviteleri, 2D sitotoksisite testi ve 3D sferoid oluşumu/büyüme testi ile MIA PaCa-2 pankreas kanseri hücre hattında değerlendirildi. En yüksek fenolik içeriğe sahip *T. rubra* çiçek ekstresi FRAP ve CUPRAC testlerinde en yüksek antioksidan kapasiteyi gösterdi. En yüksek flavonoit içeriğine sahip olan *T. rubra* brakte ekstresi DPPH testinde en yüksek antioksidan aktiviteyi gösterdi. Tüm ekstreler, 0.125–1 mg/mL konsantrasyon aralığında LPS ile indüklenen nitrit üretimini %50'den fazla inhibe etti. *T. tomentosa* brakte ekstresinde en yüksek NO inhibisyonu tespit edildi. Ek olarak, tüm ekstreler, IL-6 salımı ve PGE_2 üretimi üzerinde güçlü inhibitör etki gösterdi. *T. rubra* türünde brakte, çiçek ve çiçek durumu ekstreleri, 2D sitotoksisite deneyi ile sırasıyla 0.16 ± 0.01, 0.21 ± 0.01 ve 0.24 ± 0.05 mg/mL IC_{50} değerleri ile en yüksek sitotoksik aktiviteyi gösterdi. Benzer şekilde, *T. rubra* brakteleri, 3D sferoid deneyi ile %50 azalma ile en güçlü sferoid boyut değişikliğini gösterdi. Sonuç olarak, araştırılan *Tilia* türlerinin enflamasyona karşı güçlü koruyucu aktivite gösterdiğini ve özellikle *T. rubra* çiçek ve braktelerinin umut verici antikanser aktivite gösterdiğini ortaya koymaktadır.

Anahtar kelimeler: Antioksidan aktivite, anti-enflamatuvar aktivite, antikanser aktivite, *Tilia* türleri, 3D sferoid modeli

1. INTRODUCTION and PURPOSE

Oxidative stress is considered one of the main causes of many inflammatory diseases such as metabolic and cardiovascular disorders, neurogenerative illnesses, and cancer (1–3). Medicinal plants are rich in phenolic compounds with antioxidant capacities, which neutralize free radicals and protect cells from oxidative stress. Dietary intake of antioxidants has been clearly linked with improved health and strongly suggested for protection against the development of many oxidative stress-related diseases including inflammatory diseases and cancer and low risk of disease, additionally preventing oxidative damage to key biomolecules that may affect cell function. This protective role is mainly attributed to their phytochemical content and may be associated with the antioxidant capacities of phytochemicals (1–4).

Cancer is the second leading cause of death worldwide. It is causing about 10 million deaths in 2020 worldwide, or one in six deaths (5). Behavioral and dietary risk factors such as insufficient physical activity, elevated body mass index, alcohol, and tobacco use, and consuming low fruit and vegetable and factors such as ethnicity, lifestyle, and diet differences have a significant impact on the expression and progression of this disease. In recent years, due to their potent antioxidant properties and their importance in preventing oxidative stress-related diseases such as neurogenerative diseases, cardiovascular diseases, and cancer, dietary polyphenols have attracted attention. Additionally, dietary polyphenols have been shown to modulate the activity of various enzymes and cell receptors. High mortality and incidence make this disease important public health and economic issue. The most common methods for cancer treatment are radiation therapy and chemotherapy; however, these methods adversely affect the healthy cells and cause various side effects. Natural products provide an excellent source for bioactive secondary metabolites against malignant disease. compounds are suitable for the synthesis of safer anticancer drugs due to their diverse chemical compositions including secondary metabolites. Therefore, efforts are being made to search for effective naturally occurring compounds as anticancer drugs (6). Furthermore, several studies have reported the link between inflammatory diseases and oxidative stress. Although nonsteroidal anti-inflammatory drugs (NSAIDs) are effectively used against inflammatory diseases, their side effects such as gastro-toxicity necessitate for safer alternatives (7).

Plants have been used as important sources of drugs since ancient times. According to WHO even today, nearly 80 percent of people use herbal remedies (8). Numerous phenolic compounds found in the human diet have been analyzed for their potential benefit in cancer prevention. A diet rich in fruits and vegetables has been shown to reduce the risk of cancer formation. Phenolic compounds are one of the most important groups of secondary plant metabolites and with their safety and low toxicity have gained general acceptance. In recent years, they have attracted more attention for their potential in the prevention and treatment of oxidative stress-related diseases by inhibiting oxidative damage to proteins, DNA, and lipids which may cause mutation, apoptosis, and carcinogenesis (9,10). The identification of chemopreventive activities of herbal remedies has become an important research issue (11).

Among herbal remedies, one of the most known and widely used ones are the species *Tilia* (Tiliaceace) which have been commonly used for therapeutic purposes for centuries. *Tilia* has prominent significance in phytotherapy and widespread use in Turkish folk medicine as well. *Tilia* flowers and inflorescences (flowers and bracts mixture) are consumed as herbal teas traditionally, such as diaphoretic, diuretic, expectorant, and sedative agents due to its wide range of bioactive compounds (12). The therapeutic potential of the species has been associated with phenolic content, especially flavonoids. The inflorescences of *T. platyphyllos* Scop., *T. cordata* Miller. and a hybrid *T. vulgaris* Heyne. are listed as officinal species in European Pharmacopoeia. However, *T. rubra* and *T. tomentosa* are also utilized in Turkish folk medicine for similar purposes (8,13,14).

Several studies established antioxidant, anti-inflammatory and anticancer activities of different *Tilia* species (15,16). However, a comparative evaluation of different *Tilia* species from Flora of Turkey regarding their antioxidant capacities, and their protective effect against inflammation and anticancer activities have not been studied. Additionally, anticancer potencies have not been investigated with a three-dimensional (3D) spheroid formation/growth assay. 3D spheroid formation/growth assays have become an important biological tool in recent years, for research of tumor cells, that uses methods and materials more similar to the *in vivo* microenvironment of tumor cells including cell-to-cell and cell-to-extracellular matrix interactions. It is

considered a bridge between animal studies and two-dimensional (2D) monolayer cell cultures (17).

The present work aims to investigate chemically characterized hydroalcoholic extracts prepared from different parts of *T. cordata*, *T. tomentosa*, *T. rubra,* and *T. platyphyllos* in terms of their antioxidant activities (DPPH, FRAP, and CUPRAC), their protective effects against inflammation (nitric oxide determination and IL-6 releasing inhibition and PGE_2 production inhibition) and to demonstrate their anticancer activities with 2D cell cytotoxicity assay and 3D spheroid formation/growth assays.

2. LITERATURE REVIEW

2.1. Oxidative Stress, Inflammation and Cancer

Oxidative stress may directly cause damage to cellular biomolecules and indirectly cause damage by activating several transcription factors that may induce the expression of genes including pro-inflammatory cytokines, growth factors, chemokines, and cell cycle regulatory molecules. In this sustained oxidative/inflammatory environment, direct damage to cellular biomolecules and activation of inflammatory pathways may lead a normal cell transformed into a tumor cell (1).

2.1.1. Oxidative Stress

Cells can generate free radicals also called oxidants; reactive oxygen species (ROS) and reactive nitrogen species (RNS) from both endogenous and exogenous sources. When oxygen molecule is used to generate energy as ATP (adenosine triphosphate) consequently it produces free radicals. These byproducts generally resulted from the cellular redox process (18). ROS and RNS are generated from endogenous and exogenous sources. Endogenously they are generated as a result of mitochondrial oxidative metabolism such as superoxide anion (O_2^-) in which molecular oxygen (O_2) acts as the final electron acceptor. Oxidant molecules can be produced also in inflammation, immune cell activation, infection, ischemia, mental stress, and aging. The molecular oxygen is reduced at the level of the respiratory chain, which is located in the membranes of mitochondria. Although mitochondria are the major site of ROS production, fatty acid metabolism and enzymatic processes including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, nitric oxide synthase, and cytochrome P450 reductase are other endogenous sources. In hypoxic conditions, during respiratory chain reaction, nitric oxide may also be produced and further leads to toxic carbonyl products such as malondialdehyde. Exogenous sources of free radicals include water and air pollution, alcohol, heavy or transition metals (Cd, As, Hg, Pb), cigarette smoke, an industrial solvent, and certain drugs such as gentamycin, cyclosporine, and radiation (1,19,20).

High concentrations of free radicals generate oxidative stress and cause a deleterious process that damages cell membranes and other important biomolecules such as protein, lipids, or DNA. Degenerative and chronic diseases like cancer, cardiovascular

and degenerative diseases and autoimmune diseases are all significantly influenced by oxidative stress (21). When the balance between oxidant molecules and antioxidant defense systems is impaired oxidative stress occurs within the cell. In order to maintain this fine balance our bodies have several antioxidant defense mechanisms either naturally produced endogenous enzymes such as superoxide dismutase, glutathione S-transferase, catalase, and non-enzymatically acting endogenous antioxidants such as glutathione, N-acetyl cysteine, metal binding proteins including ferritin, transferrin and externally supplied dietary antioxidants such as beta carotene, vitamin C and vitamin E. Both endogenous and exogenous antioxidants act as free radical scavengers and balance ROS generation, by detoxification and preventing their accumulation in the body. Therefore they can improve the immune defense and lower the risk of oxidative and nitrosative stress-mediated degenerative diseases and cancer (18,22).

As a consequence of cellular metabolism, reactive oxygen species (ROS) including free radicals such as hydroxyl radicals (OH•) and superoxide ions ($O_2^{\bullet -}$) and non-radicals such as hydrogen peroxide (H_2O_2) produced by living organisms. The main endogenous sources of oxidant molecules are normal aerobic respiration, peroxisomes, and stimulated polymorphonuclear macrophages and leukocytes. Exogenous sources of oxidant molecules include ionizing radiation, pesticides, organic solvents, certain pollutants, and tobacco smoking. ROS take part in normal cell processes at low to moderate concentrations; however, at high concentrations, they produce some undesired modifications to cell components such as lipids, proteins, and DNA. Antioxidants such as warriors have an important role in protecting against these harmful modifications and damages. Oxidative stress is a term used to describe an imbalance between oxidants and antioxidants, a shift in favor of oxidants. Oxidative stress contributes to various pathologic circumstances such as atherosclerosis, neurogenerative diseases, cancer, hypertension, acquired immunodeficiency syndrome, ischemia, and diabetes. All aerobic organisms are structured with enzymatic and non-enzymatic antioxidants that defend against oxidative damage and inhibit the negative consequences of ROS. However, in pathological circumstances, this natural defense system may be overwhelmed. Hence, dietary intake of foods rich in antioxidants becomes important. Therefore, it is important to search for the determination of the natural antioxidants (23,24).

2.1.2. Inflammation

The history of inflammation dates back to the ancient Greek and Egyptian cultures. Hippocrates in the 5th century used edema term to describe inflammation. He also considered inflammation as a start for the healing of injured tissue. Aulus Celsus, the first writer, described the main signs of inflammation as rubor (redness occurring due to hyperemia), calor (increased heat due to increased blood flow to the affected tissue, and metabolic activity of inflammation mediators, and dolor (having pain) in the 1st century AD. Galen, who was a physician and surgeon, introduced the fifth sign of inflammation as the loss of function of the affected tissue (25,26).

Inflammation is a host defense mechanism to harmful stimuli such as toxins, traumas, injuries, pathogens, or irradiation. During infection or injury, inflammation starts as an essential immune response to maintain tissue homeostasis. It is a defense mechanism to eliminate pathogens or irritants and to boost tissue repairing. Inflammation can be divided into two major types: acute inflammation and chronic inflammation. During acute inflammation, the cellular and molecular events usually minimize infection or injury efficiently. This process presents to maintain and restore tissue homeostasis and resolve acute inflammation. However, if the inflammation cannot be controlled, it can become chronic inflammation and can cause many chronic inflammatory diseases that persist for years such as rheumatoid arthritis, type 2 diabetes, neurogenerative and cardiovascular diseases, and cancer. These diseases are identified by a lengthened duration of active inflammation leading to tissue destruction (27–29).

Acute inflammation is initiated by specialized immune cells present in the tissue. These cells include mast cells, macrophages, and non-immune cells. These cells recognize pathogens or injuries via pattern recognition receptors (PRRs). PRRs are surface receptors that can recognize molecules found in pathogens, and they can also identify the molecules that are exposed when tissue is damaged. When PRRs are activated with a molecule from a pathogen or exposed to tissue injury, the specialized immune cells start to action such as releasing of mediator which can attract additional immune cells to migrate to the injury area and further stimulate the acute inflammatory response (30).

A chemical signaling cascade begins to mend injured tissues as soon as there is tissue damage. Leukocyte chemotaxis from the bloodstream to the injury site is triggered

by this chemical signaling. At the site of injury, activated leukocytes release cytokines that trigger inflammatory responses. The inflammatory response is the coordinated activation of numerous signaling pathways that modulate the levels of inflammatory mediators in both local tissue and inflammatory cells drawn from the bloodstream. This response can be summarized as follows: 1) Damaging stimuli detected by pattern-recognition receptors; 2) inflammatory pathways activated 3) inflammatory markers released; 4) inflammatory cells recruited to the site of damage. PRRs can recognize both pathogens known as pathogen-associated molecular patterns, PAMPs, and endogenous signals activated by tissue-damaging known as danger-associated molecular patterns, DAMPs. PRR family includes Toll-like receptors (TLRs), retinoic acid-inducible gene, retinoic acid-inducible gene like receptor, C-type lectin receptors, and NOD-like receptors. TLRs are the most extensive research family of PRRs that plays a role in the activation of the inflammatory response in mammalians (27–29).

2.1.2.1. Inflammatory Mediators

The inflammatory response is actively influenced by and can be adjusted by a number of chemical mediators from the circulatory system and inflammatory cells. The chemical mediators that are released include peptides such as bradykinin, vasoactive amines such as histamine and serotonin, and eicosanoids such as thromboxanes, leukotrienes, and prostaglandins. The mediators released by specialized immune cells mainly belong to the cytokine family. These are important protein groups that provide cell signaling. Besides the cytokine family, some small molecules act as mediators such as nitric oxide. The acute inflammatory response provides clearing the foreign agent or damaged cells quickly, allowing tissue for repair and resolution of the inflammation (30).

Pathogens, toxic compounds, and cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β and interleukin-6 (IL-6) interact with the TLRs, TNF receptor, IL-1 receptor and IL-6 receptor. Intracellular signaling pathways such as nuclear factor kappa-B (NF-κB), mitogen-activated protein kinase (MAPK), and Janus kinase (JAK) signal transducers and activators of transcription (STAT) pathways are triggered by these receptor activations. These pathways contribute to the inflammatory response by regulating pro-inflammatory cytokines and recruitment to the site of injury. Damaged epithelial and endothelial cells release factors in addition to chemokines and growth factors at the site of tissue injury that triggers inflammatory cascade. Inflammatory cells

including activated macrophages and monocytes induce the production of inflammatory cytokines such as IL-1β, IL-6, TNF-α, and inflammatory proteins and enzymes as a response to a stimulus. Pro-inflammatory cytokines facilitate inflammation while anti-inflammatory cytokines inhibit inflammation. Inflammatory cytokines, such as ILs, TNFs, TGFs (transforming growth factor) IFNs (interferon), and chemokines are produced for leukocytes recruitment to the site of damage whereas IL-10 and IL-12 act as anti-inflammatory cytokines and function as inhibitory cytokines such as inhibiting functions of activated macrophages and production of nitric oxide (27–29).

2.1.2.1.1. Nitric Oxide

Nitric oxide (NO) is generated by many mammalian cells and can act as a signaling or toxic agent. It was previously known as a toxic atmospheric pollutant since its discovery to be utilized by many cells. It is a short-lived gaseous free radical. The biological effect of this molecule is multifaceted, these effects can be deleterious, regulatory, or protective therefore NO can be described as a double-edged sword. NO is synthesized from an L-arginine molecule and this synthesis is catalyzed by the nitric oxide synthase (NOS) enzyme. Active NOS consists of two calmodulin molecules and two NOS proteins. With NADPH and O_2 as co-substrates, L-arginine is converted to NO and L-citrulline (31,32).

There are three different isoforms of NOS have been characterized. NOS I is neuronal NOS or nNOS is predominantly expressed in the peripheral nervous system and neurons in the brain. NOS III, endothelial NOS or eNOS, is predominantly expressed in endothelial cells. NOS I and NOS III are inactive in resting cells, and they are constitutively expressed. When there is an increase in Ca^{+2} concentration in the cell, the binding of NOS I and NOS III to calmodulin is stabilized and this activates the enzyme to produce NO. For instance, acetylcholine increases Ca^{+2} concentration in endothelial cells and triggers NO production. NO production ceases when the concentration of Ca^{+2} decreases. NO production by NOS I and NOS III is constitutively expressed and is transient and short lasting (31,33). Another isoform of the NOS family is NOS II, which is also called inducible NOS or iNOS. iNOS expression is not found in most resting cells. When there is an exposure to a bacterial product such as lipopolysaccharide or inflammatory stimuli such as proinflammatory cytokines; TNF-α or IL-1 and IFN-γ, the

iNOS gene is expressed in many inflammatory and tissue cells. There is no need for an increase in Ca^{+2} concentration for tight binding of calmodulin and iNOS. iNOS is therefore called as calcium-independent NOS and it can constantly produce NO at high levels for a prolonged time. In different experimental inflammation studies, it has been suggested that the production of large amounts of NO by iNOS is harmful and can contribute to injury (31,33,34).

A high concentration of NO produced by iNOS is quickly oxidized to reactive nitrogen species (RNS), nitrogen dioxide (NO_2), dinitrogen trioxide NO_2O_3, and trinitrogen tetraoxide NO_2O_4 under gaseous conditions. In aqueous systems such as biological systems, the major RNS formed is NO_2O_3. When there is combined oxidative and nitrosative stress and when high concentrations of superoxide anion O_2 and NO are formed, these two radicals form highly reactive oxidant peroxynitrite, ONOO. Peroxynitrite is considered to be responsible for the most toxicity of NO. The reduced form of glutathione, GSH plays an important role as an antioxidant or detoxifying agent against RNS. S-nitrosoglutathione GS-NO is formed by nitrosation of GSH with NO. GS-NO acts as NO and GSH reservoir (35). With the interaction of ROS such as superoxide anion (O_2^-) and NO, RNS are formed. Thus, NO is generally considered as toxic species. However, studies have also shown that NO can diminish oxidative injury in biological systems. ROS such as superoxide and peroxide are the primary sources of oxidative stress, and they are generally produced by Fenton reactions. NO can diminish Fenton-mediated ROS production and oxidative stress by direct scavenging of oxidants and producing less potent oxidants, RNS. NO can also terminate lipid peroxidation by reacting with oxy and peroxy radicals and can be protective against cell membrane compromise (36).

As seen in Figure 1, the constitutively expressed enzymes NOS I and NOS III or the inducible form of NOS, NOS II is responsible for the production of nitric oxide. NOS I and NOS III are activated by a physiological stimulus that triggers Ca^{+2} signal. NO production is rapid and transient. Actions of NO in low concentration are direct and they interact with an iron atom in the heme group of guanylyl cyclase. This interaction causes the activation of the enzyme to produce cGMP. Contrastly, in resting cells NOS II is not expressed and induced by immunological signals such as cytokines (IL-1, TNF-α) and bacterial lipopolysaccharide (LPS). NOS II produces nitric oxide at a sustained high

concentration. This high concentrated NO is unstable thus under aerobic conditions rapidly oxidized and produces reactive nitrogen oxide species. Since then, there have been several publications about the effects of NO on inflammatory responses. NO can regulate the release of various inflammatory mediators from a wide range of inflammatory cells such as leukocytes, macrophages, endothelial cells, platelets, and mast cells. It can also alter the blood flow and activity of many enzymes participating in inflammatory responses. It also modulates the leukocyte adhesion to the vascular endothelium in inflammation (35).

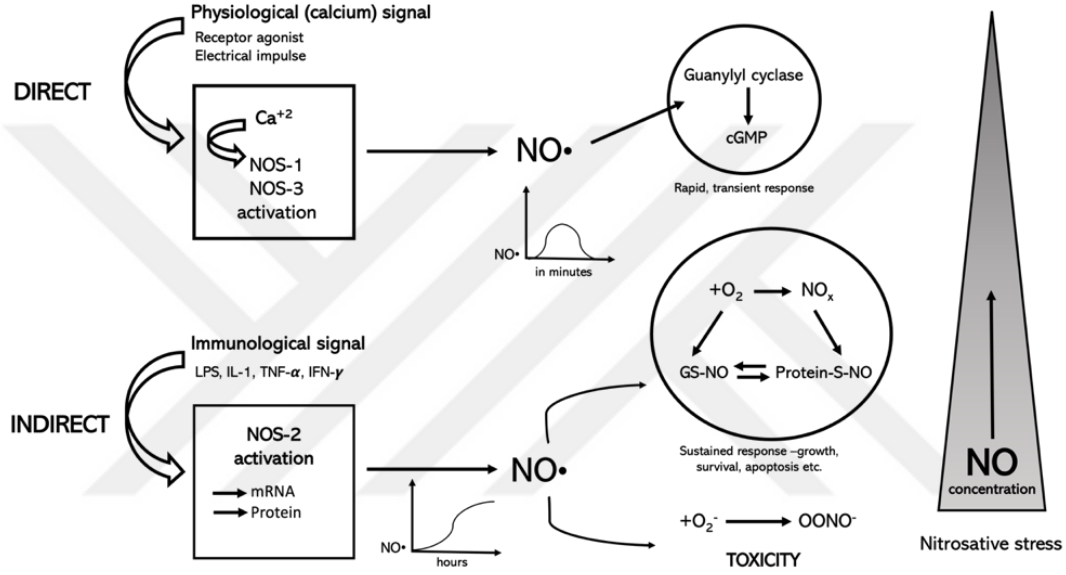


Figure 1. Nitric oxide production and signaling.

Nitroglycerin is a nitrosovasodilator that is widely used against angina. NO was generated from nitroglycerin and mediates vasodilatory effects. Vasodilation is one of the first responses to inflammation. Many inflammatory mediators such as histamine and bradykinin cause vasodilation through stimulation of endothelial NO release. NO can activate soluble guanylyl cyclase by diffusing it into the vascular smooth. This activation leads to an increment of intracellular cGMP levels and relaxation of the smooth muscle (37). NO has been shown to have an influence on various immunomodulatory cytokines production by macrophages. It inhibits the production of macrophage-derived cytokines and can also modulate their actions. For example, a study showed that aspirin administration to rats resulted in TNF-α dependent apoptosis in gastric mucosa cells, while administered NO-releasing aspirin markedly diminish the apoptosis and protected

gastric mucosa cells against damage. In recent years, for a stronger anti-inflammatory effect NO-releasing drugs have been developed. Similarly, in mast cells, so-called alarm cells that alert the immune system in case of the presence of harmful substances or pathogens, NO produced by mast cells diminishes the release of inflammatory mediators. These inflammatory mediators include histamine, platelet-activating factor, leukotrienes, serotonin, heparin, prostaglandins, and TNF. Interestingly, the presence of IL-1 has been shown to up-regulate the rate of NO release from mast cells. Additionally, a hallmark feature of inflammation is the infiltration of leukocytes to the site of tissue injury is also influenced by NO. Also, it has been shown that NO can inhibit the expression of adhesion molecules in neutrophils. NO donors have been shown to suppress the adherence of leukocytes to the vascular endothelium. Also, platelet has a crucial role in inflammatory responses. They can release many proinflammatory mediators such as lipoxins, thromboxane, and serotonin. Platelets have also capable of regulating the angiogenesis process (growth of new blood vessels). NO diminishes the adherence of platelets to vascular endothelium and plays an important role in down-regulating the response against inflammation (37). On the other side, nitric oxide and its derivatives have harmful effects like alteration of DNA, mutation of tumor suppressor genes, induction of lipid peroxidation, depletion of antioxidant stores, and in cancer, it has been shown to cause hypoxia that induces angiogenesis (32).

NO has multifaceted biological effects. Among its protective roles, it can act as an antioxidant, provide inhibition of leukocyte adhesion, and protects against TNF toxicity. NO has also a regulatory role in vascular tone, vascular permeability, and cellular adhesion. Deleterious effects of NO include enzyme function inhibition, susceptibility to metal toxicity, induction of DNA damage, and lipid peroxidation and it can cause depletion of antioxidant stores (32). Nitric oxide has also therapeutic potential for inflammatory diseases. It has been shown that the addition of nitric oxide-releasing moiety to a nonsteroidal anti-inflammatory drug enhanced NSAID's efficacy and potency while decreasing toxicity in experimental models (38).

Nitric oxide (NO) is produced as a by-product during the conversion reaction of l-arginine to l-citrulline by NOS. In response to pathogen infection such as exposing lipopolysaccharide, an inducible form of NOS (iNOS) produces high levels of NO. Expression of iNOS is activated by signaling pathways namely, the nuclear factor kappa-

B (NF-κB), MAPKs that phosphorylates ERK (extracellular-regulated kinase), JNK (NH2-terminal kinase) and p38 transcription factors, and STAT1. iNOS can aggravate inflammatory circumstances thus it is considered a good point for anti-inflammatory activity (39).

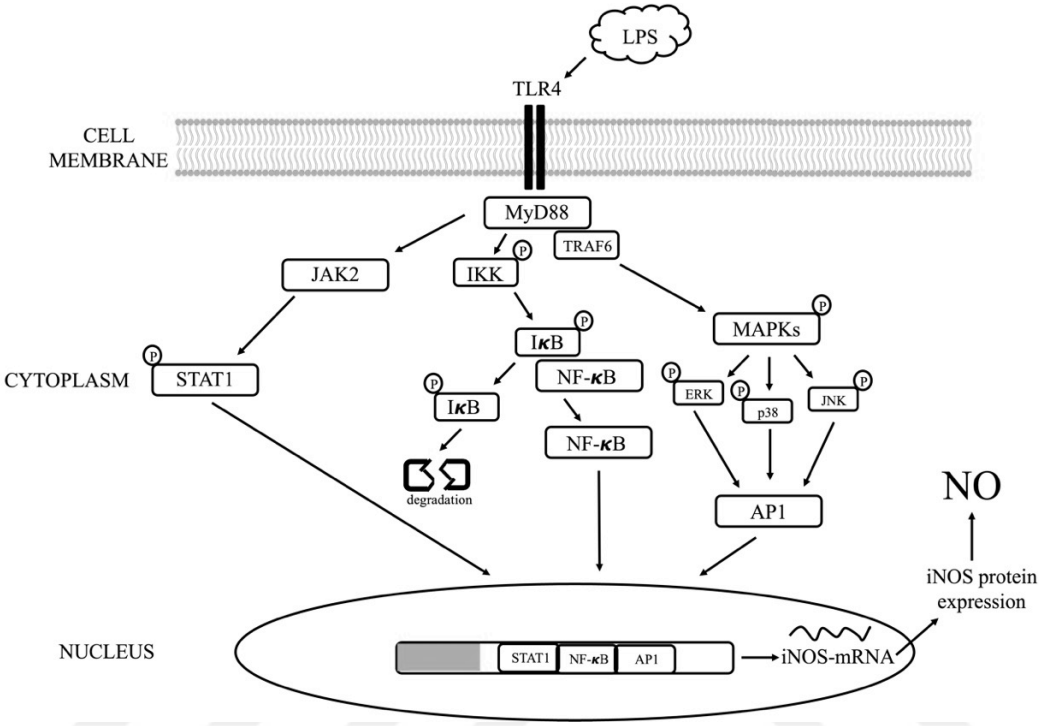


Figure 2. LPS induced NO production

As seen in Figure 2, the iNOS signaling pathway is activated by the binding of toll-like receptor 4 (TLR4) with lipopolysaccharide (LPS). Then this pathway is transduced via the NF-κB as well as STATs, JAK, and MAPKs (ERK, p38, and JNK). Other protein components involved in iNOS signaling shown are the MyD88 gene (myeloid differentiation primary response 88), IκK (IκB kinase), and IκB, AP-1, ERK, and JNK (39).

2.1.2.1.2. Prostaglandin E_2

Prostaglandins (PGs) are bioactive lipids from the prostanoid family, a subfamily of eicosanoids, and are derived from the oxidative conversion of 20-carbon saturated fatty acid arachidonic acid (AA). AA is generally found in membrane phospholipids and their hydrolysis is catalyzed by phospholipases (PL). As shown in Figure 3, when hydrolyzed and freed, AA rapidly cyclized and oxidized by cyclooxygenase (COXs) enzymes also

known as PG endoperoxide synthase to form an unstable product endoperoxide-containing PG (PGG_2) and then reduced to PGH_2. COX family has two main isoforms, one is COX-1 found in the majority of cells, which is constitutively active and responsible for essential functions like protection of gastric mucosa and, the other is COX-2 which is present in certain areas such as kidneys and some regions of CNS. COX-2 production may be induced greatly with response to an inflammatory stimulus or growth factors. In tissues that are not inflamed, the PG levels are extremely low however, as a response to an inflammatory reaction their production amount and nature are significantly altered. Once PGH_2 is formed, it quickly transforms into other prostanoids by related synthases. The main prostaglandins produced in the human body are PGE_2, PGI_2, PGD_2, and $PGF_{2\alpha}$. PGs are widely distributed throughout the body for maintaining homeostasis (40–42). PGs serve as vasodilators at the first stage of the inflammatory response to enable the recruitment of monocytes and neutrophils to the site of inflammation.

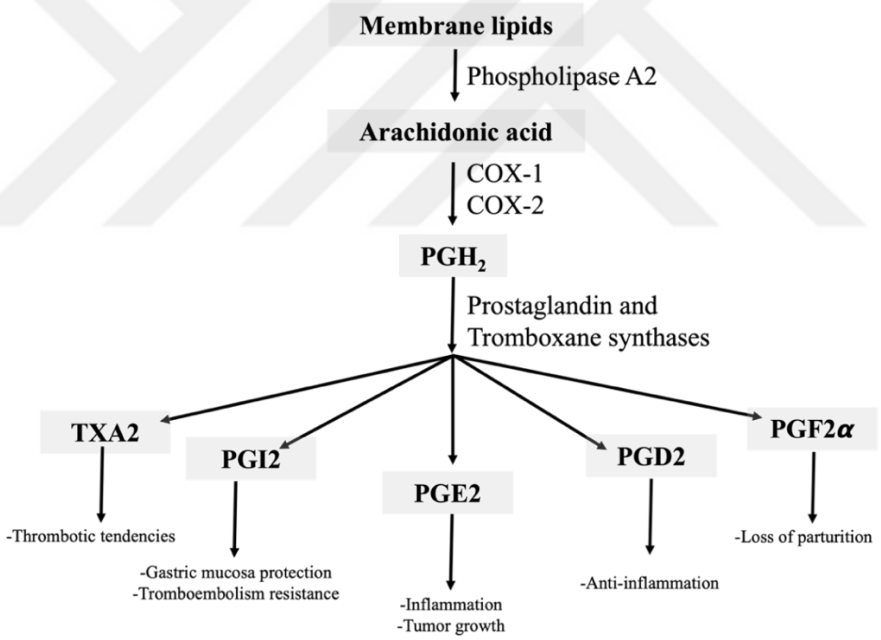


Figure 3. Prostaglandin synthesis and main functions

As one of the most prevalent PGs generated, PGE_2 is well known for its vast range of actions in the body. It is the key facilitator of numerous biological processes including the control of immunological responses. It has been also shown to regulate blood pressure, particularly in overconsuming salt, COX-1 derived plays a role in the integration of gastric mucosa. Also, numerous pathological disorders have been linked to abnormal

PGE_2 production or breakdown. It has special importance in inflammation as it plays role in the development of inflammatory symptoms including redness, swelling, and pain. PGE_2 has vasodilatory functions and increases the permeability of blood vessel walls and its action on peripheral sensory neurons results in pain (41). PGE_2 has multifaceted roles in inflammatory as it may act as pro-inflammatory or anti-inflammatory. For instance, it has been shown to increase oxidative damage caused by LPS, apoptosis of eosinophils, and hyperalgesia in inflammation. As anti-inflammatory actions, it has been shown to inhibit T-cell proliferation and suppress cytokine generation by macrophages (43).

2.1.2.1.3. Interleukin-6

The interleukin-6 is a member of the IL-6 family of cytokines that have pleiotropic activities. Several immune cells, such as macrophages, dendritic cells, mast cells, and B cells can generate IL-6 (29). Non-immune cells including endothelial, fibroblast and epithelial cells may also release IL-6. IL-6 levels are extremely low in a normal environment, but they can increase thousands of times in an inflammatory situation. The rapid and transient production of IL-6, in response to infections and tissue damage, promotes the host defense by enhancing acute phase responses, hematopoiesis, and immunological responses. IL-6 is produced at the site of inflammation and is essential for the acute phase response, which is characterized by the production of acute phase proteins. Additionally, IL-6 and its soluble receptor sIL-6R control the transition from acute to chronic inflammation by leucocyte infiltration alterations from polymorphonuclear neutrophils to macrophages or monocytes. Additionally, IL-6 stimulates T- and B-cells, in chronic inflammation. Even though transcriptional and posttranscriptional mechanisms tightly regulate the expression of IL-6', dysregulated continuous IL-6 production has a pathological impact on chronic inflammation and autoimmunity (44).

In addition to being engaged in the control of the hepatic acute phase response, B-cell stimulation, the balance between regulatory and effector T cells, metabolic regulation, and other brain functions. Some autoimmune inflammatory disorders, such as rheumatoid arthritis, Crohn's disease, and asthma have high levels of IL-6. Additionally, it has been demonstrated that IL-6 influences Type 1 T helper and Type 2 T helper cells (29).

2.1.3. Cancer Development

Cancer is a multistage process that involves initiation, promotion, and progression stages (1). One of the leading causes of death in the world is cancer disease. Cancer has both hereditary and environmental origins, according to its etiology. Most human malignancies (90%) are caused by environmental factors, primarily through lifestyle factors such as tobacco use, nutrition, and UV radiation), with the remainder of cancers being caused by infections and chemical exposure. Smoking (25–30%), a poor diet, obesity (30–35%), infections (15–20%), radiation (10%), and a lack of physical exercise are all lifestyle factors that increase the risk of developing cancer. Cancer is a multi-stage disease that involves uncontrolled cell proliferation and mutations. Numerous studies have been conducted to understand how cancer develops. Based on the molecular, cellular, and pathologic aspects of the transformation of a normal cell into a neoplastic lesion, the multistage processes leading to cancer development involve initiation, promotion, and progression stages (1). The transformation process begins with initiation, which happens when a normal cell experiences a genomic DNA mutation. The interaction of chemical and physical carcinogens with genomic DNA can result in the development of an initiating cell, including UV light and radiation. Genotoxic agents are physical and chemical carcinogens that directly damage DNA and cause mutations. Additionally, initiated cells can also be produced by mutations resulting from incorrect DNA repair. Unrepaired DNA caused by oxidative stress appears to be a primary contributor to spontaneous mutations. After initiated cells develop, exposure to chemicals and physiological endogenous factors can cause the initiated cell to proliferate specifically in clones through a process called tumor promotion. In order to clonally develop a focused lesion of pre-neoplastic cells, tumor promotion entails the selective proliferation of an initiated cell. An epigenetic process called promotion does not involve damaging DNA. The alteration of gene expression that leads to a rise in pre-neoplastic cell populations through cell division and/or a decrease in apoptosis is a defining characteristic of the promotion stage. Some preneoplastic cells undergo further genetic alterations after several cell divisions, which leads to the development of benign and/or malignant neoplasms (45). The multistage of carcinogenesis was summarized in Figure 4.

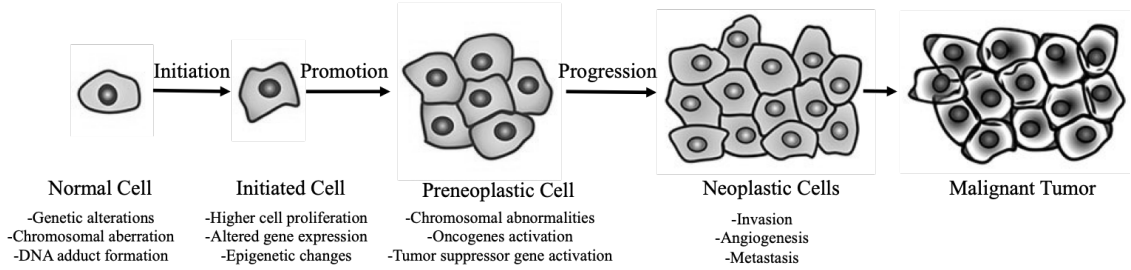


Figure 4. Multistage of cancer

Chemoprevention has multiple intervention strategies including pharmacological drugs to prevent, slow, and reverse cancer formation at different stages, generally in its main three stages of initiation, promotion, and progression. Mutagens and mitogens are associated with more than 90% of malignancies. Thus, to prevent or reverse carcinogenesis, chemoprevention strategies should aim to be concerned with substances that decrease apoptosis and increase proliferation and cell maturation or differentiation. As seen in Figure 5, there are two classes of conventional chemopreventive agents, blocking and suppressing agents. Blocking agents act on the initiation stage of cancer by inhibiting procarcinogen-derived carcinogenesis formation or preventing ultimate electrophilic and carcinogenic substances to react with target molecules such as proteins and DNA. On the other hand, suppressing agents can block the expression of initiated cells in the promotion and progression stages. The understanding of chemoprevention techniques has greatly advanced, particularly concerning signal transduction pathways involved in carcinogenesis (11,46).

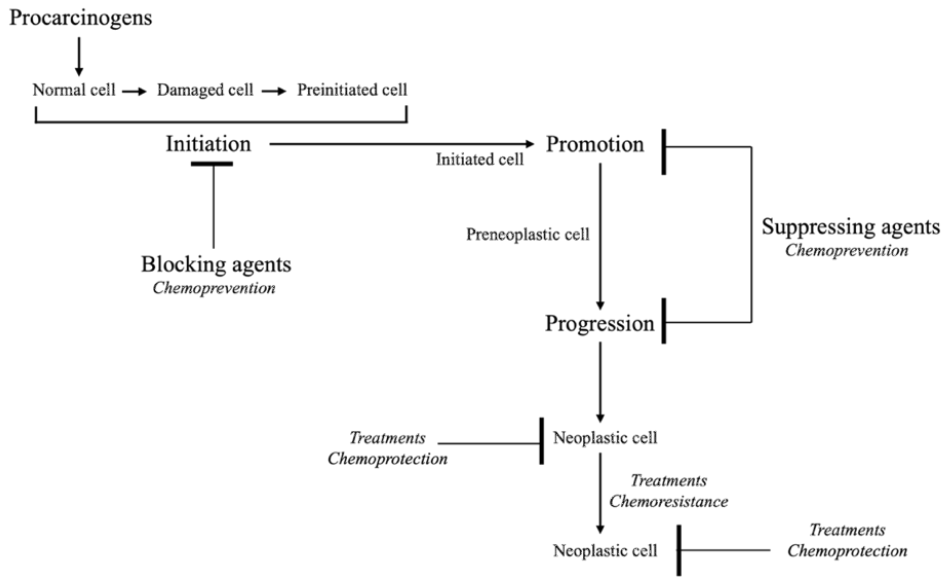


Figure 5. Chemopreventive and chemoprotective actions in cancer disease.

2.1.4. Relationship Between Oxidative Stress, Inflammation and Cancer

Oxidative stress interacts with all multistage processes of cancer and inflammation. ROS may directly damage DNA in initiation stage by introducing structural alterations and gene mutations into the DNA. ROS may block cell-to-cell communication, induce abnormal gene expression and modify secondary messengers in the promotion stage, and this may result in increase in cell proliferation and decrease in apoptosis of the initiated cells. Oxidative stress may also contribute progression of cancer by adding DNA alteration to initiated cells (1). Moreover, as seen in Figure 6 inflammation involves an enhanced ROS generation by immune and inflammatory cells at the site of inflammation as response to pathogens generated by host defence mechanism. As a part of inflammatory response, produced ROS facilitates the clearance of pathogen however when produced for excessive amount and prolonged periods leads to oxidative stress and diseases associated with chronic inflammation. Additionally, ROS may effect redox-sensitive signalling pathways thus may enhance inflammatory response (1).

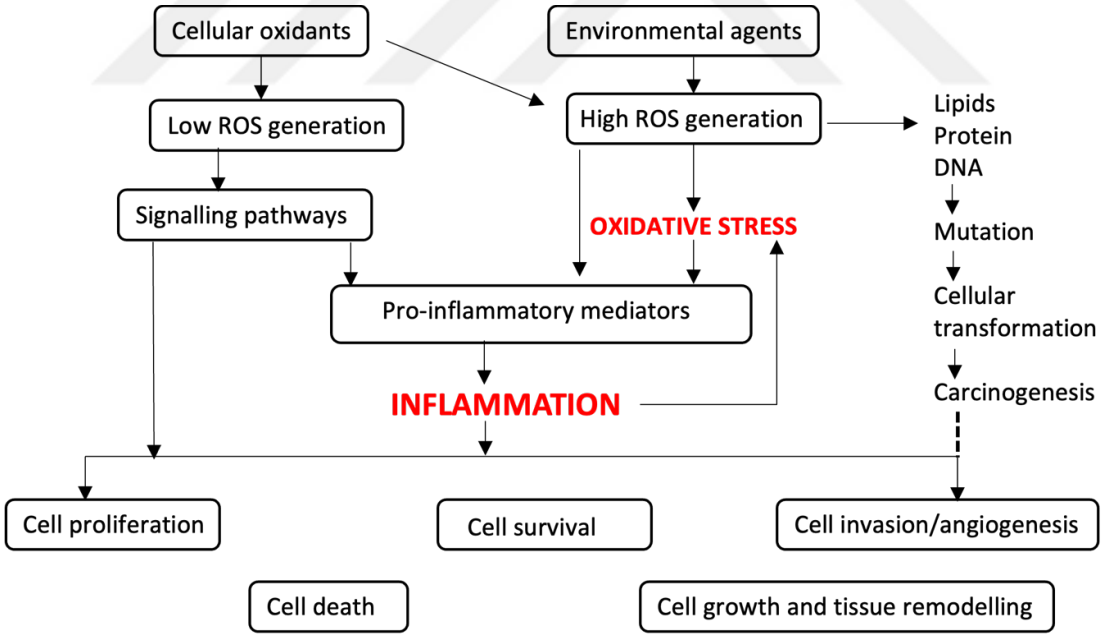


Figure 6. Interrelation between oxidative stress, inflammation and cancer

Redox-sensitive transcription factors including nuclear factor kappa-B, AP-1 and nuclear factor like 2 (Nrf-2) have been observed to be ROS regulated. They are activated by ROS and translocate into nucleus to turn on the expression of specific genes. For

instance, a transcription factor NF-κB when in an inactive form present as bounded to a cytosolic protein such as IκB (nuclear factor of kappa in B-cells inhibitor alpha) in cytoplasm. ROS or oxidant molecules cause modification in this bound like phosphorylation of the bond between NF-κB and IκB by a serine kinase leads to degradation and release NF-κB. Released NF-κB then translocated into nucleus and induce expression of inflammatory proteins (21).

The activation of transcription factors continues with inducing expression of growth factors, cellular adhesion molecules (CAMs), cytokines like (TNF-α and β, (IL-1 to IL-15 and chemokines like TGF-α and β by the altered genes. On the other hand, kinases which are enzymes facilitate phosphorylation such as p38 MAPK, JNK and protein kinase C can be activated by ROS. This activation leads to production of chemokines and pro-inflammatory cytokines which induce transcription factors and recruitment of immune cells such as leukocytes and macrophages. Consequently, cytokines and chemokines bind to their specific receptors epidermal growth factor receptor to generate reactive oxygen species an in turn excessive ROS production during inflammatory response may further aggravate oxidative stress-inflammation cycle. Indeed, it has been shown with many studies that inflammatory response and ROS production is interrelated (21).

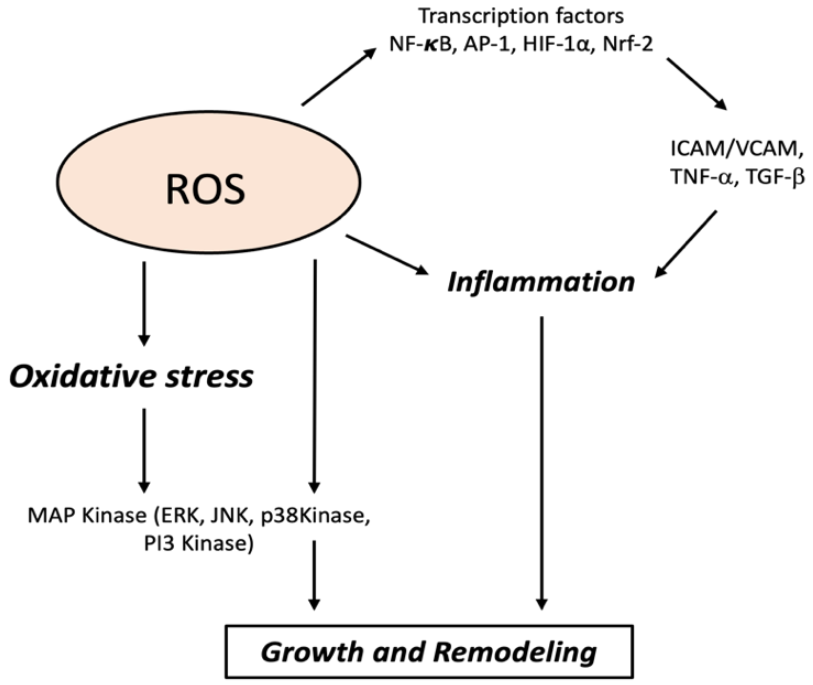


Figure 7. Interrelation between ROS and signalling pathways.

Reactive oxygen species have been reported to activate several transcription factors such as NF-κB, AP-1, Nrf-2 and hypoxia inducible factor- 1α: As seen in Figure 7, these transcription factors induce expression of many genes including pro-inflammatory genes leading to induction of proteins (ICAM/VCAM, interleukins) and transforming of growth factor (TGF-β). Also, the tyrosine kinases (MAPK, PI3K, p38MAPK, ERK and JNK may be modified by reactive oxygen species. They may all lead to cellular growth and remodelling (1).

ROS and NOS produced within cell such as inflammatory and immune cell has two faces. Excessive amounts of ROS and NOS produced create an oxidative stress and have damaging effect to biomolecules, when low levels of produced they initiate biological processes by inducing activation of signalling pathways. ROS and NOS has physiological roles in signal transduction as secondary messengers. ROS and NOS signalling has been linked to cellular growth, proliferation and survival. Phases of the cell cycle are significantly regulated by their impact on the intracellular redox cycle. Targeting of cell cycle regulators such as retinoblastoma, pro-apoptotic kinases such as c-Jun N-terminal kinase and MAPK can greatly influence cell cycle phases. Additionally, important alternation of redox balance may impact on cell survival including cell proliferation and cell death, which are critical in proliferative diseases like cancer. Moreover, accumulation of free radicals has direct toxicity on cellular structures such as membranes, proteins, lipids, ion channels, nucleic acids and enzymes (18,21).

Lipid peroxidation due to free radicals cause cell membrane impairment. In addition to this lipid peroxidation causes toxic carbonyl products or reactive aldehydes formed such as 4-hydroxynonenal and malondialdehyde. These toxic carbonyl products may interfere with genetic material and forms toxic DNA adducts. As a result of cell membrane impairment inflammatory damage and cell death may occur. Oxidation of protein thiols by oxidants may lead to denaturation, aggregation and precipitation. Ion channel damaging may disrupt ion exchange and interfere with important cellular ion homeostasis such as calcium homeostasis. In addition to these DNA sugar backbone and base pairs are susceptible to oxidative and nitrosative damage. The interaction may cause DNA mutations such as DNA-protein or DNA-DNA cross-linking, point mutations, single or double-strand breaks and sister chromatid exchanges, mutations in tumor-suppressor genes and proto-oncogenes, thus promoting neoplastic transformation. The

guanine base present in DNA is the most susceptible region to oxidation. In the presence of ROS, guanosine may oxidize to 8-oxoguanosine, and results double-strand breaks when inserted during DNA replication. Overall, cumulative oxidative and nitrosative damage by ROS and NOS to cellular structures may lead to transformation of normal cells to cancerous cells. Several of these proteins have regulatory roles of transcription factors so modification in their structure may lead to altered function. The biological outcome includes a loss in transforming activity such as mutagenicity and genotoxicity (2,18,21).

2.2. Botanical Chapter

2.2.1. *Tilia* Genus

Tilia trees are great trees up to 25-30 m high with a large crown. Their bark is fissured and greyish black. The inflorescence of the *Tilia* flower is yellowish green. Usually, two to seven flowers form the inflorescence (47,48). The leaves of *Tilia* trees are generally heart-shaped, and the bark is smooth and grey. They bloom 5 petalled yellow-white flowers in June-July. The used parts of the plant are inflorescences "Flores *Tiliae*", the bark "Cortex *Tiliae*" and coal prepared from its wood "Carbo Ligni *Tiliae*" (49–51). Especially fresh and dried flowers of *Tilia* trees are used as medicinal parts. Flowers are yellowish white in color and arranged in clusters of 5 to 11. The ovate-lanceolate or oblong-shaped calyx is 5-sepaled. The plant has one superior ovary with numerous stamens. The fruit of *Tilia* trees is one seeded and pear-shaped (52).

Tilia inflorescence consists of bract and flowers. Inflorescences are collected with their bracts are full bloom, dried, and preserved under low-moisture conditions. *Tilia* flowers are registered in 1986 German (DAB 9), 1962 Belgian, 1972 French, 1949 British, 1965 Italian, 1972 Swiss, 1958 Dutch, 1967 Hungarian, 1954 Polish, 1968 Romania, 1961 Russian Pharmacopoeia, and 1930, 1948 TK. Swiss Pharmacopoeia (1972) It is also registered as a tea formulation in French Pharmacopoeia (1965) (50,51).

Tilia trees are deciduous trees with a narrow crown that can grow up to 30-40 m in height. Until 15-20 years old, their bark is smooth and crack-free. In the following years, it cracks in the longitudinal direction and becomes pale gray or brownish gray. The buds are 2-3 scaly and they have an alternating arrangement. The leaves of the plant are

simple, the base of the leaves is asymmetrical with long stems and auricles. The base of the lead is circular-ovoid-triangular shaped. The margin of the leaves is toothed or serrated. Between species, the simple or starry hairs on the leaves show distinct differences. Bract tongue or machete-shaped, usually 5-15 cm long and 1-2.5 cm wide, hairy or hairless and whole-sided, rounded-tipped, linear-narrow-elliptical, pedunculated or sessile. The cymose bears varying numbers of flowers depending on the species and it emerges from the midrib of the bract. Flower of the plant is small, good smelled, and nectar-bearing. It has 5 parts the bowl and crown. The crown is pale or bright yellow or white yellow. Stamens are 25-60 in groups. The ovary is sessile and has 5 eyes, and the stigma has 5 lobes. The fruit is unopened with mostly 1, sometimes 2, rarely 3 seeds; globose, ovate-inverted ovoid, keeled or flat, thin or thick crusted. The fruits are usually dense, brown, short, and have star hairs in bunches (52).

Tilia flowers are used in urban planning with their decorative appearance and pleasant smell. *T. cordata* Mill. and *T. platyphyllos* Scop., the flowers of the species are the most medically acceptable. However, flowers of other species are used in the same way in our country. The officinal species can be found rarely. Therefore, the more abundant species *T. rubra* and *T. tomentosa* are commonly used instead of the species included in the European Pharmacopoeia (53,54). There are 23 species that spread in Europe, West and East Asia, and North and Central America, and 4 of them naturally grow in Turkey. The diagnostic key of these taxa is given below (52):

1. The underside of the leaves is covered with silvery-white star hairs. .....***Tilia tomentosa***

1. The hairs on the underside of the leaves have hair bundles either along the veins or at the junction of the veins.

2. Last year shoots are bare or with star hairs; leaves slightly wrinkled, glabrous, or sparsely simple hairy on both sides or only on the underside; flowers with 38- 46 stamens.....***Tilia platyphyllos***

2. Last year her exile is bare; leaves smooth or slightly wrinkled, glabrous on both sides, except for small groups of hairs in the axils of the veins on the underside; flowers 20-45 stamens

3. The leaves are 38-70 (-90) mm long, 37-68 mm wide, circular, the base is more or less symmetrical heart-shaped, the tip is suddenly narrowed and elongated; the underside is distinctly dull bluish with clustered reddish-brown hairs in the vein axils, sparsely hairy in the area outside these clusters and on the veins; a number of flowers in cymose (4-) 5-8 (-30); the number of stamens is 20-32; fruit (5-) 6-7 mm and thin peel.....***Tilia cordata* subsp. *cordata***

3. The leaves are (50-) 66-128 mm long, (40-) 52-106 mm wide, ovate, asymmetrical at the base, more or less heart-shaped or flat like cut, and the tip is slowly narrowed and elongated; grayish or light green on the underside and light-brown hairs clustered in the vein axils, glabrous outside these clusters; the number of flowers in cymose (2-) 3-5 (-6); stamen number 39-45; fruit (7-) 10-11 mm and thick peel .....***Tilia rubra* subsp. *caucasica***

2.2.1.1 *Tilia cordata* Miller subsp. *cordata* (Small-Leaved *Tilia, syn: T. sylvestris* Desi.)

It spreads in Western Asia and Europe. In our country, there are several localities around some streams that flow into the Black Sea on the Istanbul-Tekirdağ border. *T. cordata* trees can grow up to 30-37 meters in height. The shoots are olive green, green or red with star hairs or glabrous. Buds are visible with 2 scales. Leaves of the tree are 38-70 mm long and 37-68 mm wide and mostly circular. Base of the leave is slightly asymmetrical heart shaped. The edges of the leaves are flat at the base and serrated outside the base. The upper surface is dull dark green in color and the lower surfave is matte blue or pale green with sparse stellate hairs. Its petiole is 21-46 mm long and glabrous or sparsely hairy. Bracts are light yellowish-green and glabrous. The flowers emerge midrib of the bracts. Flowers bloom in May-June (52,55).

2.2.1.2 *Tilia rubra* DC. subsp. *caucasica* (Caucasian *Tilia, syn: T. dasystyla* subsp. *caucasica*)

In the forests on the northern and southern slopes of Turkey and the Greater Caucasus, in the part from near Novorossiysk in the west to Dagestan and Northeastern Azerbaijan; In the Lesser Caucasus, it spreads dispersedly in Armenia, Georgia, and the Elburz Mountains bordering the Caspian Sea in the north of Iran. In Turkey, it is found

in the Southern Marmara and Main Aegean Sections, as well as the Black Sea Region and Erzurum-Kars Sections. They are trees that can grow up to 35-40 meters in height. The shoots are mostly red in the first year, rarely green, glabrous or very sparsely hairy. In the second year it becomes grayish brown. The buds are hairless, pale red or reddish green, with 2-3 visible scales. The leaf is 50-128 mm long and 40-106 mm wide and ovoid, heart shaped. The edge of leave is straight at the base, serrated or serrated outside the base. The upper surface is green and glabrous, the lower surface is grayish green. Petiole 26-56 mm long, swollen at the base, mostly glabrous. Bracts are light green, rounded tip, glabrous and wavy underside. The flowers bloom in July. The petals are light yellow in color (52,55).

2.2.1.3 *Tilia platyphyllos* Scopoli (Large-Leaved Tilia)

Tilia platyphyllos spreads in Europe, Turkey, Ukraine, Caucasus, and Iran. It is naturally found around the Black Sea, Marmara and Antalya. These trees can grow up to 25-40 meters in height. The shoots are bright green, and mostly red in winter, and glabrous or very sparsely starry-haired. The buds are bare and have 2-3 visible scales. Its leaves are 59-120 mm long and 59-100 mm wide and circular in shape. Its base varies in shape from asymmetrical heart-shaped to flat like a beveled cut. The edge of the leaves is smooth at the base and serrate outside the base. The upper surface of the leaves is dark-green and usually slightly wrinkled. Leaf blade glabrous or sparsely simple hairy along the main vein or lateral veins; the underside is light green, glabrous or sparsely-densely simple, with white hairs, sometimes in the axils of the main veins, these hairs may be a little denser or clustered. Petiole 15-48 mm long, glabrous or sparsely pubescent. Bracts are light green, sessile, or with a 10-27 mm long stem, rounded or slightly pointed. Flowers are large with pale yellow petals often slightly curled back and sweet-smelling. It blooms at the end of June. Fruit 8-12 mm long, globular-inverted ovoid; dense, soft, white hairy; it has a more or less thick shell; when dry, it is usually pentagonal, vertebrate, or invertebrate (52,55).

2.2.1.4 *Tilia tomentosa* Moench (Silver *Tilia,* syn: *T. argentea* Desf. ex DC)

Tilia tomentosa Moench. spread in the Balkans, Hungary, Ukraine, and Turkey. In Turkey, it is found in the Marmara, Western Black Sea, Aegean, and Adana sections. These trees can grow up to 35-40 meters in height. The shoots are green and hairy in the

first year. The buds have 2-3 scales; the outer edges of the scales are dense, brown, soft, star haired. The leaf is 50-130 mm long and 55-107 mm wide, circular or broadly ovoid. The base is heart-shaped and flat; its edges are serrated, and the upper surface is dark green. The lower surface of leaves is densely silvery hairy. The petiole is 25-56 mm long. Auricle 14-22 mm long, oblong, pinkish green, and membranous. The flowers bloom in late June-early July. Flower and flower stems are dense and softly hairy. The sepals are light green and covered with white star hairs. The petals are lanceolate and yellow. Fruit 6-10.5 mm long, ellipsoid, or slightly ovoid (52,55).

2.2.2. Geographical Distribution

Tilia is a genus in the family of Tiliaceae with about thirty species of big trees. *Tilia* species are native throughout most of the temperate and subtropical regions of the Northern hemisphere. They can be profusely found in Europe. They are also native to Asia and eastern North America (50,51). Four species of *Tilia* are generally recognized as native in Europe. Caucasian *Tilia (T. rubra*), silver *Tilia* (*T. tomentosa),* small-leaved *Tilia* (*T. cordata)* and large-leaved *Tilia* (*T. platyphyllos). Tilia cordata* and *Tilia platyphyllos* are distributed over the whole continent of Europe. *Tilia rubra* is distributed only in the Balkans. The fourth species *Tilia tomentosa* is native to Europe and Asiatic Turkey. Species registered in the pharmacopeias are *T. platyphyllos* Scop. (Large-leaved *Tilia*), *T. cordata* Miller (Small-leaved *Tilia*). The inflorescences of *Tilia platyphyllos* Scop., *T. cordata* Miller. and a hybrid *T. vulgaris* Heyne. are approved as officinal species in European Pharmacopoeia. (50,55,56).

In Turkey, of these species, *T. cordata* does not found. *T. platyphyllos* is rarely encountered. Silver *Tilia* (*T. tomentosa* Moench.), grows widely in the Marmara region and *T. rubra* DC. (Caucasian *Tilia*) grows in the Black Sea region and these species are popularly used as drugs (51,57). They also grow wild or purposely planted in gardens in Europe and North America but the material originates mainly from Romania, former Yugoslavia, Turkey, Bulgaria, and a part of China (58). The geographical distribution of *Tilia* species and their subspecies is summarized in Table 1.

Table 1. Geographical distribution of *Tilia* species and their subspecies.

Europe and Western Asia

1. *T. cordata* Mill.
1.2. *subsp. sibirica* (Bayer)
2. *T. dasystyla* Steven
2.1. *subsp. caucasica* (V. Engl)
2.2. *subsp. multiflora* (Ledeb.)
3. *T. platyphyllos* Scop.
3.1. *subsp. pseudorubra*
3.2. *subsp. cordifolia* (Besser) C.K. Schneid
4. *T. tomentosa*

North and Central America

22. *T. americana L.*
23. *T. caroliniana Mill.*
23.1. *subsp. occidentalis* (Rose)
23.2. *subsp. floridana* (small) E.
23.3. *murray subsp. heterophylla*

Eastern Asia

5. *T. amurensis* Rupr.
6. *T. nobillis* Rehder&Wilson
7. *T. henryana*
7.1. *T. miqueliana* Maxim
8. *T. japonica* (Miquel) Bayer
9. *T. kiusiana* Shiras
10. *T. maximowicziana* Shiras.
11. *T. panicostata* Maxim.
11.1. *subsp. dictyonecuta* (V. Engler)
11.2. *subsp. yunadensis* (Diels)
12. *T. concinna* Pigot
13. *T. chinensis* Maxim.
14. *T. collidonta* Chang H
15. *T. chingiana* Hu&Cheng
16. *T. mandshurica* Maxim.
17. *T. mongolica* Maxim
18. *T. subsp. toquertii* (C.K. Schneid)
19. *T. endochrysea* Hand-Mazz
20. *T. oliveri* Szyszl.
21. *T. tuan* Szyszyl.
21.1. *subsp. tristis* Pigott
21.2. *subsp. oblongifolia* (Rehder)

2.2.3. Chemical Components of *Tilia* Genus

Medicinally used part of *Tilia* are dried flowers or inflorescences of *T. cordata* Miller, *T. platyphyllos* Scop and *Tilia* x vulgaris Heyne or mixture of these. (59) The major constituents of the dried inflorescences are flavonoids (1-5%), mucilage (7-10%), essential oil (0.05%) (53,56,60). Among flavonoids (1–5%): mainly quercetin glycosides such as rutin, quercitrin, isoquercitrin, hyperoside, and with kaempferol glucosides such as astragalin and tiliroside. Also, the presence of phenolic acids such as caffeic, protocatechuic, chlorogenic and *p*-coumaric acids, tannins such as procyanidin dimers B-2 and B-4 in dried inflorescences of *Tilia* sp. were reported (60).

In *Tilia* inflorescences, there are more than 70 identified essential oil compounds, which gives the drug its characteristic odour. Main constituents of essential oils present in *Tilia* inflorescences were reported to be farnesol and its acetate, geraniol, linalool, camphor, carvacrol, eugenol, carvone, germacrene, menthol, citral, geranyl acetate, 1,8-

cineole, limonene and citronellol. Fresh *Tilia* inflorescences has a more pronounced pleasent floral scent of honey, however this odour is weakened after drying (60–62). Additionally, it contains significant amounts of mucilage, and with this feature it is often associated with coating the pharyngeal mucosa with mucilages thereby provides the relief of cough (63). Particularly, bracts of *Tilia* spp. contains a complex of mucilages (7-10%). Mainly arabinogalactans and uronic acid units are present in dried drug. The mucilage is composed of 5 fractions, mainly d-galactose, L-rhamnose, L-arabinose, uronic acid units and small amounts of glucose, xylose and mannose (60).

2.2.4. Traditional Medicinal Usage

Tilia flower has been used traditionally for therapeutic purposes since the Middle Ages due to its valuable medicinal properties. *Tilia* flower is approved as a cough reliever associated with cold and against cold symptoms by the Commission E. Also, the use of *Tilia* flowers as a diaphoretic and antispasmodic agent is indicated (58,60) The infusions of inflorescences are used in diseases of upper respiratory tract and fever for its diaphoretic action. Infusions of flowers are used against mild digestive disorders and atherosclerosis. The essential oil component of *Tilia* is used for its calming effect (61).

Additionally, The German Standard Licence indicates infusion of *Tilia* flowers for relieving irritation of respiratory tracts caused by cough. Furthermore antispasmodic, hypotensive, diuretic, diaphoretic and mild astringent, peripheral vasodilatory, anti-inflammatory, sedative, antidepressant, and antimicrobial effects of *Tilia* species have been reported in several studies. Furthermore, it is used against hysteria, sinus headache, insomnia, migraine, and arteriosclerotic hypertension traditionally (57,58,64). Moreover, The German Commission E has approved the use of *Tilia* flower infusion in the treatment of colds. Diaphoretic activity may be due to an increment in the response of the sweat glands to heat stimuli. Flavonoids and *p*-coumaric acid in plants induce diaphoresis for the treatment of feverish colds and chills. However, some references argue that diaphoresis is due to ingestion of a hot liquid such as *Tilia* tea (65,66). In a clinical trial, researchers investigated *Tiliae* flowers for their diaphoretic action in uncomplicated catarrhal disease patients. Patients with uncomplicated catarrhal disease inhaled vapor made with *Tiliae* flowers in water. Control group with the same patient number inhaled vapor from colored water without *Tiliae* flowers. In the group that inhaled *Tiliae* flower vapor, they all experienced a certain relief and improvement in their condition while the

control group experienced improvement for a little period. According to the author, inhalation of vapor made with *Tiliae* flowers has a notable diaphoretic effect on patients. However, statistical analysis of the data was not done thus an objective comparison is not possible (60).

2.2.5. Biological Activity Studies on *Tilia* species

Tilia species has great importance in phytotherapy for many biological activities such as antioxidant activity, anti-inflammatory activity, and diaphoretic activity. Biological activity studies conducted on *Tilia* species in literature were summarized below.

2.2.5.1. Antioxidant Activity

Edible or non-edible plants are great sources of natural antioxidants with a wide variety of phenolic compounds. With their antioxidant activity and protective effect against many diseases, they become indispensable in alternative medicine. *Tilia* is a member of alternative medicine which has many properties and is used for many purposes as traditional medicine (67).

Akyuz et al. evaluated (67) antioxidant capacity in three different parts of *Tilia rubra* For the investigation of antioxidant activity, DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging and CUPRAC (cupric reducing antioxidant power) assays were done. The DPPH scavenging activity of extracts were ranging between 0.106-.231 mg/mL. The strongest antioxidant potency was found in the leaves of the plant which was the highest total flavonoid and total phenolic content containing part. Results were similar in the CUPRAC assay. Leaves of the plant showed the highest CUPRAC value, meaning the strongest antioxidant activity. Yildirim et al. (24) compared water extracts of black tea, sage, and *T. tomentosa* flowers in terms of their antioxidant activity by the thiocyanate method. Although there were no statistically significant differences in investigated plants' antioxidant activities, tea samples had shown to be more effective than the *Tilia* sample and the *Tilia* sample was found more effective than sage extract in antioxidant activity. In another study, 70% ethanolic extracts of *T. platyphyllos* were analyzed for their phenolic composition, inhibition of lipid peroxidation, and free radical scavenging activities with scavenging effects on DPPH

radicals, reducing power by ferricyanide Prussian blue assay, β-carotene bleaching inhibition, and lipid peroxidation inhibition and by thiobarbituric acid reactive substances assay. *T. platyphyllos* showed a more potent antioxidant activity compared to other plants investigated with higher abundance in phenolic compounds (16). In 2020, Pavlović et al. (15) analyzed inflorescences of %80 ethanolic extract of officinal *Tilia* spp. from different sources with the DPPH-HPTLC method to evaluate their antioxidant activity. Compounds that showed radical scavenging activity were determined as chlorogenic acid, astragalin, quercitrin, hyperoside, tiliroside, and caffeic acid. Among these compounds caffeic acid exerted the highest antioxidant capacity. In 2014, Akyüz et al. (67)evaluated phenolic composition and antioxidant capacity in three different parts of 100% methanolic extracts of T. rubra. The DPPH scavenging activity measured in the blossom, leaf, and trunk of the plant was 0.116 ± 0.38, 0.231 ± 0.43, and 0.004 ± 0.10 mg/mL expressed in the term of IC_{50}. The IC_{50} value represents the concentration of phenolic compounds that are required to inhibit 50% of free radicals. It is expressed in mg/mL and the lower IC_{50} value indicates higher antioxidant activity. Results of the study showed that the strongest antioxidant activity was obtained with *Tilia rubra* leaves. Additionally, in 2021, Mitic et al. (68) showed the potent antioxidant capacity of 50% ethanolic extract of *T. cordata* flowers extracted with an optimized process for 120 minutes at 55 °C.

2.2.5.2. Anti-inflammatory Activity

Flos *Tiliae* is used traditionally in the symptomatic treatment of cold and flu and inflammation of the mucosa. There are some studies that support the anti-inflammatory action and the usage of *Tilia* flower infusion in the cold, tonsillitis, and pharyngitis, where the mucosa is inflammed and irritated (69). Flos *Tiliae* contains high amounts of mucilages, and this is thought to be a reason for coating pharyngeal mucosa with *Tilia* mucilages and thereby relieving cough. Nevertheless, preparing *Tilia* flower infusion with plant material closed in sachets will not perform a sufficient mucilage extraction for this activity. Therefore, the possible anti-inflammatory and antibacterial activities of phytoconstituents like flavonoids and phenolic acids cannot be ruled out. In bacterial-associated common cold, it has been shown that a patient's inflammatory response to some strains of bacteria may be the reason to develop a sore throat. Therefore it is highly probable when direct contact with pharyngeal and tonsil mucosa that compounds from

Tilia flower extract can modify the response of neutrophils which generates an inflammatory response (69).

Toker et al. (70) studied two main glycosides, from the leaves of *T. argentea* (*T. tomentosa)* Desf. Ex DC (Tiliaceae) Silver *Tilia* for their activity against inflammation. To evaluate anti-inflammatory activity, a carrageenan-induced paw edema model in mice was used. Both compounds were shown to exhibit anti-inflammatory activity at 50 mg/kg dose without any acute toxicity and gastric damage. In another study, to evaluate the antioxidant, and anti-inflammatory activities of *T. platyphyllos* and *Equisetum giganteum* activity *in vitro*, a macrophage cell line RAW 264.7 was used. Hydroethanolic extracts of aerial parts of the plant (leaves, flowers, and inflorescences) were used. To evaluate the protective effect against inflammation of both plant extracts was performed by measuring nitric oxide (NO) production. In a microplate reader, by measuring optical density the nitrite levels produced were determined. As positive control, dexamethasone was used. *T. platyphyllos* was found to have a higher amount and content in total flavonoids, most abundant respectively, protocatechuic acid and (-)- epicatechin and quercetin 3-*O*-glucoside. Moreover, it showed higher antioxidant and higher anti-inflammatory action than *E. giganteum* (16). In another study, Flores-Sanchez et al. (71) evaluated a different species from Tiliaceae genus, *T. americana var. mexicana* which is used against anxiety and inflammatory processes in Mexican folk medicine for its anti-inflammatory activity. The chemical composition of methanolic leaves and callus extracts was assessed with high-performance liquid chromatography (HPLC) and scopoletin was determined as an anti-inflammatory compound. 12-O-tetradecanoylphorbol-13-acetate induced ear edema was performed to demonstrate the anti-inflammatory effect of scopoletin. Both leaf and callus extracts demonstrated a protective effect against inflammation with a median effective dose (ED_{50}) in the range of 0.38-1.73 mg per ear, respectively In 2018, Fawzy et al. (72) studied 70% methanolic extract of aerial parts of *T. cordata* to evaluate its anti-inflammatory, antinociceptive and nephroprotective activities and isolate the plant's phytoconstituents. Kaempferol, quercetin, vitexin, kaempferol 3-*O*-α-rhamnoside, quercetin 3-*O*-β-galactoside, and kaempferol 3-*O*-rutinoside was identified as phenolic compounds in the plant with chromatographic and spectrophotometric methods. The 70% methanolic extract of aerial parts of *T. cordata* showed a powerful anti-inflammatory activity. After 3h of carrageenan injection, the

same decrease in the swelling was obtained with 70% methanol extract (300mg/kg) and indomethacin (72).

2.2.5.3. Anticancer Activity

Medicinal plants contain various antioxidant components, which play key roles in free radical scavenging and lipid peroxidation prevention. With antioxidant compounds, cells can be protected from oxidative damage. Secondary metabolites in plants, such as phenolics have strong antioxidant activities and have a great potential for scavenging free radicals for preventing certain diseases including cancer (73).

Barreiro et al. (74) examined aqueous, dichloromethane, and ethanolic extracts of *T. cordata* flowers regarding their anti-tumor effects on BW 5147 lymphoma cells and non-tumor lymphocytes. All extracts exerted a selective activity on tumor cells and induced apoptosis. In non-tumor lymphocytes, all extracts suppressed mitogen-induced proliferation. As a result of this study, aqueous ethanolic, and dichloromethane extracts of *T. cordata* flowers showed a concentration-dependent inhibition of both tumor and non-tumor cells. Also, lignan derivatives that are isolated from the plant have been proven to act as anti-cancer substances in the recent decade. Kim et al. (75) examined methanolic extracts ans isolated fractioned lignan glycosides from the trunk of *Tilia amurensis* Rupr. for its anti-cancer activity. The extract showed significant cytotoxicity against SK-OV-3 (ovary carcinoma), HCT-15 (colon adenocarcinoma), A549 (lung carcinoma), and SK-MEL-2 (skin carcinoma). In addition, Zarringhalami et al. (73) showed potent cytotoxic effects of water extract of *Tilia dasystyla* on the Saos-2 cancer cell line.

2.2.5.4. Other Biological Activities

Antimicrobial Activity

Flos *Tilia*e has been used as an infusion traditionally to relieve throat irritation, induce diaphoresis, and cough in cold. Vatľák et al. (76) evaluated methanolic extracts of *T. cordata* leaves for their antimicrobial activity. The highest antibacterial activity of the methanolic extract of *T. cordata* leaves was found against a gram-negative bacterium, Pseudomonas aeruginosa. Additionally, in a study from Serbia, Pavlovic et al. (15) compared antimicrobial activities of inflorescences extract of different species of *Tilia* (*T. platyphyllos*, *T. cordata* Miller, and *T. x vulgaris* Heyne). They had two groups: one

was collected from the field, other was bought from commercial samples. Both samples collected from the field and bought commercially inhibited the growth of gram-positive bacteria Bacillus subtilis, Streptococcus pyogenes, Streptococcus mutans, and Staphylococcus aureus. Moreover, methanolic extract of *Tilia cordata* flowers exhibited antifungal activity against Aspergillus niger. In the same study, %10 aqueous extracts of dried flowers have been shown to be effective against influenza virus A2 (60).

Antispasmodic Activity

The activity of the *Tilia* species on the smooth muscles has been demonstrated in several experiments. Cavadas et al. (77) detected several amino acids, including GABA in the aqueous extract of *Tilia europeae*. Viola et al. (78) isolated BDZ-R ligands from *Tilia tomentosa*. Moreover, the sedative and anxiolytic effect of different *Tilia* species has been demonstrated in several studies. Figueiredo et al. (79) also showed *Tilia* species has effects on neural cells, where flavonoids are responsible for this effect by the involvement of GABA/BDZ and 5HT1A serotonergic receptors. Aqueous extract of *T. platyphyllos* and *T. vulgaris* seeds have been reported to produce a biphasic response, first an antispasmodic effect and then a spasmogenic effect on rat duodenum *in vitro*. The spasmogenic activity was inhibited by papaverine and atropine and strengthened by acetylcholine (49). In another *in vitro* study, Cerantola et. al (80) evaluated the influence of *T. tomentosa* flower extract on isolated mice's small intestines. A significant inhibition in ileal basal tone was obtained which displays the relaxant activity of the plant extract. Phenolic and flavonoid compounds like quercetin and kaempferol in *Tilia tomentosa* flower extracts are thought to be responsible for this relaxation. Flavonoids are involved in the inhibition of calcium and potassium channels, and they are also involved in nitric oxide production. The smooth muscle relaxation is possibly due to these actions. Conversely, Al-Essa et al. (81) studied the *in vitro* direct effects of ethanolic extract of *T. cordata* Miller. on dispersed smooth muscle cells of the intestine. In the study, intestinal smooth muscles of guinea pigs which were dispersed by collagenase were investigated. A dose-dependent contraction was obtained in intestinal smooth muscle cells induced by the extract. Some of the active components with cholinergic activities found in ethanolic extract of the plant are attributed to increased contraction of smooth muscle cells of the intestine by activating muscarinic receptors.

Pancreatic Lipase Inhibitory Activity

Lipids are important human nutrients; however, elevated consumption of lipids contributes to long-term complications including obesity. Herbal medicines have the potential to inhibit the digestion of dietary lipids. Pancreatic lipase is the key determinant enzyme for the absorption of lipids in the diet. Thus, inhibition of lipases decreases lipid absorption. Slanc et al. (82) examined over hundred species of medicinal plants, fruits, and vegetables and their lipase inhibitory activity. *T. platyphyllos* extract was the most active plant extract in pancreatic lipase inhibition.

Sedative Activity

Tilia species are widely used as tranquilizers and sedatives traditionally, especially in Latin America. The infusion of the inflorescence is consumed as tea for this effect. Viola et al. (78) examined %70 ethanolic extract of *T. tomentosa* Moench inflorescences to discover the active principles responsible for this pharmacological effect. Benzodiazepines are a widely used class of psychoactive drug worldwide. Previously, a compound was identified that interacts with benzodiazepine receptor as a partial agonist in a well-studied plant used as a tranquilizer, *Passiflora coerulea*. Afterward, *T. tomentosa* was studied to learn whether the plant contains BDZ-R ligands to use against anxiety. This study concludes that various BDZ-R ligands exist in *T. tomentosa*. In another study, a widely used plant as anxiolytic and for sedation, methanolic extracts of the inflorescences of *T. americana L. var. mexicana* collected from three different regions of Mexico, were tested for anxiolytic and sedative effects. The flavonoid pattern of the samples exhibited significant differences. Despite these differences, all the samples showed anxiolytic and sedative-like responses in both *in vitro* and *in vivo* studies. This study shows that *T. americana var. mexicana* shows depressant activity in the central nervous system similar to the well-studied species of *Tilia* (83). Moreover, the aqueous extracts of *Tilia americana var. mexicana* inflorescences from stored specimens and freshly collected samples were examined and compared for their sedative and anxiolytic activities. To evaluate sodium pentobarbital (SP)-induced hypnosis induction experimental mouse models were used via oral administration of different concentrations of samples. The results showed that inflorescences of stored specimens have the same effectiveness concerning anxiolytic and sedative effects as samples freshly collected (84). The inflorescences of *T. americana var. mexicana* is a

well-studied drug regarding its sedative and anxiolytic-like activity. However pharmacological studies are lacking for the leaves of the plant. In this research, leaves of *T. americana var. mexicana* were compared regarding its sedative and anxiolytic activity with its inflorescences. They found that the leaves of *T. americana var. mexicana* were effective as the inflorescences to produce anxiolytic-like effects. Flavonoids like rutin, quercetin, and isoquercitrin are partially responsible for this action (85). In another study, *T. tomentosa* Moench bud extracts were examined for their sedative and anxiolytic activity which is used traditionally as a sedative compound for centuries. However, its mechanism of action is still unknown. To evaluate the anxiolytic and sedative action of *T. tomentosa* Moench bud extract on *in vitro* hippocampal neurons, the impact of the sample on the chloride current of $GABA_A$ receptor-activated hippocampal neurons was tested through microelectrode-arrays and the patch-clamp technique. Results of this study suggest that *T. tomentosa* Moench buds extracts mimic GABA and BDZ agonists and act as an effective sedative and anxiolytic compound (86).

Antidepressant Activity

Depression is one of the most common mental disorders, which influences more than 280 million people in the world. Treatment of depression has relied on the use of selective serotonin reuptake inhibitors and tricyclic antidepressants. These drugs are clinically effective, however, have also been shown to produce significant adverse side effects. For this reason, medicinal plants become a source for the search for alternative therapies. (87) Chávez-Morales et al. (87) examined fractions including tiliroside obtained by column chromatography from methanolic extract of inflorescences of *T. americana*. The result of this study presents tiliroside from *T.* americana showed an antidepressant activity in tail suspension test, open field test, and swimming test on the mouse.

Lymphocyte Proliferative Activity

Anesini et al. (88) investigated aqueous extract of flowers of *T. cordata* for its lymphocyte proliferative action. Aqueous extracts of *Tilia* flowers have been used against anxiety and their uses have been supported by studies. The anxiolytic effect of the extracts is attributed to flavonoid constituents. These flavonoid constituents have been shown to interact with benzodiazepine receptors with high affinity. It is known that when benzodiazepines interact with their receptors, they play a role as immunomodulators.

Peripheral type benzodiazepine binding sites are located in human circulating lymphocytes such as lymphoma cell lines. The study aimed to compare lymphocyte proliferative activities of aqueous extract of *T. cordata* flowers and selected benzodiazepine agonists by determining cyclic AMP (cAMP) levels. Cell proliferation is negatively affected by elevated levels of cAMP. As a result, the aqueous extract of *T. cordata* flowers stimulated lymphocyte proliferation by the peripheral benzodiazepine binding sites. This action supports the traditional use of *Tiliae* flos infusion in the treatment of colds, bronchitis, influenza, and chills. Moreover, Barreiro et al. (74) studied the antiproliferative action of ethanol, dichloromethane, and aqueous extracts obtained from flowers of *T. cordata* Miller. Their effects were evaluated in both normal lymphocytes and tumoral (BW 5147 lymphoma). All extracts showed a clear concentration-dependent selective action on tumoral lymphocytes, including apoptosis. In normal lymphocytes, without affecting viability extracts suppressed mitogen-induced proliferation. They did not show cytotoxicity.

2.3.6. Techniques for Determination of Chemical Composition and Biological Activity

2.3.6.1. Chemical Characterization Methods by Chromatographic Techniques

Over the past 100 years, chromatographic techniques have developed from a tool for the separation of pigments to a tool for dealing with the most complex purification and analytical problems in phytochemistry with qualification and quantification aims (89). The International Union of Pure and Applied Chemistry (IUPAC) defines chromatography as the physical method for the separation of components distributed between two phases moving in a definite direction to be separated. There are two phases in chromatography: stationary and mobile phases. The chromatography technique is applied for the resolution or separation of analytes or sample compounds to make further analysis or use. The technique is based on the principles of adsorption and partition. Analytes or sample compounds have different affinities towards the stationary phase and mobile phase. The non-polarity and polarity properties of compounds play a key role in the separation process by adsorption. With a polar analyte and polar stationary phase, the analyte traverses the column slowly as compared to non-polar analytes and elutes last. In partition, the solubility of mixtures in different solvents is key for separation. The preferential partitioning of a sample in a phase provides the separation of a mixture. As a

sample in a mixture moves along with the mobile phase it is allowed to pass through a stationary phase. The different properties between samples in a mixture cause certain molecules to stay longer in the stationary phase while certain molecules can rapidly pass through the stationary phase to the mobile phase and rapidly exit the system (90,91).

High-Performance Thin-Layer Chromatography

Regulatory agencies generally recommend fingerprint chromatography for the identification of herbal extracts (89). High-Performance Thin-Layer Chromatography (HPTLC) is the most advanced form of thin-layer chromatography (TLC) which is an accepted standard planar chromatography method in the pharmacopeias for quality control of plant materials. It is a widely applicable analytical technique that plays an important role in the identification of herbal remedies. Quantitative and qualitative determination of marker substances and detection of adulteration are also other widely used applications of this method. TLC is the only technique in which all the components of the sample can be visualized in the chromatogram. This method also has advantages in its simplicity, low cost, availability for multiple detections, and obtaining results rapidly but also has some disadvantages in lack of automation, lack of quantification accuracy, and problems in reproducibility. HPTLC is an advanced version of TLC which minimizes these disadvantages. With the automaticity, the smaller particle size of plates and shorter developing distances provide a better solution and reproducibility in the HPTLC technique (91–94).

TLC is the only chromatographic method that presents the result as an image. Also, it is the only method that includes all the components in the sample in the resulting chromatogram. In TLC sample capacity is high and results can be obtained in a very short time. Multiple detections and parallel analysis of samples are possible. It is simple, flexible, and low in cost. However, there are some lacks in TLC like the lack of automation and lack of quantification accuracy. These deficiencies sometimes constitute a problem for reproducibility. High-performance thin-layer chromatography is a developed TLC method. The plates are pre-coated with a stationary phase with an average particle size of 5μm in HPTLC and this provides more sensitive detection and better separation than normal TLC plates with an average particle size of 12μm. This method uses an automated sample application and needs a minimum sample size that provides better reproducibility (89).

High-Performance Liquid Chromatography

High-Performance Liquid Chromatography (HPLC) which is one of the most powerful tools in analytical chemistry, has the ability to identify, quantify and separate any compounds in a sample that is dissolved in liquid. It can separate thermally labile, non-volatile, and water-soluble compounds with high precision and high resolution in a short time. Additionally, a wide variety of stationary phases and mobile phases provides ease in finding suitable separation conditions (89).

Development and Validation of HPLC Methods

Inadequate characterization of the chemical profile of plants limits research reproducibility, and continuity of investigations and restrains perceiving the underlying mechanisms. This may in turn negatively affect advancements in clinical practices. The development and validation of analytical methods are key components for creating and interpreting safe and effective research. For the effective development of an analytical method, laboratory conditions must be optimized. (95)

The International Organization for Standardization (ISO) defines validation as "verification if the specified requirements are acceptable for an intended application". Method validation is the confirmation that developed analytical methods are accurate and precise for the intended purpose. International Conference on Harmonization (ICH) guideline states the required validation performance parameters as, accuracy, precision, specificity, linearity and range, the limit of detection (LOD) / limit of quantification (LOQ), selectivity/specificity, ruggedness/robustness, stability and system suitability (96).

Accuracy: Accuracy is defined as the closeness of a calculated value obtained by the method to the true value. Accuracy is obtained by analyzing the test sample in different concentrations and calculated by the difference between the mean and the known true value, along with confidence intervals. According to ICH, accuracy determinations should be done with a minimum of three different concentration levels with a minimum of nine determinations (96–98). For an assay method criteria for accuracy are determined as the mean recovery will be 100 ± 2% at each concentration in the range of 80-120% of the target concentration (96).

Precision: When an analytical method is used repeatedly to numerous samplings of a homogenous sample, the precision of a method is measured by the degree of agreement among the individual test findings. The standard deviation or relative standard deviation is typically used to express the precision of an analytical process (96–98). HPLC criteria for the precision of an assay method are determined as the instrument precision (RSD) will be ≤1% and the intra-day precision will be ≤ 2% (96).

Selectivity and Specificity: This parameter is defined by the International Conference of Harmonization (ICH) documents as the capacity to conclusively determine if an analyte contains components that may be anticipated to be present, such as contaminants and degradation products. In method validation, the terms selectivity and specificity can be used interchangeably to refer to the same idea. An analytical method's selectivity is determined by its capacity to measure the target analyte precisely and specifically in the presence of other components that may be anticipated to be present in the same matrix. Peak purity and the minimum resolution factor of two nearby peaks are two common ways that demonstrate selectivity in HPLC. For an assay method, the criteria for selectivity is determined as the analyte peak's baseline chromatographic resolution will be at least 1.5 from other sample components. (96–98).

Linearity and Range: Linearity is the ability of an analytical technique to yield test results that are directly proportional to the analyte concentration in the sample within a specified range. When testing findings are analyzed from samples with different analyte concentrations, at least six concentrations, the variance around the slope of the linear regression is calculated according to a known mathematical connection to represent linearity. HPLC criteria for linearity in an assay method are determined as the minimum specified range is 80 to 120% of the target concentration for a minimum of five concentration levels (96–98).

Limit of Detection and Limit of Quantification: The smallest amount of analyte in a sample that can be identified, but not necessarily quantified, outside of the specified experimental circumstances is known as the limit of detection (LOD). The lowest amount of analyte in a sample that can be quantitatively measured with enough precision and accuracy is called the limit of quantification (LOQ). HPLC criteria for LOD and LOQ are determined as a signal-to-noise (S/N) ratio of 3:1 is deemed acceptable with a relative

standard deviation (RSD) less than 10% for LOD, whereas for LOQ an S/N ratio of 10:1 is deemed suitable with an RSD of less than 3% (96).

Robustness: The robustness of an analytical technique is a measure of its ability to be unaffected by subtle but intentional changes in method parameters and offers a clue as to its dependability in typical conditions. These parameters include column temperature, flow rate, mobile phase additives, mobile phase composition, and pH (96–98).

Stability: To provide repeatable and trustworthy findings, the HPLC technique needs the sample, standard, and reagents to be stable for a suitable amount of time. Long-term column stability is essential for method robustness since even the greatest HPLC column will ultimately deteriorate and lose its original performance. Prior to chromatographic studies, solutes might easily break down while being prepared or stored. Research on the stability of analytes and standards should be done throughout method development (96–98).

System Suitability: Testing for system compatibility is a crucial step in many analytical processes. The idea behind the testing is that the instruments, electronics, analytical procedures, and samples to be examined constitute a complete system that can be assessed as such. The parameters for a system suitability test that must be developed for a specific operation depend on the kind of method that is being validated (96–98).

2.3.6.2. Evaluation of *in vitro* Antioxidant Capacity

Due to its capacity to reduce oxidative stress, several studies demonstrate that antioxidants are crucial in preserving human health as well as in the prevention and treatment of diseases. In order to determine how effective naturally occurring antioxidants are in the prevention and treatment of diseases associated with oxidative stress, it is crucial to measure the antioxidant activity/capacity of foods and biological samples (99).

Total Phenolic Content

Phenolic compounds can exhibit antioxidant activities in many pathways. The main pathway is due to their redox properties. Their redox properties can play an

important role in neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (100).

In 1912, Professor Otto Folin and his colleagues showed that phosphotungstic acid and phosphomolybdic acid could be useful to qualitatively detect polyphenol compounds in urine. In 1927, Folin and V. Ciocalteu proposed to use this mixture to determine tyrosine and tryptophane in alkaline conditions, which are two amino acids bearing a phenolic ring and have reducing properties. As follows, Folin-Ciocalteu (F-C) reagent constituted a sensitive and reproducible quantification method for polyphenols. The reagent is a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$). It reacts with non-phenolic and phenolic reducing substances and produces chromogens. In alkaline conditions, formed oxomolybdate and oxotungstate during redox reaction presents a blue coloration which is proportional to polyphenol concentration, and can be detected spectrophotometrically. This method demonstrates the reducing capacity of a sample, which is generally expressed in terms of total phenolic content. Nowadays, this reagent is used commercially available to quantify polyphenols in plant extracts. For instance, in Europe, it is an official procedure to quantify the phenolic content of wines (101).

The total phenolic content of different *Tilia* species was determined by Folin-Ciocalteu colorimetric method described by Güzelmeriç et al. (102) with slight modifications in this study. This assay is based on electron transfer of the yellow-colored Folin-Ciocalteu reagent which includes phosphotungstic acid and phosphomolybdic acid which interacts with phenolic compounds. This mixture results in a blue-colored mixture with a maximum absorption site at 765nm.

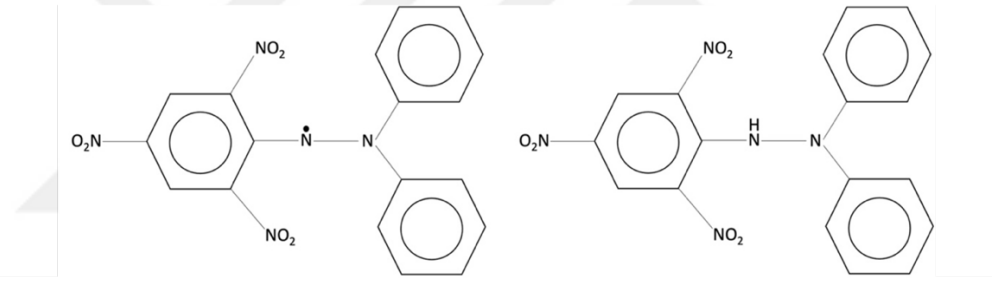
Total Flavonoid Content

Flavonoids are found in plant foods naturally and they are commonly consumed in our diet. The most important biological activity of flavonoids is their activity as an antioxidant. This activity can affect oxygen free radicals and lipid peroxidation. Such oxidative reactions are thought to be involved in chronic inflammation, cancer, and atherosclerosis (103). So far, more than 10,000 phenolic compounds have been isolated from plants and predominantly isolated phenolics are flavonoids. TFC is widely determined by aluminum chloride colorimetric assay. Al (III) forms chelates with

flavonoids to form Al (III)-flavonoids. Flavonoids have many hydroxyl groups and oxo groups which have a great affinity to metal ions, and they bind mostly at a 1:1 ratio. TFC is determined through calibration curves which are based on flavonoid standards and sample measures at the same wavelength. Among the flavonoid standards, the most used are quercetin, catechin, and rutin (104).

DPPH Radical-Scavenging Activity Assay

DPPH is a stable nitrogen free radical. It gives deep violet color in solution and this color can be measured with a UV-visible spectrophotometer in 515-530 nm wavelengths. In the DPPH radical scavenging assay, antioxidants react with radicals as hydrogen donors, and DPPH radical is reduced to the pale, yellow-colored diphenyl-β-picryl hydrazine. The discoloration amount indicates the antioxidant activity of the tested sample (105).



1: Diphenylpicrylhydrazyl (Free Radical) **2: Diphenylpicrylhydrazine (Nonradical)**

Figure 8. DPPH in radical and nonradical forms

DPPH assay is a reproducible, simple, rapid, and sensitive technique to detect the free radical scavenging capacity of samples. When DPPH solution reacts with a hydrogen donor, the molecule becomes a non-radical reduced form as illustrated in Figure 8. The color of the solution turns from deep violet to pale yellow color which is due to its picryl group.

DPPH• + AH ⟶ DPPH-H + A•

DPPH• + R ⟶ DPPH-H + R

The reaction mechanism of DPPH with antioxidants is given above. As a free radical DPPH• reacts with an antioxidant (AH) and this reaction cause A• radical (R)

formation. R• then reacts with another DPPH• molecule and finally, DPPH• is reduced to DPPH-R. This reaction causes a decrease in absorbance (106).

Ferric Ion Reducing Antioxidant Power Assay

Ferric ion reducing antioxidant power assay (FRAP) is a method for evaluating the antioxidant power of the test sample. It is a simple and automated test. At low pH, reduction of ferric to ferrous ion results in a colored ferrous-tripyridyltriazine complex formation. At low pH, if a reductant (antioxidant) is present in the test solution, ferric-tripyridyltriazine (FeIII-TPTZ) complex is reduced to the ferrous (FeII) form as given in Figure 9. It gives an intense blue color with maximum absorption at 593 nm. FRAP value of a test solution is obtained by comparing the absorbance changes at 593 nm with known ferrous ion concentration (107). The assay uses color change as an indicator as ferric ions (Fe^{3+}) reduce to ferrous ions (Fe^{2+}) colored ferrous-tripyridyltriazine complex forming, by reduction of ferric to ferrous ion with an antioxidant at low pH (4).

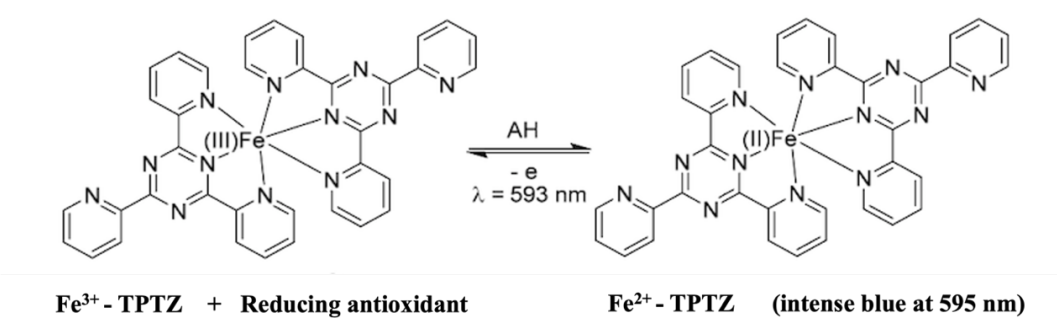


Figure 9. Reaction for FRAP Assay.

Cupric Reducing Antioxidant Capacity Assay

Cupric reducing antioxidant capacity assay (CUPRAC) assay is a colorimetric assay described by Apak et al. (108) in the early 2000s for the determination of antioxidant capacities of test samples. This assay is based on absorbance measuring of the CUPRAC chromophore forms while reduction of Cu (II) to Cu (I) in the presence of neocuproine. As a consequence of the redox reaction of antioxidants with CUPRAC reagent bis(neocuproine)copper(II) cation [Cu(II)-Nc], Cu(I)-neocuproine chelate forms, and the absorbance is recorded at 450 nm wavelength (109). Bis (neocuproine) copper (II) chelate is used as the chromogenic redox reagent. As illustrated in Figure 10, at pH

7, in the presence of a reductant (antioxidant) Cu(II)-Nc is reduced to the highly-colored Cu(Nc)2+ chelate which shows maximum absorption at 450 nm (108).

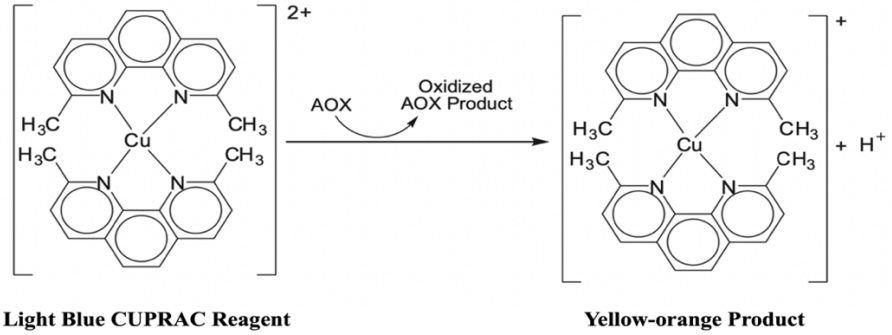


Figure 10. The CUPRAC reaction

2.3.6.3. Anticancer Activity Assessment with Two-Dimensional Cell Cytotoxicity and Three-Dimensional Spheroid Formation/Growth Assays

The cells and tissues are present in a highly complex three-dimensional environment in our body. Cell and tissue cultures have been interested since the 19th century in discovering the effects of cells isolated from tissue of interest and assessing the viability and division of cells in *in vitro* models. With cell culture studies, search for diseases such as cancer, evaluation of cytocompatibility of biomaterials, and tissue regeneration research may be performed. These experiments allow for controlling environmental variables and the repeatability of the research. Clinical trials for anticancer agents are restricted due to severe undesired effects thus, data obtained from *in vitro* assays have significant for preclinical analysis of these agents. 2D cell cultures are the traditional methods that are easy and convenient monolayered culture models and represent a well-established methodology. Cells seeded on flat dishes then adhere and generate a single layer of the adhered cells on the surface. This method has promoted important advances in biological investigations. Nevertheless, this method has some limitations including lack of cell structural architecture, different cell interactions, and biochemical signals demonstrated in *vivo* environment (110,111). The main factor for these limitations is the failure of animal models and 2D cultures to recapitulate the tumor microenvironment (TME) of humans. TME is a complex mixture of a component from cellular sources such as transformed epithelial cells, infiltrating lymphocytes, fibroblasts, mesenchymal stem cells, and endothelial cells and non-cellular sources such as

extracellular matrix, cytokines, and chemokines, a growth factor which has a significant role in cancer development and progression. Animal models are high in cost in addition to the ethical issues and poorly reflect human tumor properties (112). These limitations raised advances in scientific research for better stimulation of *in vivo* environment in *in vitro* studies. As a consequence, 3D cell culture is raised as an approach that is more similar to the *in vivo* characteristics of the cells and better recapitulates the human tumor microenvironment including interactions with extracellular matrix to cell and cell-to-cell communications. Differences between 2D and 3D cell cultures are given in Table 2.

Table 2. Differences between 2D cultures and 3D cultures

CELL CULTURE SYSTEM	ADVANTAGES	DISADVANTAGES
2D CULTURES	• Low cost; • Fast replication; • Easy to manipulate; • Long-term cultures.	• Homogenic perfusion of oxygen and nutrition; • Inadequate cell-cell, cell-ECM interactions; • More susceptible to pharmacological action; • Inadequate cell differentiation; • Faster proliferation compared to *in vivo* tumors; • Modified genetic profile compared to in *vivo* tissue.
3D CULTURES	• Heterogenic perfusion of oxygen and nutrition; • 3 different layers; proliferation, quiescence and necrosis; • Increased cell-cell, cell-ECM interactions; • Mimic penetration of drug to tumor; • Recapitulate the genetic in vivo profile. • More accurate representation of *in vivo* scenario • More suitable for drug resistance models	• High cost; • Difficulty in carrying out methodological techniques. • Static environment • More complex culture system.

3D cell cultures allow presenting physiological processes such as cell growth and its interactions with the environment in a multi-dimensional structure. This method approach is closer to *in vivo* tumor environment with exhibiting variable features such as hypoxia, pH differences, cell proliferation rates, apoptosis, and drug resistance. 3D cell culture is particularly useful in the drug sensitivity area by discovering the biological characteristics of tumor cells (17) As a result, the 3D cell culture technique has become one of the major improvements for studying tumor development and screening for anticancer drugs (110,111).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Plant Materials

Tilia cordata was collected from Şile-İstanbul, Turkey in 2019. *T. platyphyllos* was collected from Bayramiç-Çanakkale, Turkey in 2019 and *T. tomentosa* was collected from Serhat Köyü-Bayramiç-Çanakkale, Turkey in 2019. *T. rubra* was collected from Turgutlu-Manisa, Turkey in 2021. The specimens were authenticated by Assoc. Prof. Gizem Bulut. The voucher specimens for *T. cordata* Miller (MARE22872), *T. platyphyllos* Scop. (MARE22871) and *T. tomentosa* Desf. ex DC. (MARE22727) and *T. rubra* DC. subsp. *caucasica* (Rupr.) V. Engler (MARE2272a) is stored at the Herbarium of Marmara University Faculty of Pharmacy (MARE), İstanbul, Turkey. All plant parts were dried at room temperature and stored in appropriate conditions till analysis.

3.1.2. Chemicals and Solvents

2,2-diphenyl picryl hydrazyl	Sigma Aldrich; 056K1147
Methanol	Sigma Aldrich; MKBD8339
Neocuproine	Sigma Aldrich; SZE9365S
Ammonium acetate	Sigma Aldrich; 120M1890V
Copper Sulfate	Carlo Erba; 313507
Ethanol	Sigma Aldrich; 46139
Sodium acetate trihydrate	Riedel de Haen; 33450
Sodium phosphate monobasic	Riedel de Haen; 62840
Ammonium molybdate tetrahydrate	Riedel de Haen; 30590
Sulfuric acid (98%)	Riedel de Haen; 62260
Quercetin	Sigma Aldrich; 116K1836
Aluminum chloride	Merck; 8.01081.1000
Sodium carbonate	Riedel de Haen; 2217A
Folin-Ciocalteu Reagent (FCR)	Sigma Aldrich; BCBD5119
Gallic acid	Fluka; 1126184
Sodium molybdate dihydrate	Riedel de Haen;
2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ)	Fluka; BCBB447

Vanillin	Fluka; 1435805
Trolox	SigmaAldrich; BCBF4547V
Ferric chloride hexahydrate	Riedel de Haen; 41250
Ferrous chloride tetrahydrate	JT Baker; 0703801013
Ferrous sulfate heptahydrate	JT Baker; 0632701017
Folin-Ciocalteu Reagent	Sigma Aldrich; F9252
Astragalin	Sigma Aldrich; 04500585
Isoquercitrin	Sigma Aldrich; 00140585
Tiliroside	Sigma Aldrich; 79257
Quercitrin	HWI Pharma; 0074-05-80
Protocatechuic acid	SigmaAldrich; 03930590
Hyperoside	HWI Pharma; 0018-05-85
Acetonitrile	Merck
Tripyridyltriazine	Sigma Aldrich; 93285
Acetonitrile	J. T. Baker, SZBB3145V
Ultrapure water	Merck Millipore, Simplicity UV
Dulbecco's modified eagle's medium	Gibco; USA 41966-029
Penicillin-Streptomycin antibiotic	Gibco; USA 15140122
Fetal bovine serum	Gibco; USA 10500064
Trypsin	Gibco; USA 25200056
Dimethyl sulfoxide	Riedel de Haen; Germany
MTT solution	Sigma Aldrich; Germany M2128
PBS (without Ca, Mg) solution	Gibco; USA 14190094
Lipopolysaccharides from *Escherichia coli*	Sigma Aldrich; Germany L4391
Succinate dehydrogenase	
Isopropanol	Sigma Aldrich; Germany 563935
N-(1-naphthyl) ethylenediamine	Fluka; Germany
o-phosphoric acid	Merck; Germany
Sodium nitrite ($NaNO_2$)	Riedel de Haen; Germany
Sulfanilamide	Fluka; Germany
Human Pancreatic (MIA PaCa-2) Cell line	ATCC; USA
Human Dermal Fibroblasts (HDF) Cell line	ATCC; USA
RAW 264.7 Murine Macrophage Cell line	ATCC; USA

Magnetic 3D 96-well Bioprinting Kit	Greiner Bio-One; Germany
NanoShuttle^{TM}-PL	Greiner Bio-One; Germany
IL-6 ELISA Kit	Invitrogen; USA
PGE_2 ELISA Kit	Abcam; USA

3.1.3. Equipments and Instruments

Balance	Ohaus Explorer
Beaker (50, 100, 250 mL)	Isolab
Graduated cylinder (25, 50, 100 mL)	Isolab
Volumetric flasks (50, 200 mL)	Isolab
Lyophilizator	Christ Alpha 2-4 LD
Micropipette (100-1000 microliters)	Isolab
Micropipette (20-200 microliters)	Transferpette
Microplate reader	Thermo Multiskan Ascent
Refrigerator	Arçelik
Rotatory Evaporator	Buchi, Heidolph
Vortex	Heidolph Reax
Water Bath	GFL
12 well plate	TPP; Germany
6 well plate	SPL LifeScience; Korea
96 well plate	Isolab; Turkey
Bead bath	Lab Armour; Cornelius,
Biotek plate reader	OR BioTek; USA
CellQuest Flow Cytometer	Becton Dickinson; USA
Centrifuge	Thermo Scientific SL 8R; Germany
ChemidocTM XRS system	Bio-Rad Laboratory; USA
Class-II Biosafety cabinette	ESCO Class-II Airstream; Singapore
CO_2 tube	Habaş; Turkey
Deep freezer	Arçelik; Istanbul, Turkey
Eppendorf tubes (1.5- 5 ml)	Eppendorf; Germany
Falcon tubes (15- 50 ml)	Falcon; Germany
Guava® easycyte flow cytometer	Merck KGaA; Darmstadt, Germany
Incubator	Binder CB-150; Germany

Micropipette (100-1000 µl)	Pipetman
Micropipette (10-100 µl)	Pipetman
Micropipette (1-10 µl)	Pipetman
Microplate washer	BioTek; USA
pH meter	Mettler Toledo MP220; US
Plate reader	Thermo Multiscan; Finland
Powerpac universal power supply	Bio-Rad Laboratory; USA
Primovert microscope	Carl Zeiss; Germany
PVDF Membrane	GE HealthCare LifeScience; Germany
Refrigerator	Arçelik; Istanbul, Turkey
Sterile filter (0.22 µm)	GVS; Germany
Sterile serological pipette (5-10 ml)	SPL LifeScience; Korea
Sterile syringe	Hayat Lab; Turkey
T-25/ 75/ 175 Flask	TPP; Germany
HPTLC Developing Chambers	CAMAG
HPTLC Plates	CAMAG
Ultrasonic bath	Sonorex RK156BH
HPTLC system	CAMAG
HPTLC glass 20 cm x 10 cm, Si 60 F254	Merck
Agilent Zorbax Plus C18 column	Agilent Chemical
Silica gel 60 F254 Aluminium sheet	Merck
RAW 264.7 mouse macrophage cells	ATCC (Rockville, MD)
Light microscopy	Carl Zeiss Primo Vert, Germany

3.2. Methods

3.2.1. Extraction Procedure

Five grams of powdered flowers, bracts, inflorescences (1:1 bracts and flower, w/w) mixture were extracted with 80% ethanol solution (ethanol: distilled water: 80:20, *v/v*) and each sample ultra-sonicated for 30 min. Then the samples were filtered through a filter paper and a syringe filter (0.45 µm). Ethanol was completely evaporated in a rotatory evaporator under reduced pressure at 40 °C. Then the extracts were lyophilized.

For the chromatographic analysis, the hydroalcoholic extract of each sample was dissolved in methanol (MeOH) to prepare 20 mg/mL (stock) for further analysis.

3.2.2. Chromatography

3.2.2.1. High-Performance Thin-Layer Chromatography

For the analysis, a precoated HPTLC silica gel 60 F_{254} glass plate (20 x 10 cm, Merck, Darmstadt, Germany) was used. Each *Tilia* sample solution (4 µL), and a mixture of standards (equal volumes of protocatechuic acid, hyperoside, isoquercitrin, astragalin, quercitrin, and tiliroside) (2 µL) were applied on HPTLC plates by a semi-automatic sample spotter Linomat 5 (Camag, Muttenz, Switzerland). The syringe delivery speed was 100 nL/s, and the bandwidth of the analytes was 8 mm. Ethyl acetate-formic acid-water (30:3:3, *v/v/v*) solvent system was used for plate development up to a migration distance of 7 cm for 20 minutes chamber saturation. With the Camag TLC plate heater, HPTLC plates were heated at 105 °C. For the derivatization of HPTLC plates, plates were immersed respectively in Natural Product and PEG 400 solutions which were prepared according to Güzelmeriç et al. (53) to figure out chemical composition differences between samples. HPTLC plates were investigated under different wavelengths at 254 or 366 nm and photos were taken with a Camag TLC visualizer. Thus, the identification of chemical composition is confirmed by using retention factor (R_F) and band colors. For all steps, the winCATS program (version 1.4.8, Camag) was used. The mobile phase, reference compounds, and derivatization reagents were given in Table 3.

Table 3. Mobile phase, reference compounds, and derivatization reagents

Mobile Phase	Reference compounds	Derivatization Reagents
Ethyl acetate-Formic acid-Water (30:3:3, *v/v/v*) (53)	Protocatechuic acid	Natural Product
	Hyperoside	
	Isoquercitrin	
	Astragalin	Polyethylene Glycol 400 (PEG 400)
	Quercitrin	
	Tiliroside	

3.2.2.2. High-Performance Liquid Chromatography

The UV spectrum and the retention times (t_R) were investigated to support the existence of each investigated compound. To test the specificity the chromatograms of blank and the lowest concentrated standards method specificity were comparatively analyzed. The investigated compounds protocatechuic acid, hyperoside, isoquercitrin, astragalin, quercitrin, and tiliroside were not monitored on the blank chromatogram and this indicated the specificity of the developed method.

UV spectra of protocatechuic acid, hyperoside, isoquercitrin, astragalin, quercitrin, and tiliroside were compared with the UV spectra of sample test solutions to confirm the identity of compounds. The chromatographic separations were performed with HP Agilent 1260 Infinity HPLC system with a diode array detector (DAD) which has a vacuum degassing system, an automatic sampler (G1329B), a thermostatted column compartment (G4212B), and a quaternary pump. The software used was Agilent Chem Station. Agilent Zorbax Plus C 18 column (4.6×250 mm) with a 5 μm particle size was used at 25˚C to supply optimal conditions to separate investigated compounds. This newly developed HPLC method was validated according with the International Conference on Harmonisation (ICH) guidelines sensitivity [limit of detection (LOD), limit of quantification (LOQ)], linearity (calibration curve), accuracy, precision, and system suitability was performed (113).

To achieve better resolutions and good peak shape different mobile phase compositions and flow rates were tested. The best separation was obtained with the Zorbax Plus C18 column with a 1 mL/min flowing rate. 10 μL was injected and each injection was performed in triplicates for standard solutions and samples. 2 mobile phases: As mobile phase A: ultra-purified water with 1% *o*-phosphoric acid and as mobile phase B acetonitrile were used. The gradient profile was as follows: 85–15% A-B (0–3 min), 82–18% A-B (3–11 min), 80–20% B (11–16 min), 70-30% A-B (16–22 min), 50–50% A-B (22–25 min), 20-80% A-B (25–26 min) and 85–15% A-B (26–30 min). Different wavelengths were used to monitor the analytes. These values were selected according to their UVmax value. Protocatechuic acid, hyperoside, isoquercitrin, and quercitrin were monitored at 260 nm, whereas astragalin was at 265 nm and tiliroside was at 320 nm.

Each lyophilized sample (50 mg) was dissolved in 5 mL of HPLC grade methanol. The obtained sample stock solutions with a 10 mg/mL concentration were fully dissolved with the help of an ultrasonicator. Stock solutions of each sample were first diluted to 1 mg/mL with MeOH, prior to analysis. Then each sample was prepared for HPLC analysis by filtering through a 0.45 µm RC-membrane filter (Sartorius Stedim Biotech).

Initially, the stock solutions of the standards were prepared by dissolving 150 mg of each standard in 5 mL of HPLC grade methanol and obtained 300 µg/mL stock solutions were further diluted with MeOH to make a calibration curve. For each compound, seven concentration levels (0.5-50 µg/mL) were selected for the calibration curve.

3.2.2.2.1. Validation of an HPLC Method For Quantification of Key Markers

Linear concentration ranges and areas of the standard solutions were measured by using optimum developed methods at 25℃. To estimate the calibration curve linearity standard solutions were applied in seven concentrations (0.5, 1, 2.5, 5, 10, 25, and 50 µg/mL) in triplicates. The concentration in mg/mL vs. calibration curve area values were determined as linear in the range of 0.5-50 mg/mL with r^2 values in the range of 0.0992-1 for standards as shown in Table 4. LOD and LOQ values were calculated by the formulation 3.3 x (SD/S) and 10 x (SD/S).

Table 4. Linearity data of the calibration curve, LOD, and LOQ values.

Standards	Linearity range	Parameters					
		r^2	b	a	SD	LOD µg/mL	LOQ µg/mL
Protocatechuic acid	0.5-50 µg/mL	1	37.548	0.0882	2,05	0,16	0,55
Hyperoside		0.9997	26.537	-13.849	2,32	0,26	0,87
Isoquercitrin		0.9997	26.991	2.8565	2,72	0,30	1,01
Astragalin		0.9992	27.314	-12.24	2,07	0,23	0,76
Quercitrin		1	29.569	1.2067	1,83	0,19	0,62
Tiliroside		1	30.195	2.0588	2,21	0,22	0,73

[a]The calibration equation was 'y=a+bx' SD: Standart deviation; LOD: Limit of detection; LOQ: Limit of quantification

Intermediate concentrations of standards (5 µg/mL) were analyzed in triplicates during the day to evaluate the intra-day precision of the method. Additionally, to determine the inter-day fluctuations, the same concentrations were analyzed in triplicates on consecutive days. To evaluate whether the method is repeatable, the relative standard

deviations are calculated with the obtained intra-day and inter-day results. The precision of the developed method is indicated by RSD values below 5 (Table 5).

Table 5. Repeatability and interprecision data.

Standards (5 µg/mL)	Intraday precision		Interday precision	
	Average value	RSD	Average value	RSD
Protocatechuic acid	5.100±0.149	2.923	5.116±0.128	2.519
	5.012±0.098	1.950		
	5.094±0.157	3.075		
Hyperoside	4.886±0.043	0.890	4.889±0.049	0.995
	4.835±0.030	0.630		
	4.874±0.066	1.355		
Isoquercitrin	5.150±0.069	1.331	5.178±0.074	1.439
	5.106±0.048	0.936		
	5.150±0.085	1.641		
Astragalin	4.771±0.056	1.165	4.786±0.050	1.048
	4.733±0.018	0.382		
	4.761±0.053	1.117		
Quercitrin	4.994±0.061	1.222	5.003±0.053	1.065
	4.950±0.036	0.731		
	4.986±0.061	1.222		
Tiliroside	5.035±0.061	1.206	5.060±0.058	1.145
	4.998±0.031	0.616		
	5.034±0.060	1.199		

[a]The average values were expressed as µg/mL±SD; *n*=3. RSD: Relative standard deviation

Each standard solution at varying concentrations (12.5, 6.25, 3.125 µg/mL) was also applied to the calibration curves of protocatechuic acid, hyperoside, isoquercitrin, and astragalin, quercitrin, and tiliroside and analyzed in triplicates to evaluate the accuracy of the method. These results were indicated as recovery (Table 6). Obtained and the determined concentration difference was determined below 5 (0.081-3.797) (RSD).

Table 6. Recovery results

Standards	Theoretical value	Amount found	Recovery (%)	RSD
Protocatechuic acid	3.125	3.109±0.117	99.5	3.797
	6.25	6.313±0.022	101.0	0.341
	12.5	12.449±0.052	99.6	0.418
Hyperoside	3.125	3.461±0.065	110.8	1.889
	6.25	6.319±0.019	101.1	0.294
	12.5	12.245±0.010	98.0	0.081
Isoquercitrin	3.125	2.846±0.068	91.1	2.405
	6.25	5.901±0.029	94.4	0.488
	12.5	12.012±0.027	96.1	0.227
Astragalin	3.125	3,403±0.045	108.9	1.309
	6.25	6.274±0.013	100.4	0.205
	12.5	12.636±0.013	101.1	0.104
Quercitrin	3.125	3.234±0.061	103.5	1.801
	6.25	6.482±0.014	103.7	0.211
	12.5	12.901±0.020	103.2	0.158
Tiliroside	3.125	3.028±0.040	96.9	1.330
	6.25	6.098±0.011	97.6	0.188
	12.5	12.317±0.036	98.5	0.292

[a]Theoretical value of the standards were expressed as µg/mL.

[b]The average found amounts were expressed as µg/mL±SD, *n*=3. RSD: Relative Standard Deviation

3.2.3. *In vitro* Evaluation of Antioxidant Capacity

3.2.3.1. Determination of Total Phenolic Content

Total phenolic contents (TPC) of different *Tilia* species were determined by Folin-Ciocalteu colorimetric method described by Güzelmeriç et al. (102) with slight modifications. This assay relies on electron transfer of the pale yellow-colored Folin-Ciocalteu reagent which includes a mixture of phosphomolybdic acid phosphotungstic acid with phenolic compounds in *Tilia* and results in a blue coloration (114).

In the experiment, 10% (*w/v*) Na_2CO_3 and Folin–Ciocalteu reagent 1:2 diluted with distilled water were prepared. To make a calibration curve, reference solutions were prepared with gallic acid in different concentrations from 31.25 to 1000 µg/mL. Each *Tilia* sample or blank (MeOH) was diluted with distilled water up to 50 µL and in a 96-well plate mixed with 50 µL Folin–Ciocalteu reagent and 100 µL of prepared Na_2CO_3 respectively. Prepared solutions were kept in the dark at room temperature for 1 hour after vortexing. The absorbance was measured using UV Spectrophotometer (Multiscan Go,

Thermo Scientific) at 760 nm. All analyses were performed in triplicates and total phenolic contents of extracts were calculated with the slope of the gallic acid standard curve and expressed as mg of gallic acid equivalents (GAE) per g of *Tilia* samples (mg GAE/g hydroalcoholic *Tilia* extract). The obtained calibration curve is shown in Figure 11.

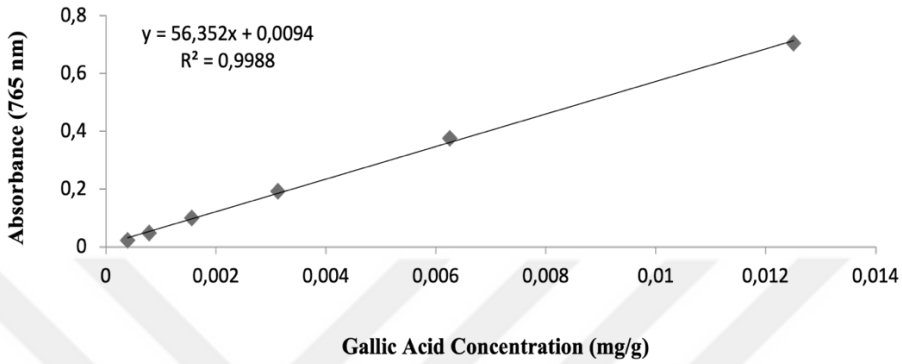


Figure 11. Total phenolic content standard curve of gallic acid concentration

3.2.3.2. Determination of Total Flavonoid Content

The total flavonoid content (TFC) was determined by performing aluminum chloride colorimetric assay in *Tilia* samples with the method described by Güzelmeriç et al. (102) with slight modifications. Quercetin was used as a reference for the determination of TFC. With serial dilutions, various concentrations of quercetin were prepared. The standard solutions of quercetin were prepared at 3.9063–125 µg/mL concentrations. The obtained calibration curve is shown in Figure 12.

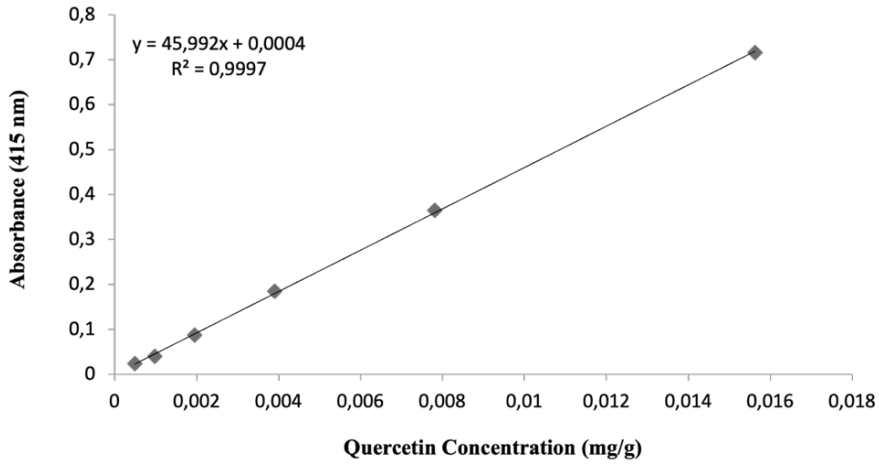


Figure 12. Total flavonoid content standard curve of quercetin concentration

30 µL *Tilia* test solution or blank (MeOH) or standard, 30 µL 10% aluminum chloride, 30 µL 1M sodium acetate was added to a 96-well plate and the volume was adjusted to 240 µL with distilled water. After 30 minutes of incubation at room temperature, the absorbance was measured at 415 nm. All analyses were performed in triplicates and the total flavonoid content of the extracts was calculated with the slope of the quercetin standard curve, and it was expressed as mg quercetin equivalents/g (mg QE/g *Tilia* extract) of hydroalcoholic *Tilia* extract.

3.2.3.3. DPPH Radical-Scavenging Activity Assay

DPPH radical scavenging activity assay was performed with the method described by Güzelmeriç et al. (102) with minor changes. Prior to analysis, 0.0394 g/l. DPPH is dissolved in methanol to prepare a 0.1 mM DPPH solution which should be stored in dark. The assay was performed with a 96-well plate. Trolox was used as a reference, and solutions were prepared in seven different concentrations, in a range of 0.003125-0.2 mg/mL in 80% methanolic solution. The obtained calibration curve is shown in Figure 13.

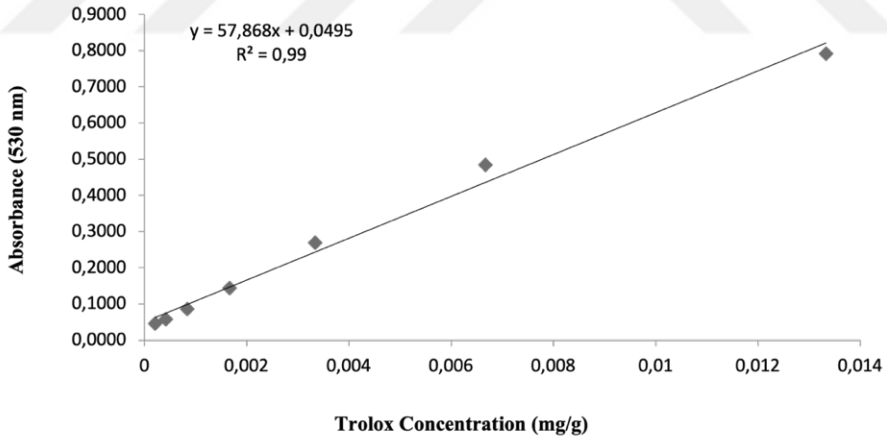


Figure 13. DPPH method standard curve of Trolox Concentration

Tilia species were diluted to 0.1 mg/mL with methanol. After dilutions, 20µl of each *Tilia* sample was mixed with 280 µL DPPH solution in a 96-well plate in triplicates and after vortexing for ten seconds stored away from light for 30 minutes. The absorbances were determined at 530 nm wavelength. All determinations were performed in triplicates. The results were presented in mg Trolox equivalent (TE)/g of hydroalcoholic extract (mg TE/g hydroalcoholic *Tilia* extract).

3.2.3.4. Ferric Ion Reducing Antioxidant Power Assay

Antioxidant capacities of the samples were measured colorimetrically by FRAP assay according to Berker et al. (115) with slight modifications. Prior to analysis solutions are prepared for FRAP assay. First, 100 mL acetate buffer solution (pH 3.6) was prepared by adding 3.1 g sodium acetate trihydrate (CH_3COONa) to 16 mL of glacial acetic acid and adjusting the volume to 1000 mL with distilled water. Secondly, 1M and 100 mL hydrochloric acid solution (HCl) was prepared with 8.211 mL of 37% hydrochloric acid solution and completed to 100 mL with distilled water. The third solution was prepared by dissolving 0.2703 g of iron (III) chloride anhydrous in 1 mL of previously prepared 1 M of HCl solution and completing the volume to 50 mL with distilled water to obtain 0.02 M iron (III) chloride hexahydrate solution. For the 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) solution, 0.156 g of TPTZ was weighed which need to be stored in a dark glass bottle and completed to 50 mL with ethanol. Finally, for the FRAP reagent is prepared which should be prepared freshly prior to analysis, 100 mL acetate buffer (pH 3.6) solution, 0.02 M 10 mL iron (III) chloride hexahydrate solution were mixed at a 10:1:1 ratio. As a reference, Trolox was used. Once the FRAP reagent was prepared, 280 µL of FRAP reagent and 20 µL of *Tilia* extracts/standard Trolox were added to a 96-well plate. After 6 minutes following the FRAP agent, the absorbances were determined at 595 nm. Calibration solutions containing 6 different concentrations ranging from 0.003125-0.1 mg/mL of 80% methanolic Trolox solution were used instead of samples to determine blank absorbances. All determinations were performed in triplicates. The results were presented in mg Trolox equivalent (TE)/g of hydroalcoholic extract (mg TE/g hydroalcoholic *Tilia* extract). The obtained calibration curve is shown in Figure 14.

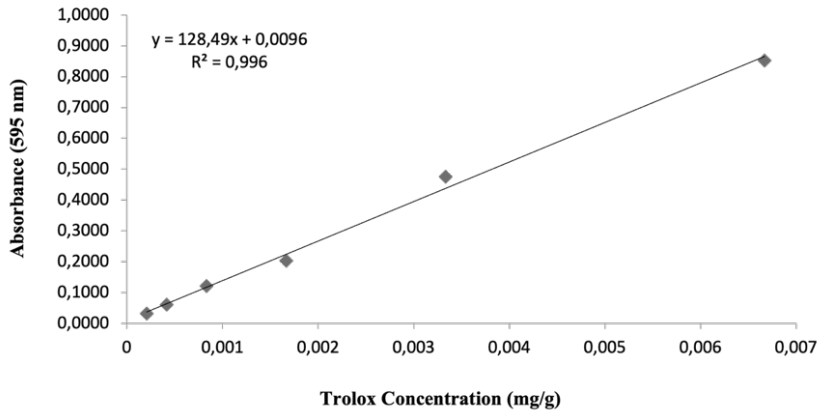


Figure 14. FRAP method standard curve of Trolox Concentration

3.2.3.5. Cupric Reducing Antioxidant Capacity Assay

Antioxidant capacities of *Tilia* extracts were determined colorimetrically with CUPRAC assay according to Apak et al. (108) with slight modifications. Before analysis copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$) solution at a concentration of 10^{-2} M, neocuproine (Nc) solution in ethanol at a concentration of $7.5x10^{-3}$ M and ammonium acetate (NH_4Ac) buffer at pH 7.0 were prepared. 10 mM $CuSO_4.5H_2O$ solution was prepared by dissolving 62.31 mg of $CuSO_4.5H_2O$ in 25 mL of distilled water. For 7.5 mM neocuproine, 39.04 mg of neocuproine was completed to 25 mL volume with methanol. For ammonium acetate buffer (pH 7) 1927 mg of ammonium acetate was dissolved in 25 mL of distilled water.

After preparation of reagents, 43 µL *Tilia* extracts were mixed with 85 µL $CuSO_4 \cdot 5H_2O$, 85 µL neocuproine, 85 µL ammonium acetate, and 51 mL distilled water. After 20 minutes of incubation at 50 °C, the absorbance was determined at 450 nm. As a blank, distilled water and as reference Trolox in 0.003125-0.2 mg/mL concentrations were used. Trolox was the calibration reference standard and was used at seven different concentrations in the range of 0.003125–0.2 mg/mL. All determinations were performed in triplicates. The results were presented in mg Trolox equivalent (TE)/g of hydroalcoholic extract (mg TE/g hydroalcoholic extract of *Tilia*). The obtained calibration curve is given in Figure 15.

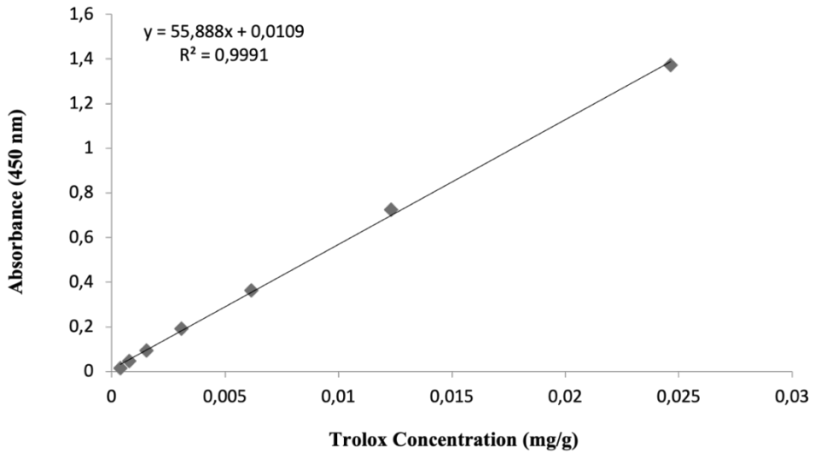


Figure 15. CUPRAC method standard curve of Trolox Concentration

3.2.4. Cell Culture Studies

RAW 264.7 mouse macrophage cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, NY, USA) enriched with 1% penicillin (10.000 units/mL) and streptomycin (10.000 µg/mL) and 10% Fetal Bovine Serum (FBS) at 37 °C at a humidified atmosphere of 5% CO_2.

3.2.4.1. Cell Cytotoxicity by MTT Assay

In living, cells, water-soluble and yellow-colored MTT is reduced to blue-violet-colored water-insoluble formazan crystals as seen in Figure 16. The absorbance of color intensity of formazan crystals solved in isopropanol is determined to calculate the number of living cells (116).

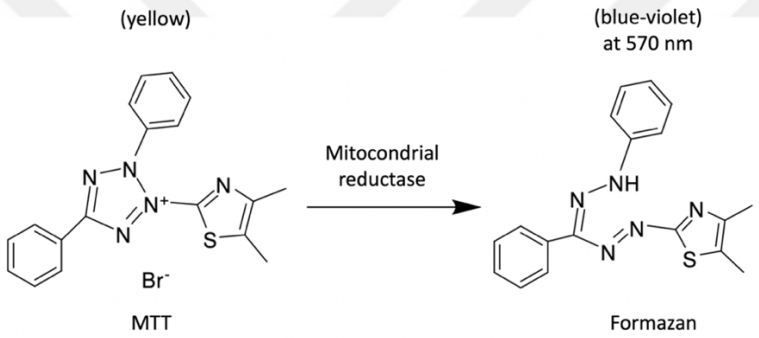


Figure 16. Structure of MTT dye and colored formazan product.

MTT colorimetric assay was used for the cytotoxicity analysis according to the method described by Aydın et al. (117). Prior to analysis, MTT stock solution (5 mg/mL) was prepared by dissolving 250 mg of MTT powder in 50 mL of PBS solution and stored at -20^{o}C with aluminum foil-covered Eppendorf tubes. For each experiment, it was freshly diluted (1:10, *v/v*). The plated cells were treated with different concentrations of *Tilia* species extracts (1 mg/mL). The plates were then incubated for 24h. After removal of medium, MTT reagent (0.5 mg/mL) was applied and incubated for another 2h. MTT is reduced in the mitochondria of viable cells by succinate dehydrogenase to form water-insoluble purple formazan crystal. Produced formazan amount is proportional to the number of viable cells. After 2h of incubation, medium removal is performed and to dissolve formazan crystals 100 µL isopropanol was added to wells. Further experiments were conducted at concentrations where the determined percent cell viability was 70% or

higher compared to the control. Then the absorbance was measured with a microplate reader (Thermo Multiscan Spectrum, Vantaa, Finland) at 570 nm wavelength. The test procedure was performed in triplicates. The cell viability in percentages was determined with the following equation:

$$\textbf{Viability of cell (\%)} = 100 \text{ x } \quad OD_{570test} / OD_{570control}$$

OD570test: Mean value of measured optical density of the tested substance
OD570control: Mean value of measured optical density of the negative control group

3.2.4.2. Determination of Nitrite Level

The anti-inflammatory activity of different species of *Tilia* was assessed by determining the oxidation metabolite of nitric oxide, and nitrite levels. Nitrite levels in the extracts were determined using a stable end product of nitric oxide (NO), Griess reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 5% phosphoric acid and 1% sulfanilamide) (118). RAW 264.7 cells were seeded in a 96-well plate at a density of 5×10^4 cells/well. Then, they were incubated at 37 °C in 5% CO_2 for 24 h. Cells were pretreated with different concentrations of *Tilia* extracts. (0.125, 0.25, 0.5, and 1 mg/mL) for 2h. After that, LPS (1 μg/mL) was used for stimulation of cells for 22 hours. The supernatant was collected, and 50 μl of the supernatant was mixed with 50 μl of Griess reagent. Then the mixture was incubated for 10 minutes in the dark. The absorbance was determined at 540nm spectrophotometrically with a microplate reader (Multiscan Ascent, Vantaa, Finland). The nitrite levels were determined by the sodium nitrite calibration curve. N(gamma)-nitro-L-arginine methyl ester (L-Name; 0.1 mg/mL) was used as positive control. For the preparation of the Griess reagent, two separate solutions were freshly prepared and mixed 1:1 (*v/v*) before each analysis. For the first solution, 300 mg of sulfanilamide (1%, *w/v*) and 1.5 mL of o-phosphoric acid are mixed, and the volume was completed to 30 mL with distilled water. For second solution 30 mg of N-(1-naphthyl) ethylenediamine (0.1%, *w/v*) completed to 30 mL with distilled water. Each solution must be kept in dark at 4 °C.

3.2.4.3. IL-6 Releasing Inhibition Assay

Tilia species were used as pretreatment on RAW 264.7 cells for two hours, followed by a 22-hour LPS (1 μg/mL) stimulation. Given the high IL-6 value of the

samples, dilution was performed five times (119). The manufacturer's recommendations were followed while utilizing a commercially available quantitative enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen, USA) to measure the quantity of IL-6.

3.2.4.4. Evaluation of Analgesic Activity with PGE_2 Levels

The suppressing ability of PGE_2 levels in LPS-activated murine macrophage cells was used to measure the analgesic effect of *Tilia* species (120). Using an ELISA kit from Abcam (USA), the release of PGE_2 from cell supernatants was measured in duplicates in accordance with the manufacturer's instructions.

3.2.4.5. Anticancer Activity

3.2.4.5.1. Two-Dimensional (2D) Cell Cytotoxicity Assay

Anticancer activity of different *Tilia* species was investigated using 2D cell cytotoxicity and 3D spheroid formation/growth assays. For this purpose, human pancreatic cancer cell line MIA PaCa-2 (CRM-CRL-1420) and human dermal fibroblast cell line HDF (PCS-201-012) were obtained from the American Type Culture Collection (ATCC, USA). Cell lines were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, 1% penicillin (10.000 units/mL), and streptomycin (10.000 µg/mL) at 37°C under a humidified atmosphere of 5% CO_2.

The MIA PaCa-2 cells and HDF cells (10.000 cells/well) were seeded on a 96-well cell culture plate. To determine 2D anticancer activity cells were left for 24 hours for incubation. After the incubation period for determining non-cytotoxic concentration, cells were treated with *Tilia* sample extracts in different concentrations (0.125-0.250-0.5-1 mg/mL) and left for incubation. After 24 hours of the incubation period, the culture medium was removed and an MTT assay reagent (0.5 mg/mL) was added to all wells and left for incubation for 2 hours at 37 °C. After 2 hours of the incubation period, the culture medium was removed and in order to dissolve formazan crystals, 100 µL of isopropanol was added to the wells. The absorbance was measured at 570 nm wavelength with an ELISA microplate reader (Thermo Multiskan Spectrum, Finland). Cell viability was calculated as percentages by the following equation:

$$\text{Viability\%} = (\text{Absorbance treatment group}) / (\text{Absorbance control}) \times 100\%$$

3.2.4.5.2. Three-Dimensional (3D) Spheroid Formation/Growth Assay

For 3D spheroid formation, the MIA PaCa-2 cells and HDF cells were used according to the 3D Bioprinting method (121). Human fibroblasts were mixed equally with pancreatic cancer cells to better stimulate the tumor environment. After cells reached 70-80% confluency, cells were treated with biocompatible NanoShuttle^{TM}-PL (Bioprinting Kit, Greiner Bio-One, Germany) and incubated overnight. After the incubation period, cells were resuspended and seeded into ultra-low attachment 96-well culture plates with a 100 µL (2.000 pancreatic cancer cells and 2.000 human fibroblasts per well) volume. Until spheroids were formed, the culture plate was placed on a magnetic drive and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C for 48 hours. Two days after the incubation period, the spheroids were photographed using light microscopy (Carl Zeiss Primo Vert, Germany). After that, spheroids were treated with flowers, bracts and inflorescences samples of *T. cordata, T. tomentosa, T. rubra,* and *T. platyphyllos* extracts with the same concentration interval tested in RAW 264.7 cells (0.125, 0.250, 0.500, and 1 mg/mL). For ten days, the treatment media was replaced with a fresh medium, and photos of spheroids were taken every 3 days. The effect of *Tilia* extracts on spheroid growth was assessed by measuring the size of the spheroids changing by using ImageJ software (Image J.2.0 software, USA). Doxorubicin was used as a reference anticancer molecule (122). All the measurements were performed in triplicated.

3.2.5. Statistical Analysis

All repeated experiments were conducted in triplicate. Statistical analysis was observed by using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA; Version 8.4.3) and Excel. Differences between groups were determined by using one-way ANOVA following the posthoc tests by Tukey. Group differences were considered to be significant at *$p<0.05$.

4. RESULTS

4.1. Results of Chromatographic Analysis

4.1.1. High-Performance Thin Layer Chromatography Results

Hydroalcoholic extracts of different parts of four *Tilia* species were analyzed by HPTLC using ethyl acetate- formic acid- water (30:3:3, *v/v/v*) as mobile phase. HPTLC chromatogram of the analyzed samples are shown in Figure 17. The R_F values and band color of standards after derivatization with Natural Product and PEG 400 were given in at 366 nm.

According to HPTLC chromatogram of hydroalcoholic extracts of samples, retention factors (R_F) of standards hyperoside ($R_F \approx 0.38$), isoquercitrin ($R_F \approx 0.42$), astragalin ($R_F \approx 0.50$),), quercitrin ($R_F \approx 0.58$), tiliroside ($R_F \approx 0.72$) and protocatechuic acid ($R_F \approx 0.88$) were determined in the blank solution mixture from bottom to top. It was determined that there was a high similarity between samples in terms of their HPTLC fingerprint. As shown in Figure 17, hyperoside ($R_F \approx 0.38$, orange band color) was detected only in *T. cordata* bracts, *T. rubra* flowers bracts and inflorescences. Isoquercitrin ($R_F \approx 0.42$, orange band color) was detected in all samples while it was mainly found in *T. cordata* flowers. Astragalin ($R_F \approx 0.50$, green band color) was found notably in *T. cordata* flowers, *T. cordata* inflorescences, *T. tomentosa* flowers, *T. tomentosa* inflorescences, T. *platyphyllos* flowers and *T. platyphyllos* inflorescences while it was mainly detected in *T. tomentosa* flowers. Quercitrin ($R_F \approx 0.58$, orange band color) was found mainly in *T. rubra* bracts, *T. rubra* inflorescences and *T. cordata* bracts. Tiliroside ($R_F \approx 0.72$, green band color) was found notably in samples *T. cordata* flowers, *T. cordata* inflorescences, *T. tomentosa* flowers, *T. tomentosa* bracts, *T. tomentosa* inflorescences, *T. platyphyllos* flowers, *T. platyphyllos* bracts and *T. platyphyllos* inflorencences. Lastly, protocatechuic acid ($R_F \approx 0.88$, blue band color) was detected in all samples, mainly in *T. cordata* bracts followed by *T. cordata* inflorescences. In addition to the investigated standards, unknown compounds were detected with intense yellow band colors in $R_F \approx 0.50$ in samples of *T. cordata* and *T. rubra*.

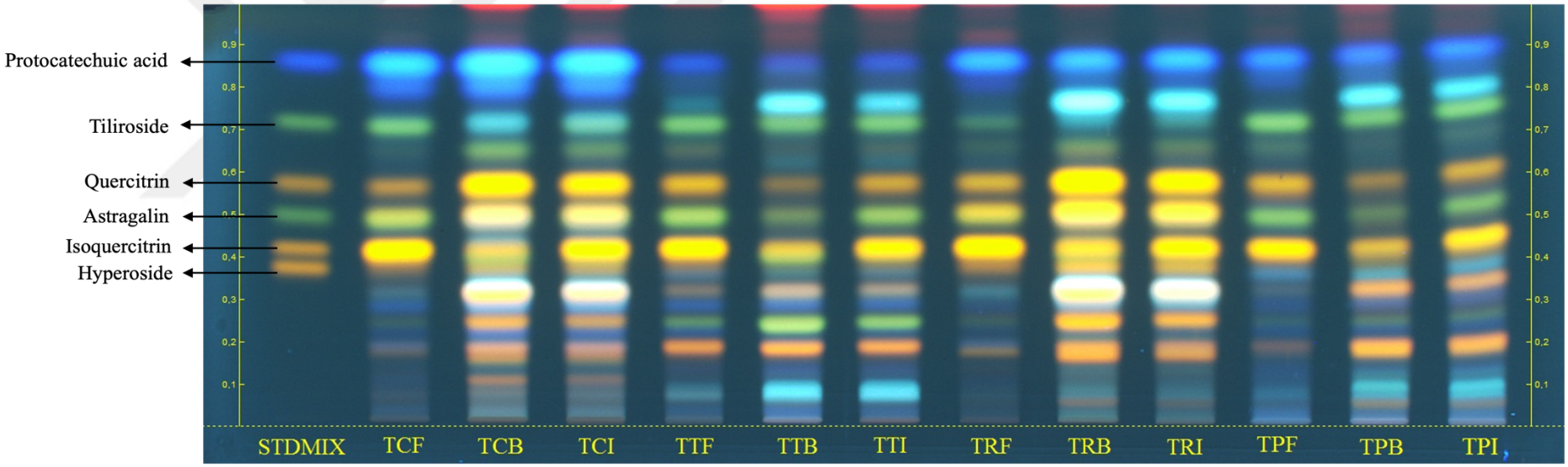


Figure 17. HPTLC chromatogram of flower, bract and inflorescence extracts of TC, TT, TR and TP.

TC: *T. cordata*, TT: *T. tomentosa*, TR: *T. rubra*, TP: *T. platyphyllos*

4.1.2. High-Performance Liquid Chromatography Results

The concentrations of investigated phenolic compounds, protocatechuic acid, hyperoside, isoquercitrin, astragalin, quercitrin and tiliroside in hydroalcoholic extracts of different *Tilia* species samples were quantified by an HPLC method. HPLC chromatograms of standard mixture and samples and t_R (retention time) values of standards were given in Figure 18-30.

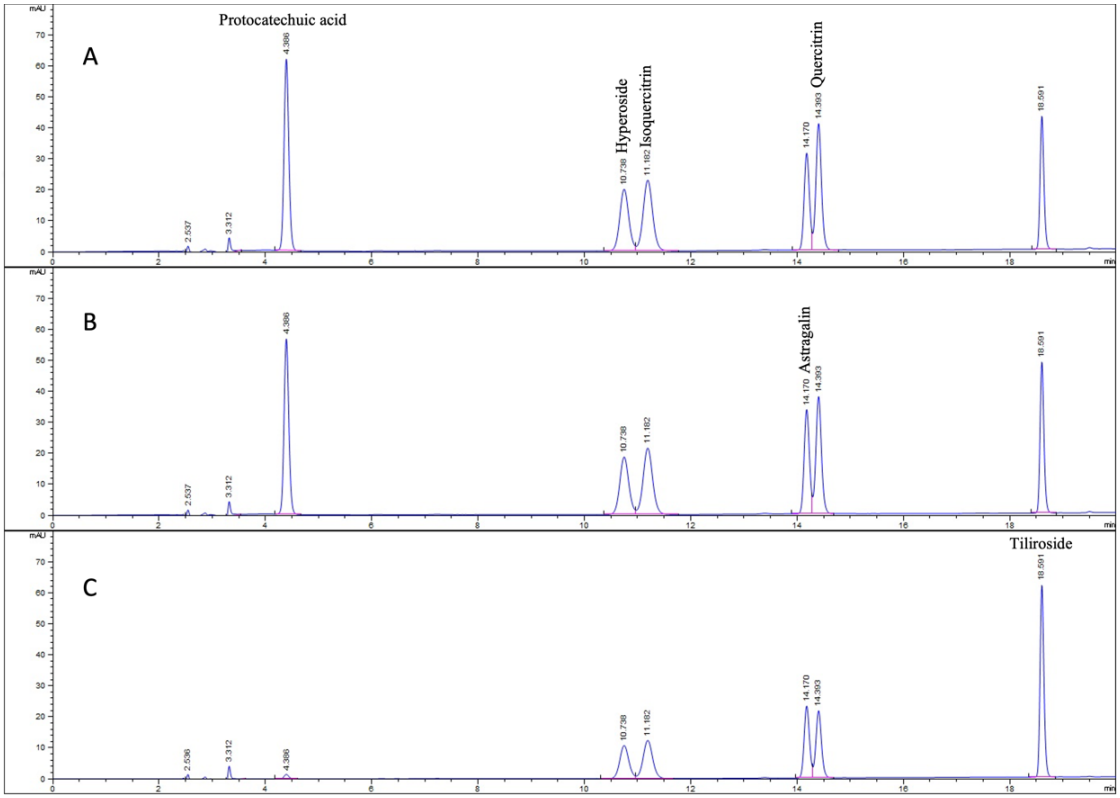


Figure 18. HPLC chromatograms of the standard mixture solution (10 µg/mL*)* at 260 nm (A), at 265 nm (B) and at 320 nm (C).

The t_R values were as follows: protocatechic acid (4.38 ± 0.01), hyperoside (10.74 ± 0.01), isoquercitrin (11.18 ± 0.01), astragalin (14.7 ± 0.01), quercitrin (14.393 ± 0.01) and tilirose (18.59 ± 0.01)

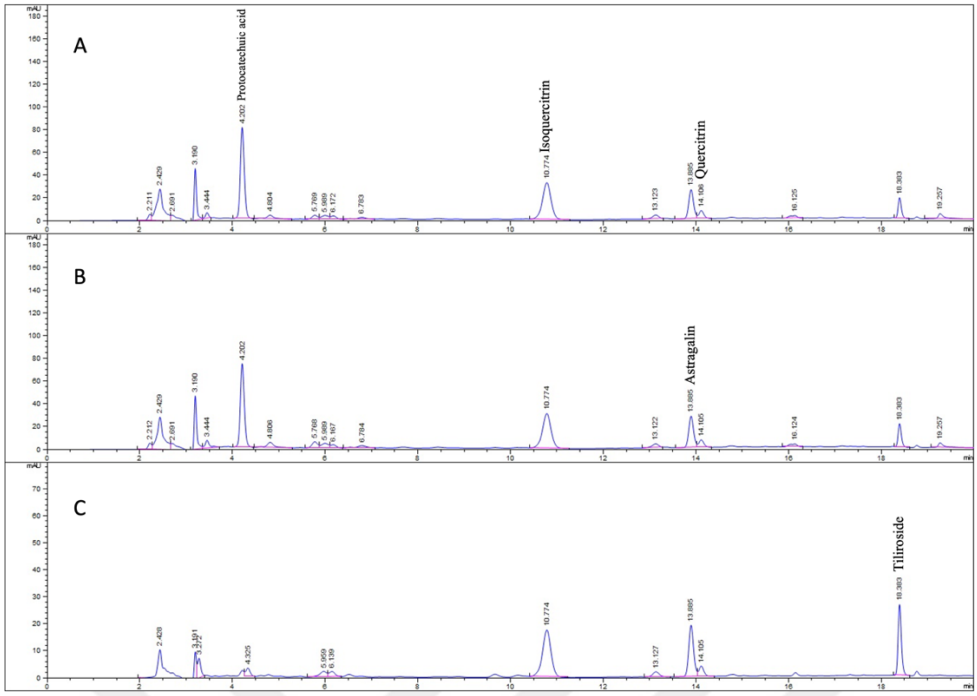


Figure 19. HPLC chromatograms of *T. cordata* flowers extract (1 mg/mL) at 260 nm (A), at 265 nm (B) and at 320 nm (C).

The t_R values were as follows: protocatechic acid (4.2 ± 0.01), hyperoside (n.d.), isoquercitrin (10.77 ± 0.01), astragalin (13.88 ± 0.01), quercitrin (14.1 ± 0.01) and tilirose (18.38 ± 0.01)

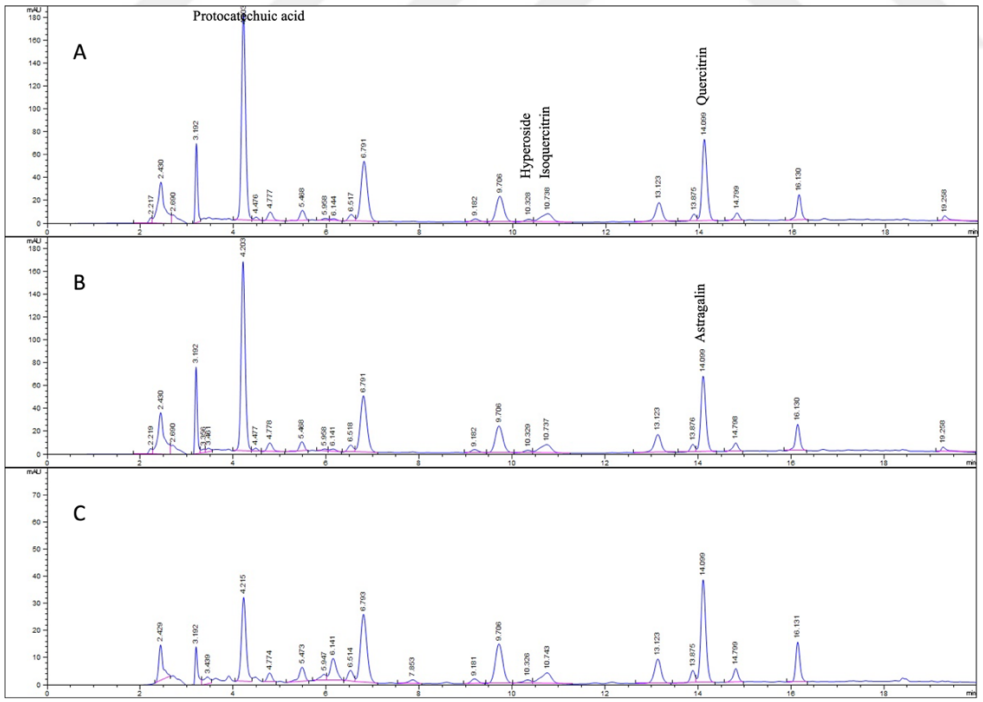


Figure 20. HPLC chromatograms of *T. cordata* bracts extract (1 mg/mL*)* at 260 nm (A), at 265 nm (B) and at 320 nm (C).

The t_R values were as follows: protocatechic acid (4.20 ± 0.01), hyperoside (10.33 ± 0.01), isoquercitrin (10.74 ± 0.01), astragalin (13.87 ± 0.01), quercitrin (14.10 ± 0.01) and tilirose (18.39 ± 0.01)

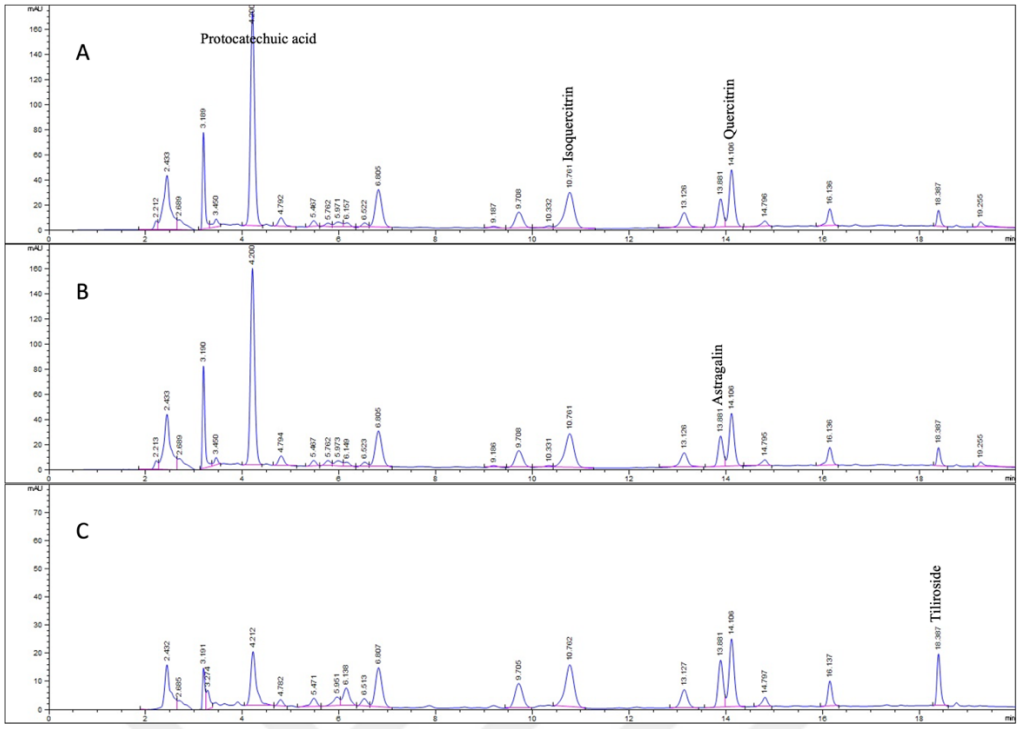


Figure 21. HPLC chromatograms of *T. cordata* inflorescences extract (1 mg/mL) at 260 nm (A), at 265 nm (B) and at 320 nm (C).

The t_R values were as follows: protocatechic acid (4.20 ± 0.01), hyperoside (n.d.), isoquercitrin (10.76 ± 0.01), astragalin (13.88 ± 0.01), quercitrin (14.10 ± 0.01) and tilirose (18.39 ± 0.01)

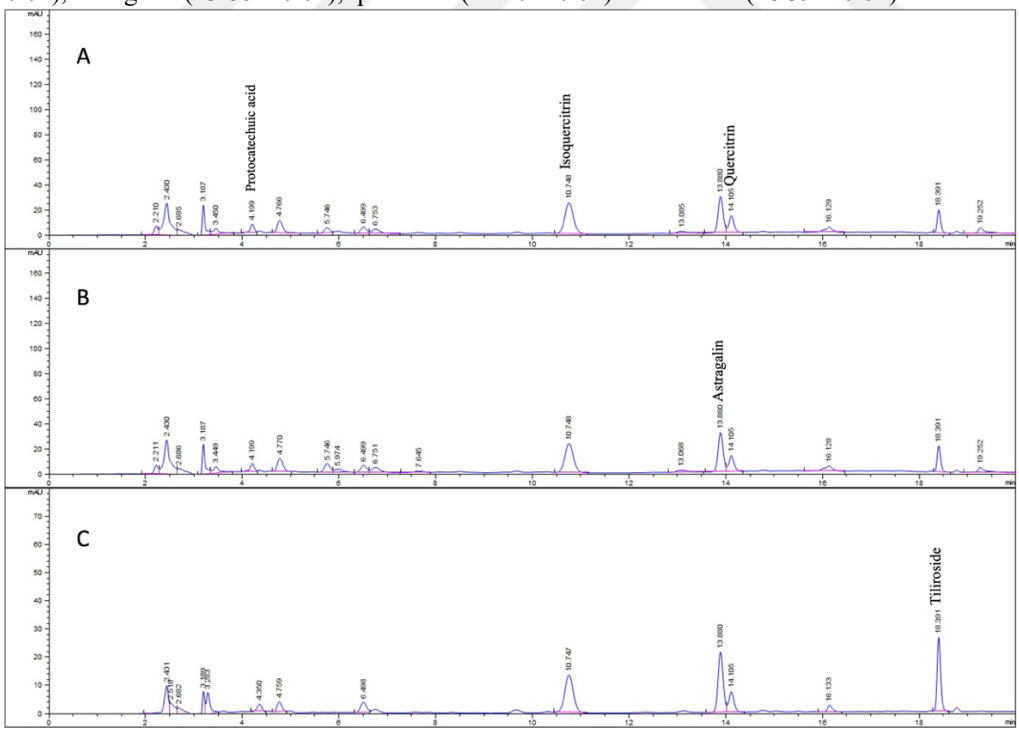


Figure 22. HPLC chromatograms of *T. tomentosa* flowers extract (1 mg/mL) at 260 nm (A), at 265 nm (B) and at 320 nm (C).

The t_R values were as follows: protocatechic acid (4.20 ± 0.01), hyperoside (n.d.), isoquercitrin (10.75 ± 0.01), astragalin (13.88 ± 0.01), quercitrin (14.10 ± 0.01) and tilirose (18.39 ± 0.01)

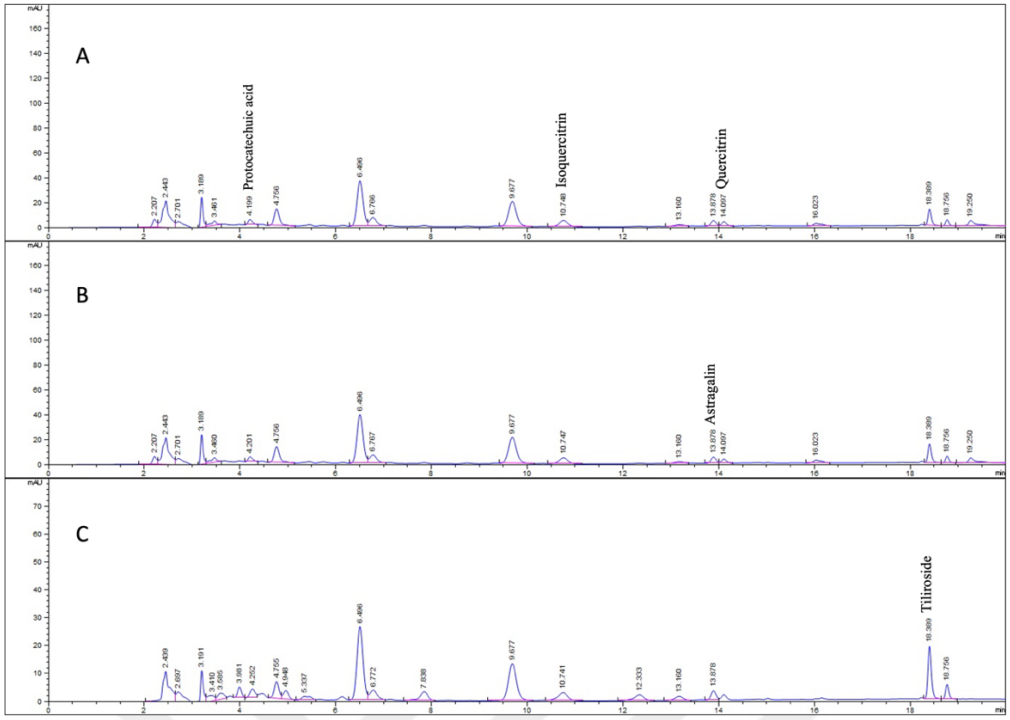


Figure 23. HPLC chromatograms of *T. tomentosa* bracts extract at (1 mg/mL) 260 nm (A), at 265 nm (B) and at 320 nm (C).

The t_R values were as follows: protocatechic acid (4.20 ± 0.01), hyperoside (n.d.), isoquercitrin (10.75 ± 0.01), astragalin (13.88 ± 0.01), quercitrin (14.01 ± 0.01) and tilirose (13.39 ± 0.01)

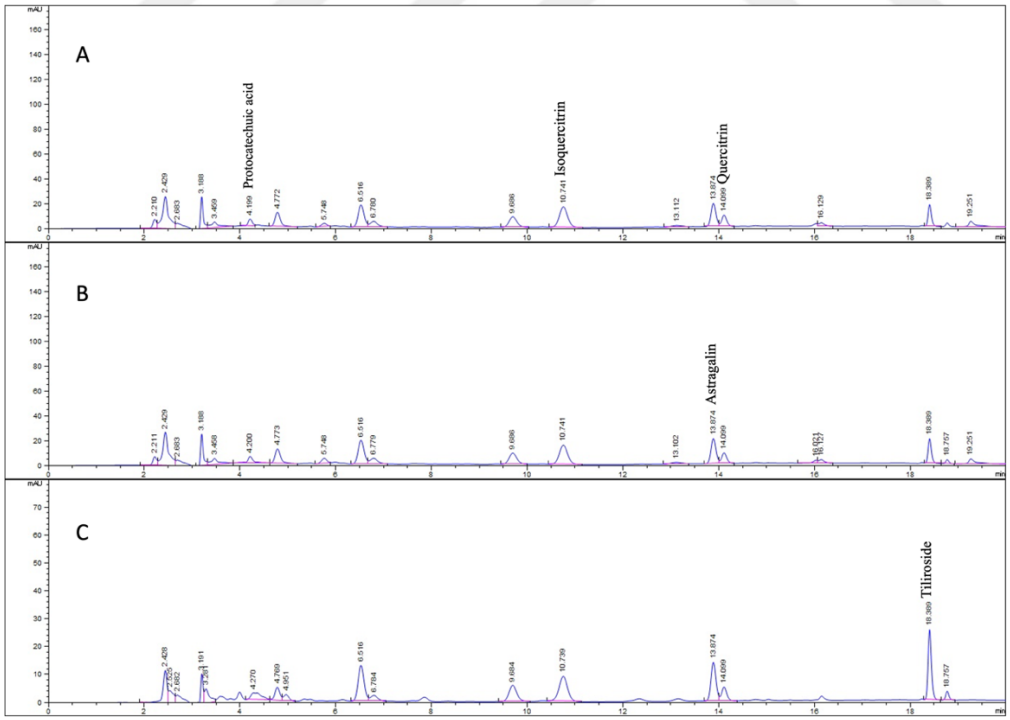


Figure 24. HPLC chromatograms of *T. tomentosa* inflorescences extract (1 mg/mL) at 260 nm (A), at 265 nm (B) and at 320 nm (C).

The t_R values were as follows: protocatechic acid (4.20 ± 0.01), hyperoside (n.d.), isoquercitrin (10.75 ± 0.01), astragalin (13.88 ± 0.01), quercitrin (14.01 ± 0.01) and tilirose (18.39 ± 0.01)

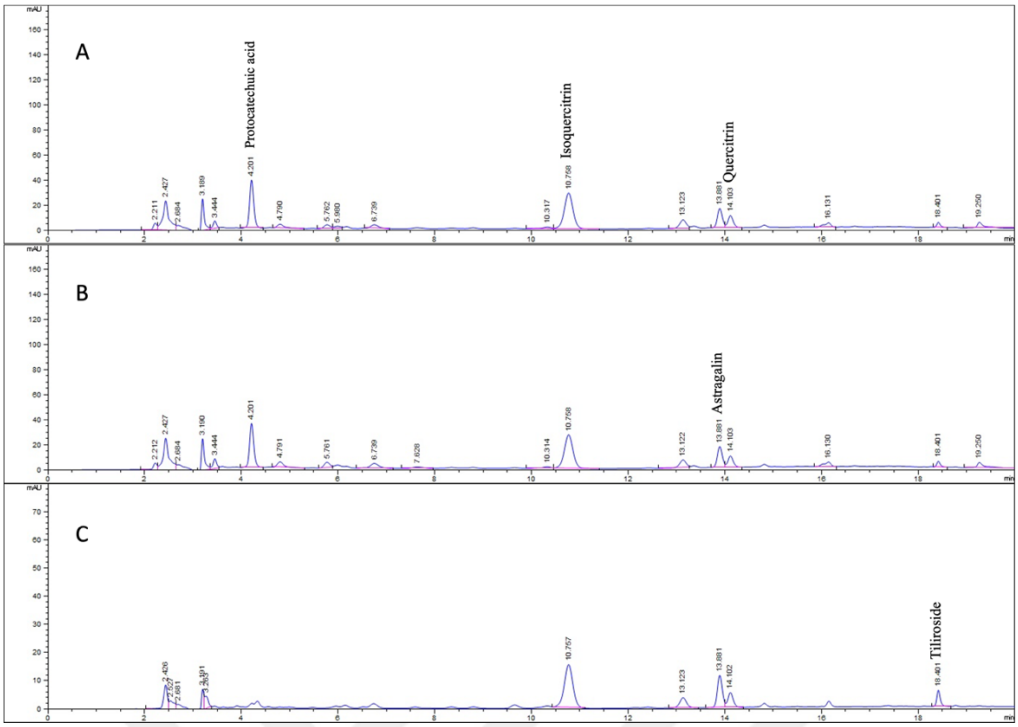


Figure 25. HPLC chromatograms of *T. rubra* flowers extract (1 mg/mL) at 260 nm (A), at 265 nm (B) and at 320 nm (C).

The t_R values were as follows: protocatechic acid (4.20 ± 0.01), hyperoside (n.d.), isoquercitrin (10.76 ± 0.01), astragalin (13.88 ± 0.01), quercitrin (14.10 ± 0.01) and tilirose (18.40 ± 0.01)

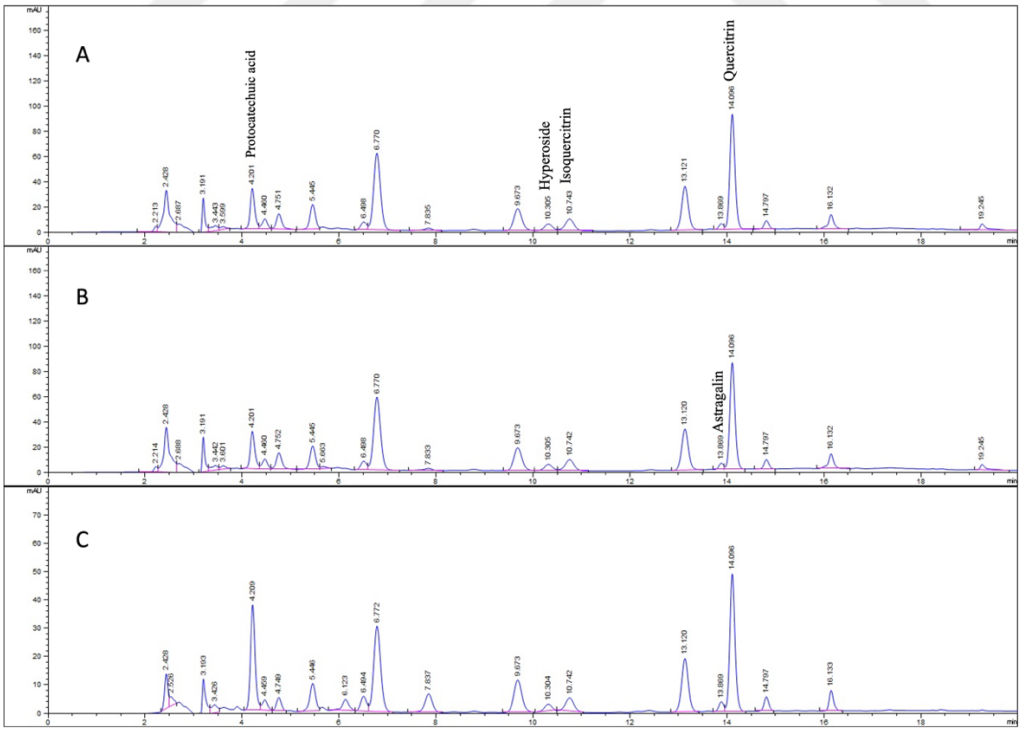


Figure 26. HPLC chromatograms of *T. rubra* bracts extract at (1 mg/mL) 260 nm (A), at 265 nm (B) and at 320 nm (C).

The t_R values were as follows: protocatechic acid (4.20 ± 0.01), hyperoside (10.30 ± 0.01), isoquercitrin (10.74 ± 0.01), astragalin (13.87 ± 0.01), quercitrin (14.09 ± 0.01) and tilirose (n.d.)

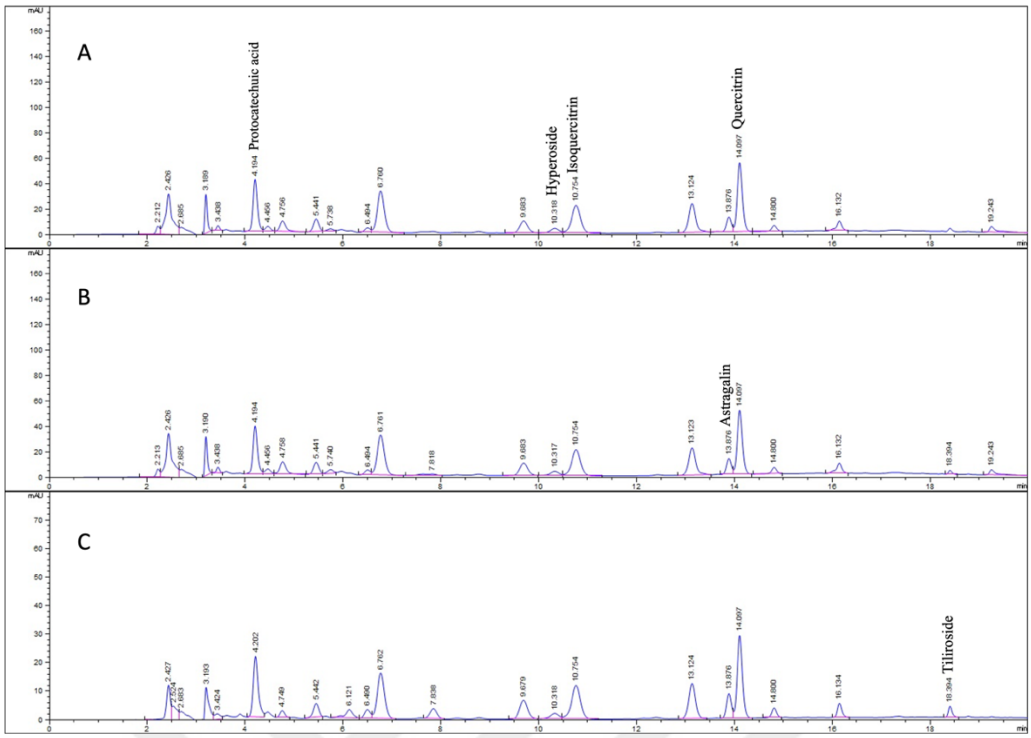


Figure 27. HPLC chromatograms of *T. rubra* inflorescences extract (1 mg/mL) at 260 nm (A), at 265 nm (B) and at 320 nm (C).

The t_R values were as follows: protocatechic acid (4.19 ± 0.01), hyperoside (10.32 ± 0.01), isoquercitrin (10.75 ± 0.01), astragalin (13.87 ± 0.01), quercitrin (14.10 ± 0.01) and tilirose (18.39 ± 0.01)

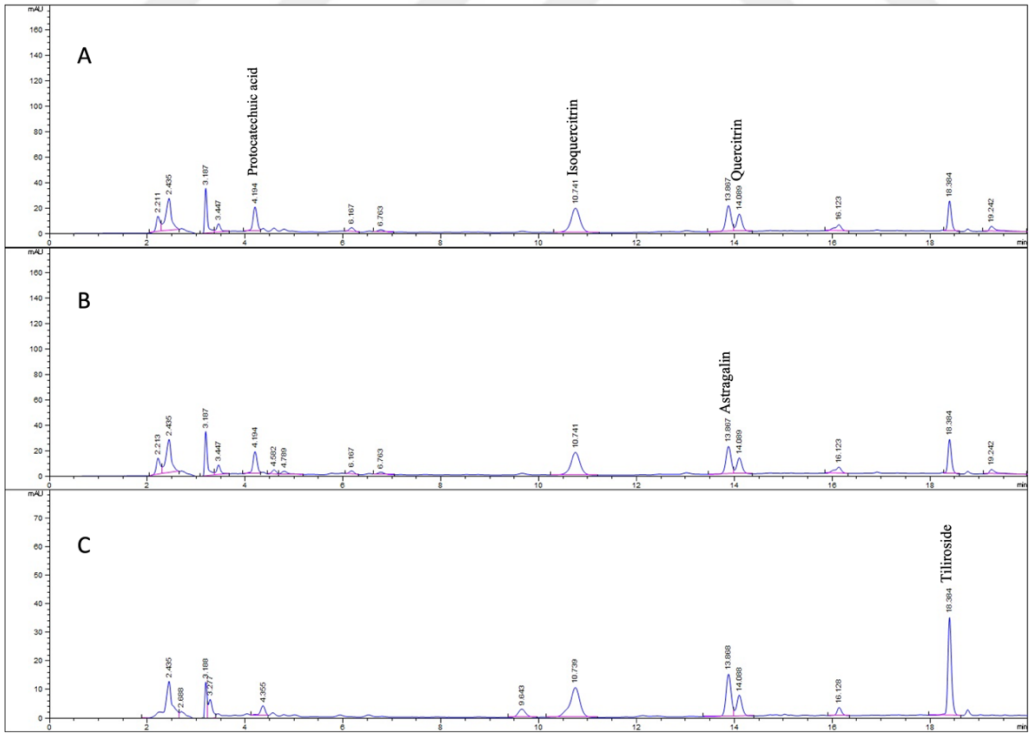


Figure 28. HPLC chromatograms of *T. platyphyllos* flowers extract (1 mg/mL) at 260 nm (A), at 265 nm (B) and at 320 nm (C).

The t_R values were as follows: protocatechic acid (4.19 ± 0.01), hyperoside (n.d.), isoquercitrin (10.74 ± 0.01), astragalin (13.86 ± 0.01), quercitrin (14.09 ± 0.01) and tilirose (18.38 ± 0.01)

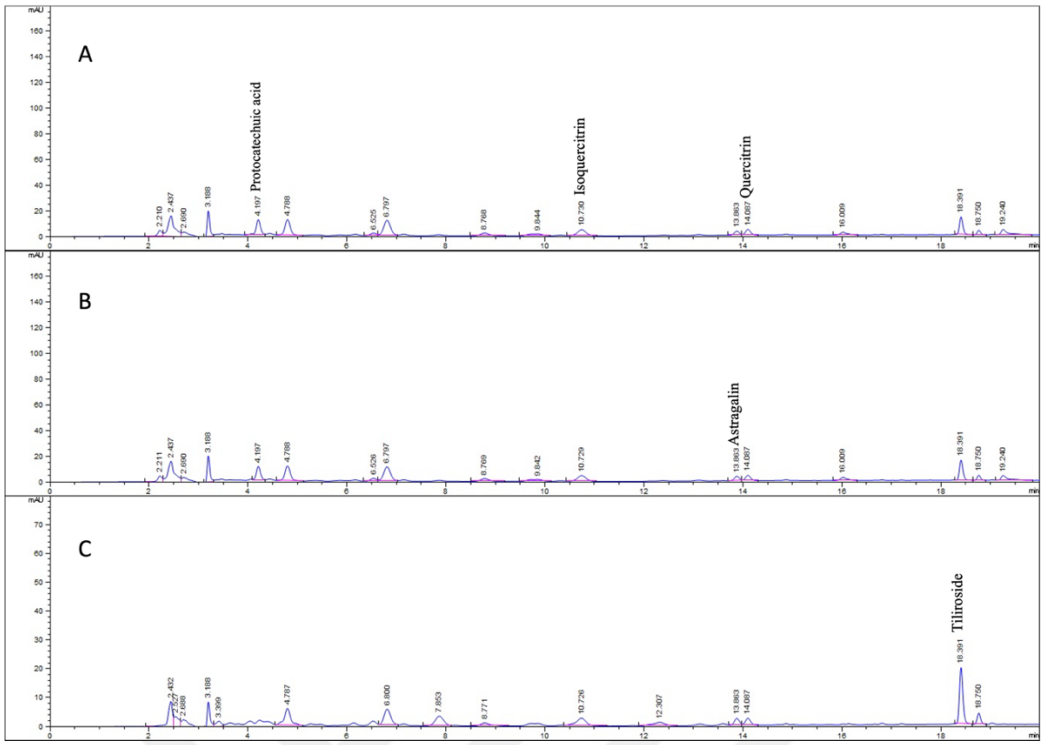


Figure 29. HPLC chromatograms of *T. platyphyllos* bracts extract (1 mg/mL) at 260 nm (A), at 265 nm (B) and at 320 nm (C).

The t_R values were as follows: protocatechic acid (4.19 ± 0.01), hyperoside (n.d.), isoquercitrin (10.73 ± 0.01), astragalin (13.86 ± 0.01), quercitrin (14.08 ± 0.01) and tilirose (18.39 ± 0.01)

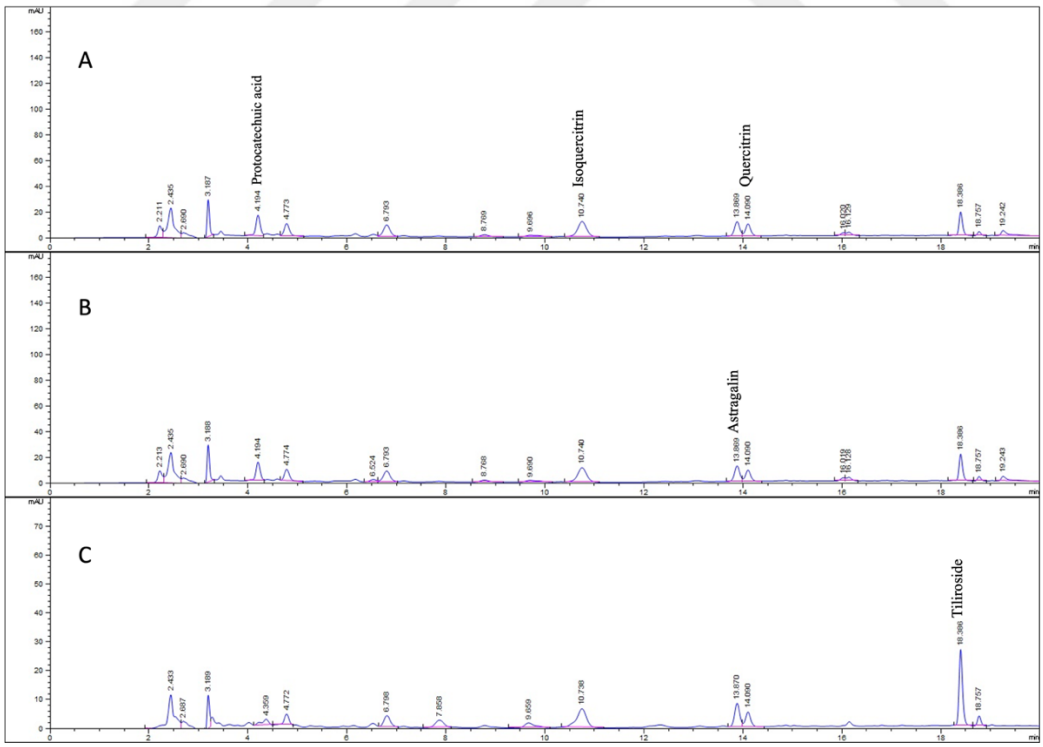


Figure 30. HPLC chromatograms of *T. platyphyllos* inflorescences extract (1mg/mL) at 260 nm (A), at 265 nm (B) and at 320 nm (C).

The t_R values were as follows: protocatechic acid (4.19 ± 0.01), hyperoside (n.d.), isoquercitrin (10.74 ± 0.01), astragalin (13.87 ± 0.01), quercitrin (14.09 ± 0.01) and tilirose (18.38 ± 0.01)

Table 7. Quantitative results of investigated standards in *Tilia* species by HPLC.

Samples	Standards					
	Protocatechuic acid	**Hyperoside**	**Isoquercitrin**	**Astragalin**	**Quercitrin**	**Tiliroside**
T. cordata flowers	13.354±0.406	n.d.	15.062±0.343	7.758±0.124	1.710±0.039	4.775±0.051
T. cordata bracts	30.260±0.816	1.744±0.026	4.729±0.110	2.119±0.046	17.871±0.211	0.244±0.020
T. cordata inflorescences	28.846±0266	n.d.	14.819±0.094	6.942±0.075	11.680±0.098	3.106±0.054
T. tomentosa flowers	1.175±0.026	n.d.	11.021±0.218	8.887±0.088	3.430±0.016	4.301±0.65
T. tomentosa bracts	1.145±0.008	n.d.	2.202±0.045	1.769±0.018	0.826±0.005	3.231±0.015
T. tomentosa inflorescences	0.985±0.036	n.d.	7.235±0.146	5.813±0.064	2.269±0.029	4.179±0.031
T. rubra flowers	6.193±0.008	n.d.	13.427±0.026	4.851±0.015	2.507±0.020	0.900±0.025
T. rubra bracts	6.675±0.171	3.006±0.041	4.443±0.112	1.727±0.026	23.128±0.333	n.d.
T. rubra inflorescences	7.456±0.508	2.280±0.021	10.158±0.168	3.636±0.053	13.667±0.189	0.590±0.014
T. platyphyllos flowers	3.116±0.097	n.d.	7.235±0.146	6.402±0.051	3.425±0.031	5.569±0.062
T. platyphyllos bracts	2.028±0.063	n.d.	2.120±0.031	1.365±0.004	1.074±0.010	3.120±0.050
T. platyphyllos inflorescences	2.675±0.090	n.d.	5.405±0.096	3.735±0.026	2.316±0.024	4.361±0.063

[a]The results were expressed as mg/g±SD; n=3, n.d: not detected

According to results in Table 7, Protocatechuic acid was detected in all samples and found predominantly in *T. cordata* bracts (30.260 ± 0.816 mg/g). The concentration in flowers and inflorescences of *T. cordata* was also higher than the other samples. Hyperoside was not detected in most of the samples however, it was found in *T. rubra* bracts (3.006 ± 0.041 mg/g), *T. rubra* inflorescences (2.280±0.021 mg/g), and *T. cordata* bracts (1.744 ± 0.026 mg/g). Isoquercitrin also detected in all samples and found predominantly in *T. cordata* flowers (15.062 ± 0.343 mg/g) followed by *T. cordata* inflorescences (14.819 ± 0.094 mg/g) and *T. rubra* flowers (13.427 ± 0.026 mg/g). Another standard, astragalin was also detected in all samples. It was predominantly found in flowers of *T. tomentosa* (8.887 ± 0.088 mg/g) followed by *T. cordata* flowers (7.758 ± 0.124 mg/g). Quercitrin was also detected in all samples, and it was predominantly found in bracts of *T. rubra* (23.128 ± 0.333 mg/g) followed by bracts of *T. cordata* (17.871 ± 0.211mg/g). Lastly, tiliroside was detected predominantly in flowers of *T. platyphyllos* (5.569 ± 0.062 mg/g) followed by *T. cordata* flowers (4.775 ± 0.051mg/g) and inflorescences of *T. platyphyllos* 4.361 ± 0.063 mg/g).

4.2. Results of *in vitro* Antioxidant Capacity Studies

4.2.1. Total Phenolic Content

In this study, total phenolic contents (TPC) of four different species of *Tilia* determined by Folin-Ciocalteu colorimetric method. The highest phenolic content determined in *T. rubra* flowers (336.31±19.89 mg of gallic acid per g of dried sample (GAE/g)) followed by *T. rubra* bracts (307.50±11.64 GAE/g) and *T. rubra* inflorescences (295.43±8.87 GAE/g). Among other species, *T. cordata* flowers (237.93±9.03 GAE/g) and *T. tomentosa* flowers (232.68±11.29 GAE/g) had the highest TPC value. In general, TPC value in flowers were found higher than in bracts except *T. cordata*.

4.2.2. Total Flavonoid Content

Total flavonoid content (TFC) determined by using aluminum chloride colorimetric method in *Tilia* samples. The highest flavonoid content determined in *T. rubra* bracts (58.61±2.85 mg of quercetin per g of dried sample (QE/g) and the difference was statistically significant. It was followed by *T. cordata* bracts (45.67±1.53 QE/g) and

T. rubra inflorescences (36.67±2.22 QE/g). *T. tomentosa* inflorescences and bracts and *T. platyphyllos* inflorescences showed similar TPC values. The lowest TPC value determined in *T. platyphyllos* flowers followed by flowers of *T. rubra* and *T. tomentosa*.

4.2.3. DPPH Radical-Scavenging Activity

The antioxidant activities of samples were evaluated with DPPH assay. *T. rubra* bracts showed the highest antioxidant activity obtained by DPPH assay with statistically important difference (727.17 ± 2.11 mg TE/g). It was followed by *T. rubra* inflorescences and *T. cordata* inflorescences. The antioxidant activity determined by DPPH assay was similar in *T. platyphyllos* flowers and bracts which was lower than *T. platyphyllos* inflorescences. The lowest activity was seen in *T. tomentosa* bracts (53.92 ± 10.01 mg TE/g hydroalcoholic *Tilia* extract).

4.2.4. Ferric Ion Reducing Antioxidant Power

The antioxidant activities of samples were evaluated with ferric ion reducing antioxidant power assay. *T. rubra* flowers showed the highest antioxidant activity obtained by FRAP assay with statistically important difference (446.05 ± 5.88 mg TE/g). It was followed by *T. rubra* bracts and inflorescences. Among other species, parts of *Tilia cordata* showed higher antioxidant activity with similar results. The lowest activity was in *T. tomentosa* bracts (32.75 ± 4.12 mg TE/g hydroalcoholic *Tilia* extract).

4.2.5. Cupric Reducing Antioxidant Capacity

The antioxidant activity of *Tilia* samples were evaluated by CUPRAC assay. According to CUPRAC assay results, *T. rubra* flowers 1192.00 ± 19.41 mg TE/g showed highest antioxidant activity with statistically important difference among other samples. It was followed by *T. rubra* bracts 1090.15 ± 56.48 mg TE/g and inflorescences 1027.02 ± 43.09 mg TE/g hydroalcoholic *Tilia* extract, respectively. Among other species, *T. cordata* inflorescences, flowers and bracts showed higher activity with similar results. The lowest activity obtained by *T. tomentosa* bracts and *T. platyphyllos* bracts with similar results (Table 8)

Table 8. TPC, TFC and antioxidant activity results of the *Tilia* samples.

Sample	TPC (mg GAE/g)	TFC (mg QE/g)	DPPH	FRAP (mg TE/g)	CUPRAC
T. cordata flowers	237.93±9.03^{C}	21.26±0.60^{E}	556.27±22.44^{CD}	302.66±8.57^{C}	787.79±78.08^{C}
T. cordata bracts	223.03±3.18^{C}	45.67±1.53^{B}	518.59±14.55^{DE}	263.72±20.62^{D}	768.38±26.23^{C}
T. cordata inflorescences	245.32±13.20^{C}	34.33±3.60^{C}	558.34±12.67^{CD}	277.13±8.84^{CD}	806.72±36.66^{C}
T. tomentosa flowers	232.68±11.29^{C}	18.93±0.79^{EF}	495.27±22.69^{E}	86.54±14.74^{G}	693.15±22.59^{C}
T. tomentosa bracts	56.55±3.67^{F}	24.31±2.80^{DE}	53.92±10.01^{H}	32.75±4.12^{H}	203.36±18.84^{F}
T. tomentosa inflorescences	165.63±12.12^{D}	22.32±0.82^{DE}	270.88±16.20^{F}	90.08±7.16^{G}	487.81±47.37^{DE}
T. rubra flowers	336.31±19.89^{A}	19.64±1.10^{EF}	561.45±19.88^{C}	446.05±5.88^{A}	1192.00±19.41^{A}
T. rubra bracts	307.50±11.64^{AB}	58.61±2.85^{A}	727.17±2.11^{A}	374.20±7.64^{B}	1090.15±56.48^{AB}
T. rubra inflorescences	295.43±8.87^{B}	36.67±2.22^{C}	638.44±14.39^{B}	340.85±17.77^{B}	1027.02±43.09^{B}
T. platyphyllos flowers	128.48±8.14^{E}	15.38±0.26^{F}	165.38±8.41^{G}	127.03±17.18^{F}	425.55±12.64^{E}
T. platyphyllos bracts	57.92±9.12^{F}	27.17±1.92^{D}	144.90±4.55^{G}	70.74±2.84^{G}	197.89±21.79^{F}
T. platyphyllos inflorescences	153.18±2.79^{DE}	23.70±0.71^{DE}	312.35±6.37^{F}	183.29±5.12^{E}	558.24±60.96^{D}

[1] Total phenolic content was expressed as mg gallic acid/g hydroalcoholic extract.
[2] Total flavonoid content was expressed as mg quercetin/g hydroalcoholic extract.
[3]DPPH radical scavenging activity was expressed mg TE/g hydroalcoholic extract.
[4]Ferric reducing antioxidant power was expressed mg TE/g hydroalcoholic extract.
[5]Copper reducing antioxidant capacity was expressed as mg TE/g hydroalcoholic extract.

4.3. Cell Culture Studies

4.3.1. Cytotoxic Effects on RAW 264.7 Cell Line

In order to show that the NO inhibitory activities of the. extracts were independent from cytotoxicity the cytotoxic effects of 0.125-1 mg/mL of *Tilia* extracts on RAW 264.7 cell line determined by MTT assay and the results were given in Table 9. According to results, samples did not show significant reduction on cell viability. Cell viability was relatively lower in *T. platyphyllos* bracts and inflorescences 55.60 ± 0.77% and 66.40 ± 0.92%, respectively. It was lower also in *T. tomentosa* flowers and bracts 67.29 ± 093% and 65.90 ± 0.91% and in flowers and inflorescences of *T. rubra* 63.18 ± 0.88 and 69.00 ± 0.96, respectively.

4.3.2. Evaluation of Inflammatory Response by Nitrite Level

In the present study anti-inflammatory activity of different plant parts of ethanolic *Tilia* extracts were assessed through determining the nitrite levels, the oxidation metabolite of nitric oxide. In this study, a well-studied anti-inflammatory agent Indomethacin and L-name were used as references. As seen in Figure 31, LPS significantly induced nitrite production of RAW 264.7 cell line compared to non-treated medium control group and ethanolic extracts of *Tilia* samples exerted a diminishing activity in LPS-induced nitrite production in a concentration-dependent manner. According to the results, 2h pre-treatment with *Tilia* samples showed significant reducing effects on nitrite level compared to 100 μM indomethacin. At 1 mg/mL concentration, highest inhibitory activity obtained with *T. tomentosa* bracts followed by *T. platyphyllos* bracts and *T. platyphyllos* inflorescences. At 0.5 mg/ml concentration, *T. tomentosa* bracts followed by *T. rubra* inflorescences exerted highest anti-inflammatory activity against LPS-stimulated nitrite production in RAW264.7 cells compared to other investigated extracts. % Nitrite inhibition of samples are given in Table 9.

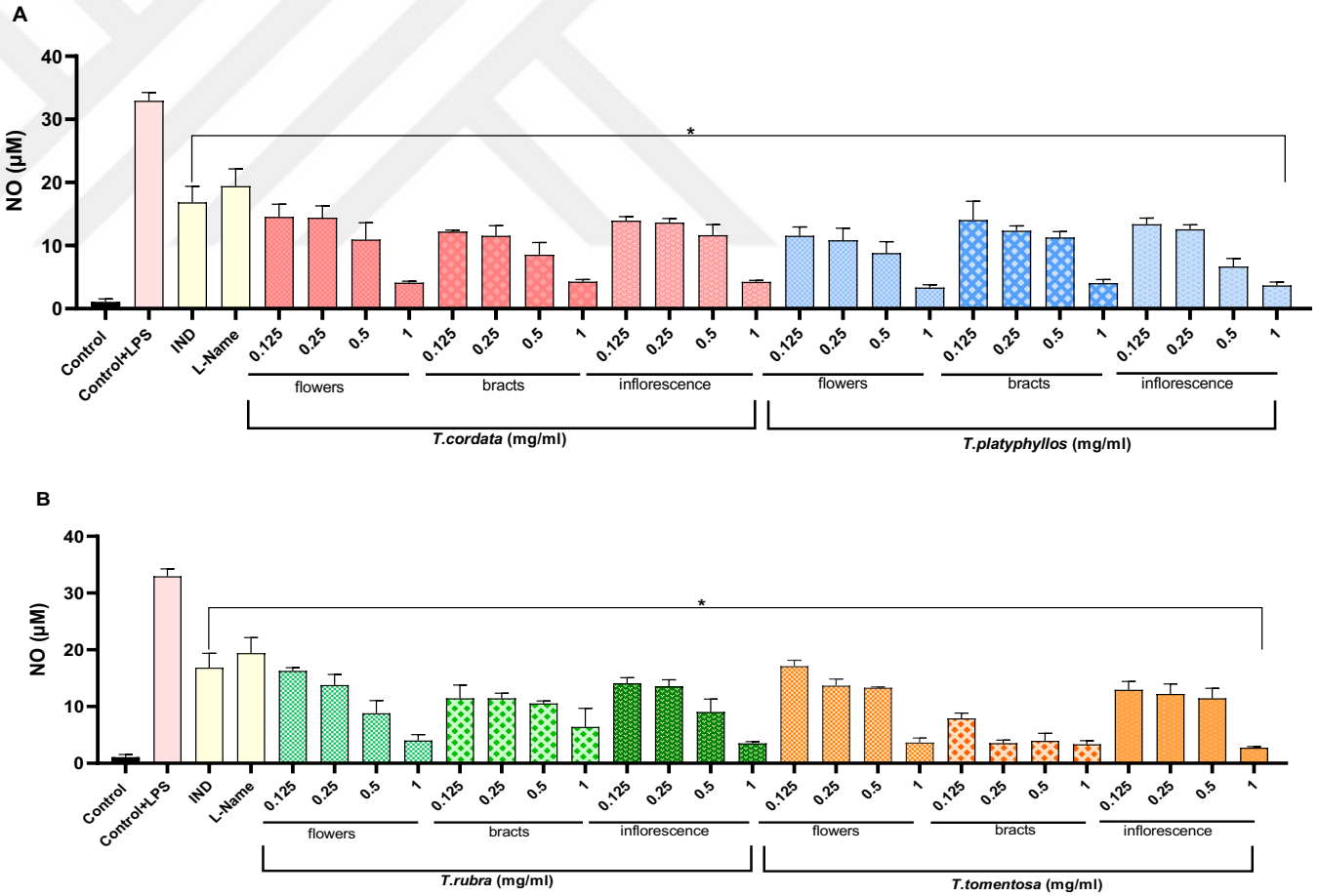


Figure 31. Nitrite levels shown by *Tilia* sp. in LPS-activated RAW264.7 cells.

Ctrl: Control group treated with DMEM; Ctrl+LPS: Control group only stimulated with LPS; IND: Indomethacin (100 µM), L-NAME: N G -nitro-L-arginine methyl ester hydrochloride (100 µM). Results were expressed as mean± SD compared to control. The significant differences between groups and Ctrl+LPS were defined with a *p<0.05

Table 9. Nitrite inhibition % of *Tilia* species in LPS-activated RAW264.7 cells

Groups	Concentration (mg/ml)	Cell Viability (%)	Nitrite inhibition (%)
Control	-	124.99 ± 1.94	-
Control+LPS	-	100 ± 0.52	-
Indometazin	100 µM	98.57 ± 1.46	50.36 ± 4.42
L-name	100 µM	98.05 ± 1.42	40.43 ± 2.76
T. cordata flowers	0.125	98.51 ± 0.67	55.80 ± 2.23
	0.25	98.77 ± 1.75	56.23 ± 4.82
	0.5	90.39 ± 0.24	66.77 ± 5.90
	1	84.67 ± 2.59	85.87 ± 2.94
T. cordata bracts	0.125	97.39 ± 3.20	62.96 ± 0.50
	0.25	93.95 ± 0.81	64.78 ± 0.48
	0.5	81.32 ± 0.46	74.08 ± 3.56
	1	74.43 ± 2.26	84.50 ± 4.31
T. cordata inflorescence	0.125	94.41 ± 0.10	57.62 ± 1.25
	0.25	87.68 ± 0.92	58.60 ± 0.66
	0.5	84.63 ± 0.10	64.66 ± 2.28
	1	80.07 ± 2.96	86.33 ± 1.47
T. platyphyllos flowers	0.125	95.85 ± 0.33	50.59 ± 0.40
	0.25	89.54 ± 1.12	58.20 ± 2.44
	0.5	87.19 ± 0.39	73.23 ± 4.76
	1	82.48 ± 3.05	85.63 ± 2.48
T. platyphyllos bracts	0.125	89.24 ± 3.11	67.27 ± 1.12
	0.25	83.91 ± 1.48	71.79 ± 1.98
	0.5	64.90 ± 2.17	-
	1	62.45 ± 0.37	-
T. platyphyllos inflorescence	0.125	92.55 ± 0.33	57.20 ± 0.14
	0.25	81.65 ± 4.23	58.88 ± 0.92
	0.5	74.12 ± 0.14	72.54 ± 2.73
	1	65.07 ± 1.16	-
T. rubra flowers	0.125	89.43 ± 3.42	64.94 ± 2.89
	0.25	89.22 ± 0.72	67.06 ± 3.69
	0.5	73.74 ± 0.57	73.23 ± 9.96
	1	67.19 ± 3.28	-
T. rubra bracts	0.125	96.22 ± 1.02	57.36 ± 4.21
	0.25	90.63 ± 1.01	62.96 ± 0.90
	0.5	89.40 ± 2.63	65.78 ± 2.51
	1	58.60 ± 4.11	-
T. rubra inflorescence	0.125	98.79 ± 1.61	62.13 ± 2.87
	0.25	96.45 ± 2.35	64.10 ± 4.88
	0.5	82.60 ± 1.49	79.69 ± 3.22
	1	64.69 ± 3.91	-
T. tomentosa flowers	0.125	101.39 ± 2.74	59.51 ± 1.54
	0.25	84.29 ± 3.10	65.95 ± 1.26
	0.5	81.27 ± 1.28	68.94 ± 2.84
	1	71.35 ± 3.60	88.29 ± 1.88
T. tomentosa bracts	0.125	100.81 ± 2.01	84.03 ± 0.52
	0.25	85.99 ± 2.46	88.95 ± 2.27
	0.5	73.50 ± 4.507	89.10 ± 0.71
	1	71.13 ± 1.81	90.11 ± 1.65
T. tomentosa inflorescence	0.125	98.85 ± 5.47	60.72 ± 1.68
	0.25	92.32 ± 0.28	62.97 ± 2.09
	0.5	90.33 ± 0.96	65.22 ± 1.49
	1	84.62 ± 1.99	89.26 ± 2.10

*(-) Non-toxic concentrations with cell viability of more than 70% cell viability were used to evaluate NO production.

4.3.4. IL-6 Releasing Inhibition

T. rubra flowers at 1 mg/mL concentration, exerted the highest inhibitory activity against LPS-activated release of IL-6 followed by *T. tomentosa* bracts at same concentration. As seen in Figure 32, in accordance with the results of nitrite reduction, all extracts showed potent IL-6 releasing inhibitory activity when compared to control, indomethacin. *T. tomentosa* bracts exhibited a high decrease in IL-6 release at 1 mg/mL concentration in LPS-activated murine macrophage cells.

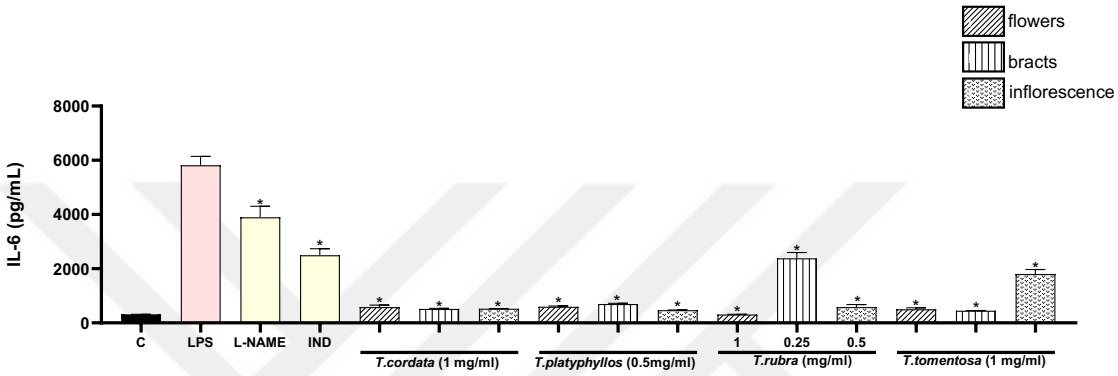


Figure 32. The effects of *Tilia* sp. on LPS induced IL-6 production in RAW 264.7 cells.

LPS: Lipopolysaccharides from E. coli; L-NAME: Nv-Nitro-L-arginine methyl ester hydrochloride; IND: Indomethacin. IL-6: interleukin-6. (*p<0.05).

4.3.5. Analgesic Activity

As seen in Figure 33, *T. rubra* inflorescences at 0.5 mg/mL concentration, exerted the highest inhibitory activity against PGE_2 production followed by *T. platyphyllos* bracts at same concentration. Minimum inhibitory effect obtained in *T. cordata* bracts at 1 mg/mL concentration followed by *T. tomentosa* inflorescences at same concentration.

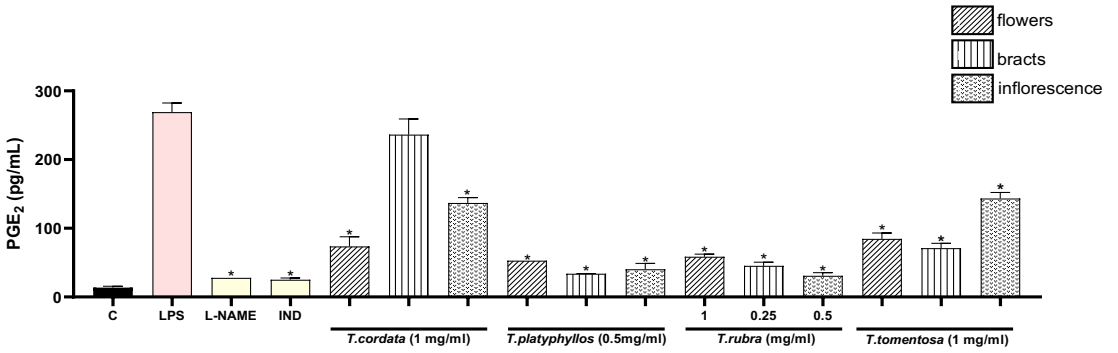


Figure 33. The effects of *Tilia* sp. on LPS induced PGE_2 production in RAW 264.7 cells.

LPS: Lipopolysaccharides from E. coli; L-NAME: Nv-Nitro-L-arginine methyl ester hydrochloride; IND: Indomethacin. IL-6: interleukin-6. (*p<0.05).

4.3.6. Anticancer Activity

4.3.6.1. Two-Dimensional Cell Cytotoxicity

In vitro anticancer activity of *Tilia* extracts were assed with 2D model on HDF and MIA PaCa-2 cell lines prior to 3D spheroid model. As seen Table 10, all extracts exerted selective cytotoxic activity on MIA PaCa-2 cell lines while at least 70% cell viability was obtained with healthy human dermal cells. Cytotoxic effects of extracts on healthy human dermal cells and pancreatic cancer cells were highly different. All extracts showed potent anticancer activity in a concentration dependent manner against pancreatic cell lines with decreasing cell viability at least 50.97%. *T. rubra* flowers (21.28 ± 3.50%) exerted highest cytotoxic activity on MIA PaCa-2 cell lines at the highest concentration following by *T. rubra* bracts (26.83 ± 1.35%).

4.3.6.2. Three-Dimensional Spheroid Formation/Growth

According to the results of the 2D cytotoxicity assay, all parts of *T. rubra* (0.125-1 mg/mL) have further investigated with 3D spheroid formation/growth assay as most potent anticancer effect was obtained with *T. rubra* (Table 11). Spheroids treated with different parts *T. rubra* extracts caused significant reduction in the growth rate of spheroids obtained, while the control spheroids kept growing up to 9 days. After 9 days of treatment spheroid size of control increased by almost 7.5 times where as flowers, bracts and inflorescences of *T. rubra* extracts inhibited MIA PaCa-2 and HDF spheroid growth in dose dependent manner. Even at lowest dose (0.125 mg/mL) all investigated parts of *T. rubra* reduced growth rate of spheroids compared to medium control. At highest dose (1 mg/mL) bracts of *T. rubra* showed strongest spheroid size change (50% reduction). Doxorubicin as a reference molecule reduced spheroid size by 70% after 9 days of treatment at 10 μM dose. Spheroids images taken every 3 days were given in Figure 34. Size and diameter changes of spheroids were given in Figure 35.

Table 10. Cell viability of *Tilia* species in MIA PACA-2 and HDF cells for 24 h.

Groups	Dose (mg/mL)	Cell Viability (%)		IC_{50} Values of MIA Paca-2
		HDF	MIA Paca-2	
Control	-	102.18 ± 2.13	104.11 ± 3.18	-
DOX	0.01 µM	97.21 ± 2.03	110.34 ± 2.93	-
	0.1 µM	88.03 ± 3.79	106.84 ± 3.13	-
	1 µM	85.46 ± 3.50	87.25 ± 4.14	-
	10 µM	73.74 ± 2.56	74.47 ± 0.75	-
T. cordata flowers	0.125	105.94 ± 1.87	87.32 ± 2.15	0.46 ± 0.01
	0.25	100.59 ± 0.23	78.15 ± 0.23	
	0.5	95.01 ± 0.43	44.47 ± 1.92	
	1	76.16 ± 1.31	36.61 ± 2.21	
T. cordata bracts	0.125	108.84 ± 0.89	99.00 ± 1.36	0.44 ± 0.00
	0.25	106.40 ± 2.20	88.13 ± 2.18	
	0.5	84.10 ± 0.63	37.42 ± 1.40	
	1	75.79 ± 1.11	32.76 ± 2.87	
T. cordata inflorescences	0.125	112.24 ± 0.18	99.32 ± 0.41	0.83 ± 0.02
	0.25	100.47 ± 4.57	85.49 ± 0.59	
	0.5	105.89 ± 1.76	73.98 ± 2.74	
	1	97.32 ± 1.36	40.11 ± 3.87	
T. platyphyllos flowers	0.125	120.56 ± 3.23	90.82 ± 2.37	0.43 ± 0.01
	0.25	111.41 ± 3.51	85.54 ± 4.52	
	0.5	111.45 ± 1.75	36.72 ± 2.13	
	1	84.99 ± 1.69	37.71 ± 7.35	
T. platyphyllos bracts	0.125	95.11 ± 0.23	86.59 ± 1.92	0.99 ± 0.01
	0.25	88.33 ± 3.01	80.94 ± 1.95	
	0.5	75.38 ± 3.13	77.82 ± 3.57	
	1	69.54 ± 2.25	50.97 ± 2.81	
T. platyphyllos inflorescences	0.125	112.19 ± 1.61	91.77 ± 0.97	0.47 ± 0.01
	0.25	94.08 ± 2.57	83.42 ± 1.74	
	0.5	93.99 ± 3.21	45.41 ± 2.72	
	1	76.26 ± 1.92	31.48 ± 2.02	
T. rubra flowers	0.125	104.21 ± 1.04	76.91 ± 2.34	0.21 ± 0.01
	0.25	101.96 ± 1.60	39.03 ± 1.76	
	0.5	97.45 ± 1.21	22.27 ± 3.52	
	1	94.77 ± 1.58	21.28 ± 3.50	
T. rubra bracts	0.125	122.7 ± 1.54	59.22 ± 0.98	0.16 ± 0.01
	0.25	93.48 ± 1.76	27.83 ± 1.01	
	0.5	89.60 ± 2.97	26.57 ± 1.87	
	1	88.27 ± 1.88	26.83 ± 1.35	
T. rubra inflorescences	0.125	110.43 ± 0.61	55.42 ± 0.90	0.24 ± 0.05
	0.25	106.71 ± 5.43	48.81 ± 2.95	
	0.5	100.74 ± 1.60	44.16 ± 2.56	
	1	97.61 ± 2.14	38.48 ± 5.78	
T. tomentosa flowers	0.125	114.20 ± 0.10	98.13 ± 0.64	0.71 ± 0.10
	0.25	110.39 ± 2.64	94.57 ± 0.76	
	0.5	107.15 ± 1.75	53.44 ± 2.76	
	1	97.39 ± 0.66	48.31 ± 1.64	
T. tomentosa bracts	0.125	106.04 ± 2.77	95.25± 1.78	0.77 ± 0.05
	0.25	96.97 ± 0.78	89.34 ± 2.23	
	0.5	99.28 ± 3.85	59.71 ± 5.09	
	1	82.78 ± 0.80	43.68 ± 1.87	
T. tomentosa inflorescences	0.125	113.52 ± 0.96	97.23 ± 1.60	0.63 ± 0.04
	0.25	99.86 ± 3.91	78.60 ± 0.56	
	0.5	102.65 ± 1.11	53.57 ± 1.45	
	1	81.79 ± 0.82	39.46 ± 0.89	

Table 11. Spheroid diameters of MIA-PaCA cells treated with flowers, bracts and inflorescences extracts of *T. rubra*

Sample	Concentrations	Spheroid diameter (square milimeters)			
		Day 0	**Day 3**	**Day 6**	**Day 9**
Controll	-	720.00 ± 25.46	1396.50 ± 38.89	4057.00 ± 251.73	5355.00 ± 149.91
DOX	10 μM	838.00 ± 171.12	680.00 ± 64.35	268.00 ± 22.63	247.00 ± 136.47
***T. rubra* flowers**					
	0.125 mg/mL	813.50 ± 19.09	1062.00 ± 59.40	1051.50 ± 2.12	1192.00 ± 76.37
	0.25 mg/mL	760.50 ± 43.13	710.50 ± 112.43	624.50 ± 70.00	628.50 ± 82.73
	0.5 mg/mL	777.50 ± 51.62	628.50 ± 70.00	584.00 ± 89.10	572.00 ± 115.97
	1 mg/mL	816.00 ± 2.83	559.00 ± 29.70	561.50 ± 54.45	560.50 ± 20.51
***T. rubra* bracts**					
	0.125 mg/mL	848.00 ± 14.14	693.50 ± 4.95	777.00 ± 19.80	938.50 ± 9.19
	0.25 mg/mL	812.00 ± 77.67	779.00 ± 81.00	731.00 ± 81.59	696.50 ± 53.67
	0.5 mg/mL	783.50 ± 13.44	753.00 ± 21.21	701.50 ± 7.78	634.00 ± 42.43
	1 mg/mL	779.33 ± 17.16	533.67 ± 67.86	507.00 ± 65.09	386.33 ± 147.70
***T. rubra* inflorescences**					
	0.125 mg/mL	826.00 ± 47.00	1203.00 ± 45.00	1467.00 ± 38.00	1625.00 ± 59.00
	0.25 mg/mL	893.00 ± 5.66	642.00 ± 46.67	573.50 ± 78.49	733.50 ± 38.89
	0.5 mg/mL	904.50 ± 16.26	612.50 ± 47.38	425.00 ± 15.56	643.50 ± 45.96
	1 mg/mL	751.00 ± 35.68	428.33 ± 79.15	353.33 ± 73.76	379.33 ± 51.48

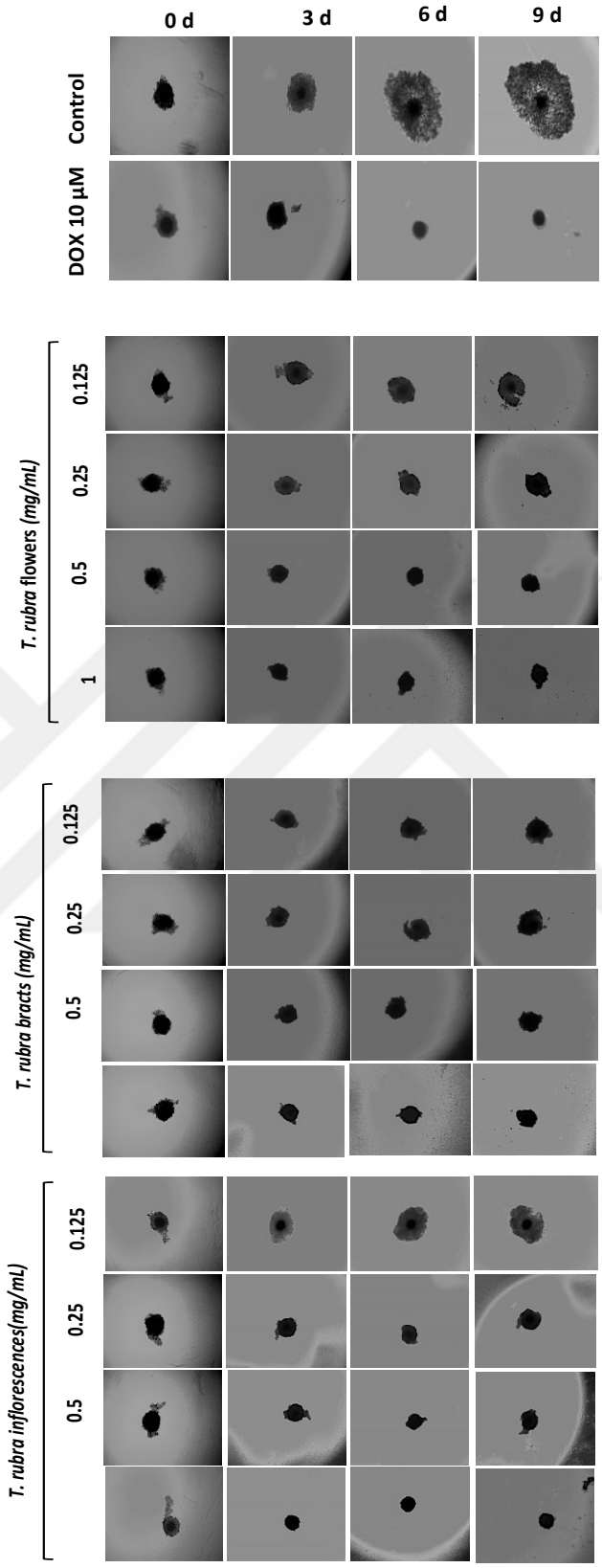


Figure 34. Spheroid images of cells treated with flowers, bracts and inflorescences extracts of *T. rubra* for 9 days.

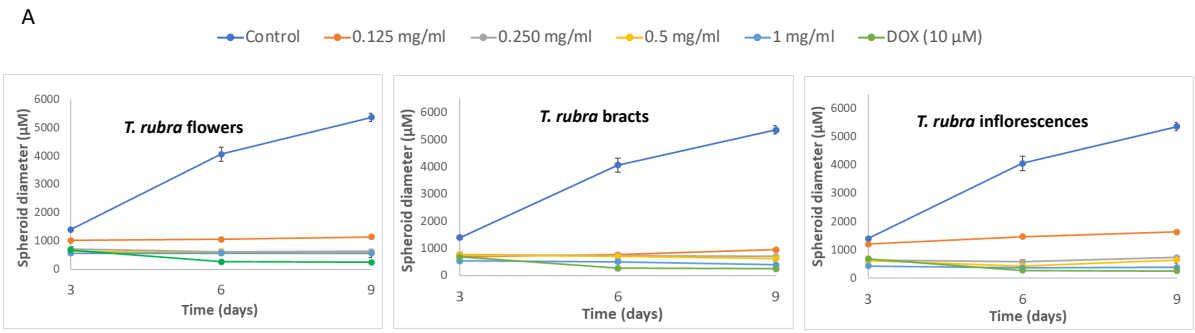


Figure 35. MIA PaCa-2 spheroid diameter of flowers, bracts and inflorescences extracts of *T. rubra* at 0.125-1 mg/mL concentration.

Additionally, as traditionally used parts of *Tilia* species are inflorescences, at highest doses (1 mg/mL) inflorescence extracts were further investigated with 3D spheroid formation/growth assay (Table 12).

Table 12. Spheroid diameter differences of cells treated with 1 mg/mL concentrations of *T. rubra, T. tomentosa, T. cordata* and *T. platyphyllos* inflorescences extracts for 9 days.

Samples (1 mg/ml)	**Spheroid diameter (square milimeters)**			
	Day 0	**Day 3**	**Day 6**	**Day 9**
***T. rubra* inflorescences**	751.00 ± 35.68	428.33 ± 79.15	353.33 ± 73.76	379.33 ± 51.48
***T. tomentosa* inflorescences**	824.00 ± 177.97	585.67 ± 31.01	561.67 ± 16.56	625.00 ± 51.18
***T. cordata* inflorescences**	816.67 ± 95.66	642.67 ± 30.66	575.67 ± 30.83	556.00 ± 15.59
***T. platyphyllos* inflorescences**	833.50 ± 7.78	1318.50 ± 10.61	1408.00 ± 12.73	1379.50 ± 4.95

Inflorescence extracts at highest doses reduced growth rate of spheroids compared to medium control. At 1 mg/mL dose inflorescences extract of *T. rubra* showed highest anticancer activiy with 50% reduction in tumor size growth followed by *T. cordata* (32%) and *T. tomentosa* (24%). Spheroids treated with inflorescence extract of *T. platyphyllos* increased in size however, growth rate was reduced almost 4 times compared to control medium. Size and diameter changes of spheroids were given in Figure 36. Spheroids images taken every 3 days were given in Figure 37.

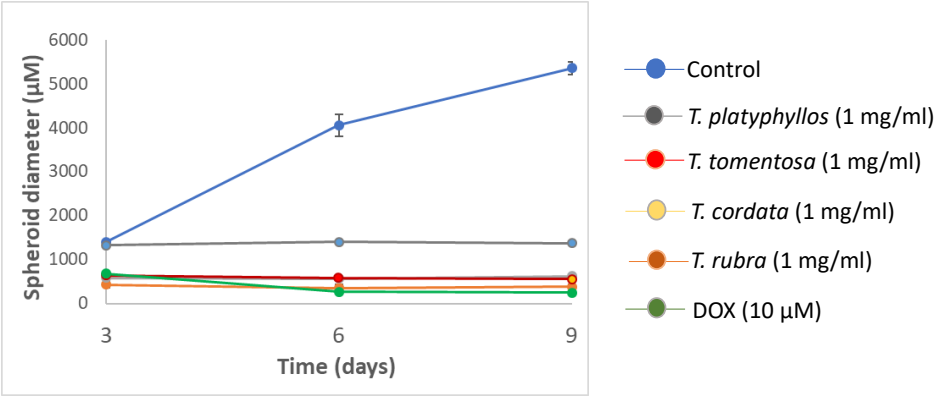


Figure 36. MIA PaCa-2 spheroid diameter differences treated with doxorubicin at 10 µm and inflorescences extracts of *T. platyphyllos, T. tomentosa, T. cordata* and *T. rubra* at 1 mg/mL concentrations

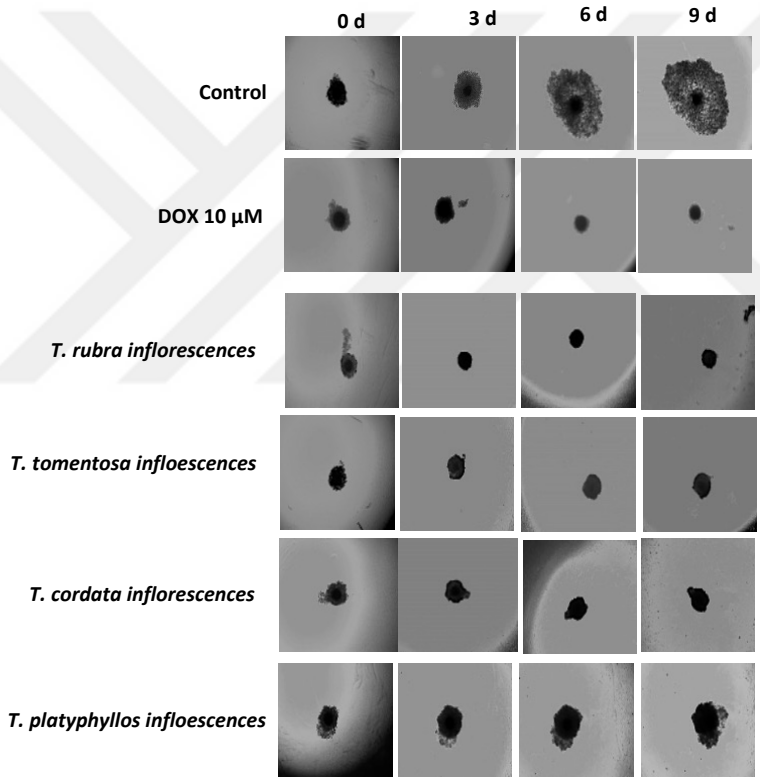


Figure 37. Spheroid images of cells treated with 1 mg/mL concentrations of *T. rubra, T. tomentosa, T. cordata* and *T. platyphyllos* inflorescences extracts for 9 days.

5. DISCUSSION AND CONCLUSION

Cancer is a leading cause of death and almost all synthetic anticancer agents are known to be toxic and adversely affect healthy cells. Therefore, discovering safe methods and non-toxic agents for the treatment of cancer could be an approach without damaging healthy tissues (123). Investigating natural drugs, mostly the wide diversity of chemical compositions of plants presents an alternative to developing safer anticancer agents. It also makes economic sense as the isolation and development of natural agents may be lower in cost as compared to new chemical discoveries. Various phytochemicals from plants have been shown to interfere with specific stages of carcinogenesis. As they can be found in nature, acquiring resistance to them is less likely they have advantages over synthetic chemical agents. It has been supported by many studies as plant-derived compounds exert cytotoxic effects and can destroy cancer cells (6,9).

When the balance between oxidant molecules and antioxidant defense systems is impaired oxidative stress occurs within the cell. Oxidative stress is regarded as one of the main causes of many inflammatory diseases, metabolic and cardiovascular disorders, neurogenerative illnesses, and cancer. Pre-clinical and clinical studies evidence strongly suggest that dietary intake of antioxidant-rich fresh fruits and vegetables has a protective effect against the development of many oxidative stress-related diseases. Increased intake of dietary antioxidants is clearly linked with improved health and low risk of disease, additionally preventing oxidative damage to key biomolecules that may affect cell function. This protective role is mainly attributed to their phytochemical content and may be associated with the antioxidant capacities of phytochemicals. Among phytochemicals, phenolic compounds are the biggest group and have drawn increased interest due to their ability to prevent and treat diseases caused by oxidative stress by inhibiting oxidative damage to proteins, DNA, and lipids which may lead to mutation, apoptosis, and carcinogenesis (2–4).

Plants have been used as important sources of drugs since ancient years. *Tilia* from the Tiliaceae family is an important herbal remedy and has widespread use in Turkish folk medicine. *Tilia* species have many biological activities mainly attributed to their rich phenolic contents (12). Traditionally *Tilia* inflorescences are preferred as a hot water infusion to benefit from many important pharmacological activities such as diuretic, expectorant, diaphoretic, and sedative activities (8,13,14). There are several *in vivo* and

in vitro studies about the antioxidant, anti-inflammatory and anticancer effects of different *Tilia* species (8,15,16). However, a comparative study regarding the biological activities of different species has not been published. In the light of this knowledge, different *Tilia* species collected from Turkey were evaluated in terms of their antioxidant capacities, their protective effects against inflammation, and anticancer activities.

Oxidative stress is known to contribute to inflammation by activating inflammatory pathways and carcinogenesis by leading to the transformation of a normal cell into a tumor cell. (1) Dietary polyphenols in herbal drugs are known to possess high free radical scavenging activity with having a highly conjugated system distinguishing with one or more phenol group and their ability to donate a hydroxyl group (124,125). They can neutralize ROS and diminish cellular oxidative stress thereby preventing oxidative damage to cellular biomolecules (2). Therefore, the major phenolic components of the extracts were characterized by HPTLC and HPLC studies. However, a comparative evaluation of total phenolic content (TPC) values between different species have not been issued. In this study, the total phenol content in flowers, bracts, and inflorescences of four species of *Tilia* was determined. The highest phenolic content was determined in *T. rubra* flowers (336.31±19.89 mg GAE/g) followed by *T. rubra* bracts (307.50±11.64 GAE/g) and *T. rubra* inflorescences (295.43±8.87 GAE/g). Among other species, *T. cordata* flowers (237.93±9.03 GAE/g) and *T. tomentosa* flowers (232.68±11.29 GAE/g) had the highest TPC value. In general, TPC values in flowers were found to be higher than in bracts except for *T. cordata*. In a previous study, Ayaz et al. (126) performed the Folin-Ciocalteu technique performed for calculating the TPC value of 70% ethanolic extract of inflorescences of *T. tomentosa* collected from 4 different regions. Determined TPC values ranged from 34.69 to 68.34 mg GA/g dry plant whereas the TPC value of 80% ethanolic extract of *T. tomentosa* inflorescences was determined as 165.63±12.12 mg GA/g in our study however, the TPC value was determined in the dry plant instead of hydroalcoholic extract. In another study, Karakaş et al. (127) performed the Folin-Ciocalteu technique to determine the total phenolic content of n-hexane maceration of *T. tomentosa* flowers 12.33 mg GA/g whereas our study determined TPC value was 20 times higher in hydroalcoholic *T. tomentosa* flowers extract. The difference can be explained by the polarity of extraction solvent and extraction method differences. In 2014, Akyüz et al. (67) analyzed different parts of 100% methanolic extract of *T. rubra* blossom extract to determine its TPC value. The TPC value calculated in blossom_was 17.372 ± 0.03 mg

GA/g of extract. According to our results 80% hydroalcoholic extract of inflorescences of *T. rubra* was almost 15 times higher (295.43±8.87 mg GA/g) however, the difference can be explained by extraction solvent difference and chemical diversity among different plant parts. In 2021, Mitic et al. (68) investigated the 50% ethanolic extract of *T. cordata* flowers for the chemical composition. The optimized extraction temperature was determined as 55 °C. TPC value was determined 17.011 mg GA/g whereas, the TPC value for 80% ethanolic extract of *T. cordata* flowers extract was higher (237.93 ± 9.03 mg GA/g). However, extraction was performed at room temperature, and 80% ethanolic extraction solvent was used in our study.

Free radical scavenging activities of flavonoids have been extensively studied and attributed to their ability to donate hydrogen with their phenolic hydroxyl groups that can stabilize free radicals. Indirectly, they can activate antioxidant enzymes, and suppress pro-oxidant enzymes. There are various studies in the literature performed to investigate the antioxidant activity of flavonoid compounds (2,128,129). Flavonoids are major constituents (1-5%) of dried inflorescences of *Tilia* species. Among flavonoid components many derivatives of quercetin such as hyperoside, quercitrin and isoquercitrin, kaempferol derivatives such as tiliroside and astragalin are present in raw material (60). Therefore, TFC values were determined in *Tilia* extracts to evaluate their contribution to antioxidant capacities. Bract extracts of *T. cordata (*45.67±1.53 QE/g) and *T. rubra* (58.61±2.85 mg QE/g) which had the highest TFC values also showed potent antioxidant activity with DPPH, FRAP, and CUPRAC assays whereas *T. platyphyllos* flower extract with the lowest TFC value showed weak antioxidant activity in all assays compared to other extracts. There are studies investigating the total flavonoid content of *Tilia* species. Lianza et al. (130) analyzed 50% ethanolic extract of *T. platyphyllos* inflorescences and they found the TFC value 91.2 ± 1.3 mg Rutin eq/mg of extract. However, the result was not comparable with our result due to extraction solvent and reference compound difference. In another study, 80% methanolic extracts of leaves of *T. platyphyllos* were dried with the infrared drying method at different temperatures and their TFC values were compared (131). The calculated TFC values for fresh leaves was 0.567 ± 0.015 mg catechin equivalent (CE)/g. The TFC values for infrared dried leaves were between 2.583 ± 0.145 and 2.790 ± 0.150 mg CE/g. Results were not comparable with our study findings since quercetin was used as a reference compound and leaves were not investigated. Different plant parts of the same plant may show chemical

diversity. In 2021, Mitic et al. (68) investigated *T. cordata* flowers with a determined optimized extraction process of 50% ethanolic extract extracted for 120 minutes at 55 °C. TFC value was determined as 12.562 mg CE/g at the optimized conditions. In this study, the TFC value for 80% hydroalcoholic extract of *T. cordata* flowers was determined as 21.26 ± 0.60 mg QE/g. However, the extraction solvent, method, selected temperature, and reference compound was different.

The phenolic profiles of extracts were investigated with HPTLC analysis. HPTLC is one of the tools for the qualitative and quantitative phytochemical analysis of herbal remedies (92). There are several publications elucidating phenolic profiles of different *Tilia* species, however, a comparative analysis between biological activities and phenolic profiles of different *Tilia* species has not been issued (67,132). In a previous study, Güzelmeriç et al. (53) analyzed phenolic profiles of four different species of *Tilia* to compare the phenolic profile of *T. tomentosa* with the *Tilia* species present in European Pharmacopeia by the HPTLC method. 80% ethanolic extracts of *T. cordata* flower, *T. cordata* bracts, *T. cordata* flowers and bracts, *T. platyphyllos* flowers, *T. platyphyllos* bracts, and *T. platyphyllos* flowers and bracts were analyzed and compared with the HPTLC fingerprint of thirteen *T. tomentosa* inflorescences. Isoquercitrin, astragalin, quercitrin and tiliroside were notably detected in all samples and hyperoside was only detected in *T. cordata* flowers. Similarly, isoquercitrin, astragalin, and quercitrin were detected in all samples in our study. However, hyperoside was absent in *T. cordata* flowers and tiliroside was absent in bracts extracts of *T. rubra* which was not investigated in the previous study.

In addition to HPTLC analysis, HPLC was performed to quantify the amount of reference compounds in the extracts. In 2014, Akyuz et al. (67) analyzed 100% methanolic, selective extract, and acidic hydrolysis extract of *T. rubra* blossoms. The main phenolics detected with all extraction methods were protocatechuic acid and gallic acid. Similarly, in this study protocatechuic acid was detected in high concentrations in all parts of the hydroalcoholic extract of *T. rubra*. In methanolic extracts of blossom sections 1369.34 µg/g protocatechuic acid, with selective extraction method, 1054.23 µg/g protocatechuic acid and in acidic hydrolysis extraction method, 1095.18 µg/g protocatechuic acid were detected whereas protocatechuic acid was determined as 6.193 mg/mL in 80% hydroalcoholic extract of *T. rubra* flowers in this study, however, in a

previous study it was determined in 100% methanolic extract and dry plant. In 2001, Toker et al. (14) studied 50% methanolic extract of three different species of *Tilia* in order to investigate phenolic profile differences of three parts of plants. In flowers of *T. platyphyllos,* isoquercitrin+rutin and astragalin were determined as the chief phenolics. Also, quercitrin was detected in lower concentrations. Similarly, according to our results main phenolic compounds determined in 80% hydroalcoholic extract of *T. platyphyllos* flowers were isoquercitrin and astragalin and quercitrin was also detected in low concentration. In bracts of the plant, quercitrin was determined as the chief phenolic whereas according to our results in *T. platyphyllos* bracts isoquercitrin and tiliroside were determined as the main phenolics. In flowers of 50% methanolic extract of *T. rubra* isoquercitrin + rutin and astragalin were determined as the main phenolics. Quercitrin was also detected. Similar to our findings quercitrin was also detected in low concentrations and the main phenolic compounds determined in 80% hydroalcoholic extract of *T. rubra* flowers were isoquercitrin and protocatechuic acid. However, protocatechuic acid content was not investigated in the previous study. In the bracts, the main flavonoid component determined was quercitrin and similarly, the main phenolic determined in *T. rubra* bracts was quercitrin (23.128 ± 0.333 mg/g) with relatively high concentrations in this study. In the methanolic extract of *T. tomentosa* flowers, the main phenolics were quercitrin, isoquercitrin + rutin, and astragalin whereas quercitrin was detected in low concentrations (3.430 ± 0.016 mg/g) and the main phenolics determined in *T. tomentosa* flowers were isoquercitrin and astragalin in our study. In the bracts of *T. tomentosa*, quercetin-3,7-dirhamnoside was determined as the chief flavonoid however it was not investigated in this study. Differences in concentrations can be explained by extraction solvent differences and developmental and environmental differences of the same species. Jabeur et al. (16) studied 80% ethanolic extracts of aerial parts of *Tilia platyphyllos* with respect to phenolic compositions and *T. platyphyllos* and most abundant phenolic acids and flavonoids detected in *T. platyphyllos* were protocatechuic acid and isoquercitrin. Similarly, in this study isoquercitrin was determined as the main phenolics in all investigated parts of *Tilia platyphyllos*. Protocatechuic acid 6.3 ± 0.1 mg/g, trans-tiliroside 1.38 ± 0.03 mg/g, cis-tiliroside 0.27 ± 0.03 mg/g, hyperoside 1.01 ± 0.02 mg/g, isoquercitrin 6.36±0.04 mg/g and quercitrin 3.5 ± 0.1 mg/g. Similarly, in our results 80% hydroalcoholic extract of *T. platyphyllos flowers* 3.116 ± 0.097 mg/g protocatechuic acid, 7.235 ± 0.146 mg/g isoquercitrin, 6.402 ± 0.051 mg/g astragalin, 3.425 ± 0.031mg/g quercitrin and 5.569 ± 0.062 mg/g tiliroside were detected while hyperoside was not

detected. The main phenolic compounds determined were isoquercitrin and astragalin. In bracts, 2.028 ± 0.063 mg/g protocatechuic acid, 2.120 ± 0.031 mg/g isoquercitrin, 1.365 ± 0.004 mg/g astragalin, 1.074 ± 0.010 mg/g quercitrin, 3.120 ± 0.050 mg/g tiliroside were detected while hyperoside was not detected. The main phenolic compounds determined were tiliroside and protocatechuic acid. Lastly, 2.675 ± 0.090 mg/g protocatechuic acid, 5.405 ± 0.096 mg/g isoquercitrin, 3.735 ± 0.026 mg/g astragalin, 2.316 ± 0.024 mg/g quercitrin and 4.361 ± 0.063 mg/g tiliroside were detected in inflorescences. The main phenolic compounds determined were isoquercitrin and tiliroside while hyperoside was not detected. However, as leaves were investigated as an aerial part in the previous study, concentrations were not comparable. Kosakowska et al. (12) analyzed inflorescences of 50% aqueous extract of *T. cordata* flowers collected from 20 different regions of Poland to identify the chemical variability in terms of their phenolic composition. Among phenolic acids, vanillic acid was determined as the main phenolic acid. Chlorogenic acid, caffeic acid, and p-coumaric acid were also detected whereas protocatechuic acid was investigated as a phenolic acid in our study. Isoquercitrin (486.7 ± 252.3 mg/100 g), hyperoside (517.9 ± 290.3 mg/100 g), astragalin (796.6 ± 474.1 mg/100 g), tiliroside (517.6 ± 177.5 mg/100 g) were also detected. In our findings, in 80% hydroalcoholic extract of *T. cordata* isoquercitrin (15.062 ± 0.343 mg/g), astragalin (7.758 ± 0.124 mg/g), quercitrin (1.710 ± 0.039 mg/g) and tiliroside (4.775 ± 0.051 mg/g) was detected and main phenolic compound detected was isoquercitrin while hyperoside was absent in *T. cordata* flower extract. The concentrations of standards were higher in our study possibly from extraction solvent difference. In 2021, Mitic et al. (68) performed the HPLC method to quantify the phenolic content of 50% methanolic *T. cordata* flowers extract. Hyperoside (0.843 mg/g) and isoquercetin (0.052 mg/g) were detected whereas according to our findings in 80% hydroethanolic extract *T. cordata* flowers 15.062 ± 0.343 mg/g isoquercitrin detected and hyperoside was not detected possibly due to extraction solvent differences. Moreover, Ferreira et al. (133) evaluated the phenolic profile of 80% hydroethanolic extract of *T. platyphyllos* aerial parts (inflorescences and leaves). Protocatechuic acid (6.899 ± 0.002 mg/g) and tiliroside (0.395 ± 0.003 mg/g) were detected in hydroalcoholic extract whereas 2.675 ± 0.090 mg/g protocatechuic acid and 4.361 ± 0.063 mg/g tiliroside were detected in inflorescences extract *T. platyphyllos* in this study. Although the extraction solvents were the same, the concentrations were different possibly due to developmental and environmental factors and plant part differences.

The presence and level of secondary metabolites in plants such as phenolic compounds, alkaloids, and terpenoids can be influenced by the changing abiotic and biotic environment. Abiotic stress can be a cause for increase production of secondary metabolites while during growth and development plants can interact with the environment and can be influenced by abiotic components from the surrounding environment such as water, soil, temperature, and chemicals. These environmental factors trigger variation in the synthesis of secondary metabolites and cause fluctuations in the intra-species variability of compounds in plants (134).

Antioxidant activities of different *Tilia* species were evaluated with three different assays. The first assay was the DPPH assay which relies on the fact that DPPH accepts a hydrogen atom in the presence of an antioxidant molecule and becomes a nonradical molecule (135). According to our findings, bract extract of *T. rubra* with the highest quercitrin concentration (23.128 ± 0.333 mg/g) also showed the highest antioxidant activity with a statistically important difference by DPPH assay (727.17 ± 2.11 mg TE/g) even presence of other investigated standards was found in low concentrations in the extract. Quercitrin has been proved to exert antioxidant activity by many studies. It can directly scavenge ROS with its two hydroxyl groups attached to a catechol ring. It has been reported that quercitrin administration decreased UVB-induced intracellular oxidative stress in HaCaT cells. Additionally, its cytoprotective effect against oxidative stress in H_2O_2-treated Chinese hamster fibroblast has been shown by research (128). There are several studies that evaluated antioxidant activities of different *Tilia* species by DPPH assay however, this will be the first study that comparatively evaluates the contribution of the phenolic profile of different *Tilia* species to the protective effect against oxidative stress. In another study, Ayaz et al. (126) performed a DPPH assay for evaluating the antioxidant activity of 70% ethanolic extract of inflorescences of *T. tomentosa*. The DPPH levels in the previous study were at least less than half of the determined value for 80% hydroalcoholic *T. tomentosa* extract in this study possibly due to extraction solvent difference. In 2017, Jabeur et al. (16) performed a DPPH assay for 80% ethanolic extract of aerial parts of *T. platyphyllos*. They determined the DPPH scavenging activity of the plant, expressed in the terms of IC_{50}, 105 ± 1 mg TE/g dry plant. However, leaves of the plant were included for investigation and the determination was performed on a dry plant instead of hydroalcoholic extract.

Secondly, the antioxidant capacity of samples was determined with FRAP assay which measures the antioxidant capacity of redox-active compounds with high sensitivity and reproducibility. This method is relatively quick, inexpensive, and simple direct measuring of the total antioxidant activity of antioxidants in a test sample (4). In this study, *T. rubra* flowers with the highest isoquercitrin concentration (15.062 ± 0.343 mg/g) showed the highest antioxidant activity with a statistically important difference (446.05 ± 5.88 mg TE/g). Isoquercitrin has been shown to scavenge ROS and RNS such as superoxide anion radicals and hydroxyl radicals. Administration of isoquercitrin was shown to reduce superoxide formation induced by phorbol 12-myristate 13-acetate in murine macrophage RAW264.7 cells (136). In this study, flower and inflorescence extracts of *T. cordata* and *T. rubra* were rich in isoquercitrin content and also showed potent antioxidant activity by FRAP assay. In a previous study, Seyhan et al. (126) performed FRAP assay for evaluating the antioxidant activity of 70% ethanolic extracts of *T. tomentosa* inflorescences collected from four different regions. The determined FRAP value was between 53.01±5.21 and 131.94± 6.05 mg TE/g dry plant. The determined FRAP value in our study was similar however as the determination made in dry plants instead of hydroalcoholic extract results were not comparable. In 2020, Kelmendi et al. (137) studied 80% methanolic extract of inflorescences of *T. platyphyllos* collected from 5 regions in Kosovo. The FRAP value determined ranges between 100.1±2.8 and 145.6 ±12.8 mg TE/g dry matter. It was suggested that the difference between antioxidant capacities of the same species of plant was environmental factor differences in different regions.

Lastly, a CUPRAC assay was performed for measuring the antioxidant capacity of *Tilia* samples. In this study, CUPRAC assay results showed that the highest antioxidant activity obtained with *T. rubra* flowers statistically important difference from other samples. It was followed by *T. rubra* bracts and inflorescences, respectively. Among other species, *T. cordata* inflorescences, flowers, and bracts showed higher activity with similar results. CUPRAC values were generally determined higher in samples, especially bract and inflorescence extracts of *T. cordata* with high protocatechuic acid content. Studies have shown that PCA has 10 times more potent antioxidant activity compared to α-tocopherol. PCA has been reported to play an important antioxidant role in inducing the antioxidant defense system of the body by upregulating endogenous antioxidant enzymes such as glutathione, superoxide dismutase, and catalase, and decreasing

oxidative damage. It has been shown to prevent and reduce oxidative stress-related damages to various organs such as protection against oxidative damage induced cell damage and apoptosis in rat cardiac muscle. More recently, PCA has been shown to decrease gastrointestinal mucosa damage caused by ketoprofen-induced oxidative stress (138). In 2014, Akyuz et al. (67) evaluated the phenolic composition and antioxidant capacity of different parts of 100% methanolic extract of *T. rubra* blossoms. The CUPRAC values determined were almost the same with leaves and 2 times higher than in trunks. Interestingly, flowers extract of *T. tomentosa* showed similar antioxidant activity to all parts of *T. cordata* extracts. This situation can be explained by higher tiliroside concentration detected in *T. tomentosa extracts*. Tiliroside was confirmed as a free radical scavenger against various ROS by many assays. It has shown 7.2% antioxidant activity compared to α-tocopherol in linolenic acid oxidation assay. Tiliroside has been shown to inhibit xanthine oxidase and increase the superoxide dismutase (SOD) level. Its antioxidant potential is due to both radical scavenging activity and SOD level increasing ability (139). However, *T. cordata* flowers extract showed potent antioxidant activity with also with DPPH and FRAP assays. This can be explained by synergistic effects of other phenolic compounds in *T. cordata* flowers extract as investigated standards were detected relatively low in *T. tomentosa* flowers extract.

One of the main aims of our study was to assess the protective effects of *Tilia* species against inflammation and assess the contribution of the phenolic profile of extracts to this biological activity. Inflammation is a complex system including immunological, physiological, and behavioral events in response to exposure to harmful stimuli such as pathogens, tissue injury, extreme temperature, and metabolic disorder. The main purposes of inflammation are to remove unwanted stimuli and reestablish tissue homeostasis. However, when the response is inappropriate to this acute inflammation, it will become chronic and may drive many important inflammation-related diseases including diabetes, rheumatoid arthritis, and cancer. During chronic inflammation, inflammatory mediators are released and controlled by intracellular signaling cascades. Inflammatory response to LPS activates and triggers NF-κB and MAPKs pathways which causes overexpression of various inflammatory mediators such as interleukins, inducible nitric oxide synthase, PGE_2, and TNF- α (140). With this knowledge, the protective effects of *Tilia* species against inflammation were assessed with determination of nitrite levels

in LPS-induced RAW 264.7 murine macrophage cells pretreated with *Tilia* species and determined IL-6 releasing inhibition and PGE_2 production inhibition activities.

Natural products, especially phenolic compounds with their chemical diversity have gained attention for their anti-inflammatory activities. The presence of phenols may be the cause of the reduction of NO generation in macrophages since phenolics have been shown in numerous studies to be potent anti-inflammatory agents and to be crucial in the prevention of oxidative stress and inflammation (124,141). In this study, the protective effect of different species of *Tilia* against inflammation was assessed by determining the nitrite levels (118). The anti-inflammatory activity of *Tilia* species have been reported in previous studies however a comparative study between different species has not been investigated. All extracts effectively reduced inflammation by preventing NO generation dose-dependently. LPS significantly induced nitrite production of RAW 264.7 cell line compared to a non-treated medium control group. According to the results, % nitrite inhibition was over 50 % for all studied concentrations (0.125-1 mg/mL). At 1 mg/mL concentration, the highest inhibitory activity was obtained with *T. tomentosa* bracts followed by *T. platyphyllos* bracts and *T. platyphyllos* inflorescences. At 0.5 mg/mL concentration, *T. tomentosa* bracts followed by *T. rubra* inflorescences exerted the highest anti-inflammatory activity against LPS-stimulated RAW 264.7 cells compared to other investigated extracts. In another study, Toker et al. (70) reported that leaves of *T. tomentosa* at 50 mg/kg inhibited inflammation in paw edema model stimulated with carrageenan. Similarly, extracts of *T. tomentosa* with high tiliroside content also showed a potent protective effect against inflammation with nitrite assay inhibiting nitrite production by 89-90%. The anti-inflammatory activity of this extract may be attributed to high tilirose content since the anti-inflammatory activity of tiliroside has been confirmed by several studies with *in vivo* and *in vitro* models. It has been shown to inhibit the production of TNF-α, and NO in LPS-induced murine peritoneal macrophages and has been shown to suppress the overproduction of NO in a dose-dependent manner (139). Moreover, Fawzy et al. (72) reported that 70% methanolic extract of *T. cordata* aerial parts has shown anti-inflammatory activity against carrageenan-induced rat paw edema test equal to indomethacin at 300 mg/kg dose. Similar to our results inflorescence extracts of *T. cordata* showed a potent protective effect against inflammation this can be explained by high isoquercitrin and quercitrin content of samples as attenuation effects of isoquercitrin on LPS- induced expression of inducible nitric oxide synthase (iNOS) was

shown in rat peritoneal macrophages (139) and ability of quercitrin to inhibit the NF-κB pathway, thus, decreasing inflammatory cytokines including TNF-α and NO in LPS-stimulated RAW264.7 cells has been reported (128). In 2017, Jabeur et al. (16) from Portugal evaluated hydroethanolic extracts of *T. platyphyllos* aerial parts through measuring the inhibition of NO production in RAW 264.7 cell line. Extract concentration provides 50% of inhibition of nitric oxide (NO) production was determined as 225 ± 22 µg/mL. In this study, all investigated extracts showed more than 50% inhibition even at 125 µg/mL. Moreover, Flores-Sánchez et al. (71) evaluated a different species of *Tilia*, *T. americana var. mexicana,* for its anti-inflammatory activity. Both leaf and callus extracts of plant showed an effective anti-inflammatory activity against tetradecanoylphorbol-13-acetate induced ear edema.

To support the anti-inflammatory activities of *Tilia* species, IL-6 releasing inhibition assay was also performed. IL-6 is an important pro-inflammatory cytokine. According to findings, *T. rubra* flowers at 1 mg/mL concentration, exerted the highest inhibitory activity against LPS-activated release of IL-6 followed by *T. tomentosa* bracts at the same concentration. In accordance with the results of nitrite reduction, *T. tomentosa* bracts exhibited a high decrease in IL-6 release in LPS-activated RAW 264.7 cells. All extracts showed potent anti-inflammatory activity in IL-6 releasing inhibition assay. Phenolic compounds present in extracts may contribute to anti-inflammatory activities with their antioxidant capacities they can also suppress inflammation through interfering with signaling pathways (2). For instance, hyperoside has been shown to inhibit NF-κB activation through suppressing the degradation of IκBα in an LPS-induced inflammation model and reduce the production of IL-6 by peritoneal macrophages of mice (129). Protocatechuic acid (PCA) and astragalin have been shown to inhibit the expression of IL-6 (138,142). Moreover, astragalin has been shown to mitigate inflammation by reducing the expression of IL-6 in the murine model of mastitis by inhibiting IkBα degradation of inhibition the nuclear translocation of NF-κB (140).

Prostaglandin E_2 is a pro-inflammatory cytokine and a lipid mediator in inflammation. Following a harmful stimulus, PGE_2 is released from tissue resulting in localized edema and an increase in pain. The potential analgesic activity of the *Tilia* extracts was assessed in this study. To our knowledge, the effects of *Tilia* species on PGE_2 production were investigated for the first time. It is generally known that inflammation,

which includes the signs of redness, swelling, pain, and fever, is brought on by the release of large amounts of PGs in reaction to the inflamed tissue (143). In this case, the analgesic action on PGE_2 generations was assessed for extract doses that also exerted anti-inflammatory effects in the NO assay. *T. rubra* inflorescences at 0.5 mg/mL concentration, exerted the highest inhibitory activity against PGE_2 production followed by *T. platyphyllos* bracts at the same concentration. All extracts showed potent analgesic activity in a concentration-dependent manner. Most potent analgesic activity was obtained in *T. cordata* bracts followed by *T. tomentosa* inflorescences at 1 mg/mL concentration. For instance, isoquercitrin concentration was highest in *T. rubra* inflorescences with the highest analgesic activity which has been shown to decrease PGE_2 production in LPS-stimulated RAW264.7 cells (136). Moreover, tiliroside has also been reported to suppress PGE_2 production, inhibit COX-2 protein expression and inhibit NF-κB-dependent gene expression in HEK293 cells. (140).

Anticancer activity of dietary polyphenols has been extensively studied and their protective activity against oxidative stress is thought to be useful in preventing and treating cancer (144). Therefore, *Tilia* extracts that are rich in phenolic content were evaluated for their cytotoxic effects on human pancreatic cell lines firstly by 2D cytotoxicity assay. All extracts showed potent anticancer activity in a concentration-dependent manner against pancreatic cell lines with decreasing cell viability by at least 50%. *T. rubra* bracts (21.28 ± 3.50%) exerted the highest cytotoxic activity on the MIA PaCa-2 cell line at 1 mg/mL. followed by *T. rubra* flowers. Although anticancer effects of different *Tilia* species have been demonstrated in different cancer cell lines, their cytotoxic activity on MIA PaCa-2 pancreatic cancer cell lines has not been investigated. For instance, Barreiro et al. (74) demonstrated three different extracts of *Tilia cordata* Mill flowers had antiproliferative activities on BW 5147 lymphoma tumor cells. All extracts exerted a selective effect on tumor cells and caused apoptosis. In non-tumor lymphocytes, all extracts suppressed mitogen-induced proliferation. As a result of the study, aqueous, dichloromethane and ethanolic extracts of *T. cordata* flowers showed a dose-dependent inhibition of both tumor and non-tumor cells. The dichloromethane extract was determined as most active with an IC_{50} that was 11 and 1000 times lower than the IC_{50} values for the ethanol and aqueous extracts. In 2017, Jabeur et al. (16) assessed antitumor activity of 70% ethanolic extract of *T. platyphyllos* aerial parts using four tumor cell lines, HeLa (cervix cancer), HepG2 (hepatocellular carcinoma), NCI-H460 (lung

carcinoma) and MCF-7 (breast adenocarcinoma). Ellipticine was used as a positive control. HeLa 195 ± 15 µg/ml, HepG2 173 ± 13 µg/mL, MCF-7 224 ± 19 µg/mL and NCI-H460 247 ± 22 µg/mL, while IC_{50} values for ellipticine was 0.91 µg/mL for HeLa, 1.1 µg/mL for HepG2, 1.2 µg/mL for MCF-7 and 1.0 µg/mL for NCI- H460. Moreover, Carmona et al. (145) demonstrated a different species from Tiliaceae, *T. americana* var. *mexicana* exerted anticancer activity with K-562 leukemia cell line leaves and inflorescences of plant extracted with ethyl acetate were assessed for anticancer activity against different tumor cell lines including U-251, PC-3, K-562, HCT-15, MCF-7 and SKLU-1. Ethyl acetate extracts of leaves and inflorescences of *T. americana* var. *mexicana* did not inhibit growth in the tested tumor cell lines. However, a significant antitumor activity for the K-562 cell line with growth inhibition of 50.3% was observed in the *T. americana* var. *mexicana* inflorescences. In addition, Zarringhalami et al. (73) showed potent cytotoxic effects of aqueous extract of *Tilia dasystyla* on the Saos-2 cancer cell line with an IC_{50} value of 0.58 ± 0.01 mg/mL. The result of these studies demonstrates the anticancer efficacies of *Tilia* species with different extractions and on different cell lines. However, *T. rubra* with the highest anticancer was not investigated. Phenolic compounds present in herbal remedies have been reported to exert anticancer activity by several mechanisms such as carcinogen inactivation, cell cycle arrest, anti-proliferation, inhibition of angiogenesis, and induction of apoptosis (146).

All parts of *T. rubra* showed potent anticancer activity compared to other investigated species. Flowers, bracts and inflorescences extracts exerted highest cytotoxic activity on MIA PaCa-2 cell lines with IC_{50} values of 0.21 ± 0.01, 0.16 ± 0.01 and 0.24 ± 0.05 mg/mL, respectively. This effect may be attributed to high isoquercitrin content as isoquercitrin has been shown to have an inhibiting effect on angiogenesis that contributes to the growth of tumor and metastasis with *in vivo*, in a rat aortic ring assay (136). As all extracts showed potent anticancer activity this may be attributed to the phenolic contents of extracts. *In vitro* studies showed the decreasing effect of quercitrin on the transactivation of AP-1 and NF-κB transcription factors that participate in cellular signaling cascades and are approved to be in charge of initiation, promotion, and progression stages. Besides topical administration of quercitrin has been shown to inhibit AP-1 transaction by inhibiting phosphorylation of MAPK. Furthermore, by modulating immune response it has been shown to inhibit proliferation and induce apoptosis in lung carcinoma cells (128). In this study, *T. rubra* bracts extract with the highest quercitrin

concentration also showed the highest anticancer activity on MIA PaCa-2 pancreatic cell lines in a concentration-dependent manner. Furthermore, PCA has been shown to exert anticancer activity on lung, breast, liver, ovarian, and colon cancer. PCA has been shown to have a dose-dependent cytotoxic effect on HCT-15 colon cancer and MCF-7 breast cancer cell lines. Moreover, it has been shown to reduce cell viability and promote apoptosis and autophagy of SKOV-3 and OVCAR-3 ovarian cancer cells. It has been also shown to inhibit the formation and mutation of DNA adducts *in vitro*, inhibit the expression and recruitment of cytokines, expression of phase II enzymes, blocking vascular endothelial growth factor-induced angiogenesis by interfering with signaling pathways (138). This is also compatible with the findings of our study as the samples containing high concentrations of PCA also showed potent anticancer activity against MIA PaCa-2 pancreatic cell lines. IC_{50} values were determined as 0.44 ± 0.02 and 0.46 ± 0.01 for bract and flower extracts of *T. cordata.* According to our results, bract extract of *T. rubra* with the highest hyperoside content also showed the highest anticancer activity. Hyperoside has been shown to exert anticancer activity against lung cancer, gastric cancer, colon cancer, pancreatic cancer, ovarian cancer, cervical cancer, and breast cancer. For instance, in a previous study hyperoside was found to prevent lung cancer development by deactivating the NF-κB signaling pathway which accelerates cancer cell proliferation. Additionally, hyperoside administration was reported to inhibit B-cell lymphoma factor 2 and B-cell lymphoma factor x overexpression in lung cancer cells and increased the expression of antitumor factors such as p53 and p27 (129). Moreover, astragalin content is considered a contributor to anticancer activities of extracts. Astragalin has been reported to have the ability to inhibit the proliferation of different cancer cells including skin (SK-MEL-2 and HaCaT), hepatocellular (H22 and HepG2) leukemia (HL-60), and lung (A549) cancerous cells. It has been shown to inhibit NF-κB activation stimulated by TNF-α in H1299 and A549 cancerous cells. Additionally, astragalin has been shown to significantly suppress the proliferation of hepatocellular cancer cells in HepG2 cells (142).

3D *in vitro* cancer models provide more reliable results and demonstrate *in vivo* characteristics of the cells and better recapitulate the tumor microenvironment. Anticancer activities of different *Tilia* species have been investigated however, a 3D spheroid formation/growth assay was not performed with *Tilia* species. Therefore, according to the results of the 2D cytotoxicity assay, the extracts prepared from all parts

of *T. rubra* at different doses (0.125-1 mg/mL) and inflorescences of other species at the highest doses (1 mg/mL) were also investigated with 3D spheroid formation/growth assay, as the most potent anticancer effect was obtained with *T. rubra* and traditionally used parts of *Tilia* species are inflorescences. After 9 days of treatment spheroid size of the control medium increased by almost 7.5 times and flowers, bracts, and inflorescences of *T. rubra* extracts inhibited MIA PaCa-2 and HDF spheroid growth in a dose-dependent manner. Even at the lowest dose (0.125 mg/mL) all investigated parts of *T. rubra* reduced the growth rate of spheroids compared to medium control. At the highest dose (1 mg/mL) bracts of *T. rubra* showed the strongest spheroid size change with 50% reduction. Comparable to doxorubicin at 10 μM dose which reduced spheroid size by 70%. In addition, inflorescence extracts of all investigated species were evaluated for their anticancer activities by 3D spheroid formation/growth assay at the highest doses (1mg/mL). All species reduced the growth rate of spheroids compared to medium control. Similar to our results obtained by 2D cell cytotoxicity assay, at 1 mg/mL dose inflorescences extract of *T. rubra* showed the highest anticancer activity with a 50% reduction in tumor size growth followed by *T. cordata* (32%) and *T. tomentosa* (24%). Although spheroid size seems to grow 2 times in cells treated with inflorescences extract of *T. platyphyllos*, it reduced growth rate 4 times when compared to medium control.

In conclusion, this study was performed to comparatively evaluate different parts of hydroethanolic extracts of chemically characterized different *Tilia* species regarding their antioxidant capacities, protective effect against inflammation, and anticancer activities. Additionally, anticancer activities of different *Tilia* species were assessed with a 3D spheroid growth assay for the first time. Protective effects of *Tilia* species against inflammation have been investigated with nitrite determination. To support the anti-inflammatory activity of *Tilia* extracts, IL-6 releasing inhibition and PGE_2 production inhibition assays were performed for the first time. Nitrite inhibition assay results show that all extracts effectively reduce inflammation by preventing NO generation dose-dependently. Even at the lowest dose over 50% nitrite inhibition was obtained in all species. At the highest doses, all extracts showed inhibitory activity on nitrite production by 85-90%. The results of this study show that all investigated *Tilia* species rich in phenolic content have cytotoxic effects in MIA PaCa-2 pancreatic cancer cell lines in a dose-dependent manner with decreasing cell viability by at least 50%. *T. rubra* flowers with 0.16 ± 0.01 mg/mL IC_{50} value exerted the highest cytotoxic activity on MIA PaCa-

2 cell lines followed by *T. rubra* bracts extract with 0.21 ± 0.01 IC_{50} value. Overall, among other species investigated all parts of *T. rubra* with the highest total phenolic content exerted the highest antioxidant activities with a statistically important difference and highest anticancer effect in 2D cytotoxicity and 3D spheroid formation/growth assay. This study highlights a potential use for *Tilia* extracts, particularly *T. rubra* species in the prevention and treatment of pancreatic cancer. Since the cytotoxic effects of *T. rubra* on MIA PaCa-2 pancreatic cell lines, future studies on other cancer cell lines might be performed. Additionally, combination treatments with known anticancer agents such as doxorubicin and animal models might be beneficial for further elucidation. Moreover, as the *Tilia* species preferred as hot water infusion traditionally, a comparative analysis to determine the hot water infusion method efficacy may be performed. The attention to herbal medicines has increased due to their safe toxicological profiles compared to synthetic drugs (6). On the other hand, standardized herbal extracts provide consistent quality and effects with well-defined constituents. Despite promising biological activities of *the Tilia* species lack of a standardization method may be considered a limitation since this can affect biological activities and reproducible results.

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7. CURRICULUM VITAE

Kişisel Bilgiler

Adı	Gamze	**Soyadı**	Yüksel
Doğum Yeri	İstanbul	**Doğum Tarihi**	
Uyruğu		**TC Kimlik No**	
E-mail		**Tel**	

Öğrenim Durumu

Derece	Alan	Mezun Olduğu Kurumun Adı	Mezuniyet Yılı
Doktora	Farmasötik Toksikoloji	**Yeditepe Üniversitesi**	2022
Lisans	Eczacılık Fakültesi	**Yeditepe Üniversitesi**	2016
Lise	--	**Ataşehir Anadolu Lisesi**	2011

Başarılmış birden fazla sınav varsa(KPDS, ÜDS, TOEFL; EELTS vs), tüm sonuçlar yazılmalıdır

Bildiği Yabancı Dilleri	**Yabancı Dil Sınav Notu (#)**
İngilizce	YDS: 80

İş Deneyimi (Sondan geçmişe doğru sıralayın)

Görevi	Kurum	**Süre (Yıl - Yıl)**
Eczacı	Seval Eczanesi	3

Bilgisayar Bilgisi

Program	**Kullanma becerisi**
Microsoft Word	Çok iyi
Microsoft Excel	Çok iyi
Mendeley	İyi

*Çok iyi, iyi, orta, zayıf olarak değerlendirin

Bilimsel Çalışmaları

SCI, SSCI, AHCI indekslerine giren dergilerde yayınlanan makaleler

-
-

Diğer dergilerde yayınlanan makaleler

Sipahi, H., Aydoğan G., Helvacıoğlu S., Charehsaz M., Güzelmeriç E., Aydın A. "Antioxidant, antiinflammatory and antimutagenic activities of various kinds of Turkish honey." FABAD Journal of Pharmaceutical Sciences 42, no. 1 (2017): 7.

Uluslararası bilimsel toplantılarda sunulan ve bildiri kitabında (*Proceedings*) basılan bildiriler

Yüksel G., Özhan Y., Şen B., Güreşçi D., Güzelmeriç E., Sipahi H. "Comparative Evaluation of Protective Effects of Different *Tilia* Species Against Oxidative Stress and Inflammation" 11th International Congress of the Turkish Society of Toxicology, Antalya, Turkey from 2-5 November 2022. (Poster Presentation Accepted)
-

Hakemli konferans/sempozyumların bildiri kitaplarında yer alan yayınlar

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Diğer (Görev Aldığı Projeler/Sertifikaları/Ödülleri)

Doktora Bursu, Yeditepe Üniversitesi, Ağustos 2017
Üniversite Öğrencileri Yurt İçi Araştırma Projeleri Destek Programı, TÜBİTAK, Mart 2016
Yüksel G, "Yeditepe Üniversitesi Eczacılık Fakültesi, 2016 Mezunları Dönem Üçüncülüğü, Yeditepe Üniversitesi, Haziran 2016
Lisans Başarı Bursu, Yeditepe Üniversitesi, Haziran 2015