T.C.

YEDITEPE UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

DEPARTMENT OF PHARMACEUTICAL TOXICOLOGY

EVALUATION OF *SIDERITIS CONGESTA IN VITRO* AND *IN SILICO* FOR PROTECTIVE EFFECTS AGAINST INFLAMMATION AND ANTI-MUTAGENIC ACTIVITY

MASTER OF SCIENCES THESIS

ZEYNEP ÇOKÇEKEN

Istanbul-2022

T.C.

YEDITEPE UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

DEPARTMENT OF PHARMACEUTICAL TOXICOLOGY

EVALUATION OF *SIDERITIS CONGESTA IN VITRO* AND *IN SILICO* FOR PROTECTIVE EFFECTS AGAINST INFLAMMATION AND ANTI-MUTAGENIC ACTIVITY

MASTER OF SCIENCES THESIS

ZEYNEP ÇOKÇEKEN

SUPERVISOR

Prof. Dr. Hande Sipahi

CO-SUPERVISOR

Assist. Prof. Gülçin Tuğcu

Istanbul-2022

THESIS APPROVAL FORM

Institute : Yeditepe University Institute of Health Sciences

Program : Pharmecutical Toxicology Master Program

Title of the Thesis : Evaluation of *Sideritis Congesta In Vitro* and *In Silico* for Protective Effects Against Inflammation And Anti-Mutagenic Activity

Owner of the Thesis : Zeynep Çokçeken

Examination Date : 16 December 2022

This study has approved as a Master Thesis in regard to content and quality by the Jury.

Chair of the Jury:	Prof. Dr. Ahmet Aydın Yeditepe University
Supervisor:	Prof. Dr. Hande Sipahi Yeditepe University
Co-Supervisor:	Assist. Prof. Gülçin Tuğcu Yeditepe University
Member/Examiner:	Prof. Dr. Yüksel Kan Konya Selçuklu University
Member/Examiner:	Assoc. Prof. Muhammed Hamitoğlu Yeditepe University

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 .....

Prof. Dr. Bayram Yılmaz

Director of Institute of Health Science

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 which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

04 Jan 2023

Zeynep Çokçeken

ACKNOWLEDGEMENTS

First of all, I would like to thank and express my special gratitude to my mentor Prof. Dr. Hande Sipahi for all her support, guidance and patience during my time. Secondly, I would like to thank all my supervisors Prof. Hande Sipahi, Assoc. Prof. Muhammed Hamitoğlu and Assist. Prof. Gülçin Tuğcu who always supported and motivated me during this experience. Furthermore, I want to especially thank Prof. Dr. Yüksel Kan, for providing plant extracts and for being our inspiration point about the *Sideritis congesta*. I would like to express my gratitude to Prof. Ahmet Aydın for his guidance, passion and support throughout my journey in science and toxicology. Working with in harmony with Yağmur Özhan, Gözdem Karapınar and Makbule Kübra Doğan was an excellent experience so I would like to express my gratefulness to them. Finally, my deepest heart-felt gratitude is to my family, supporting with their uninterruptible love and concern.

TABLE OF CONTENTS

THESIS APPROVAL FORM ii

DECLARATION iii

ACKNOWLEDGEMENTS iv

TABLE OF CONTENTS v

LIST OF TABLES vii

LIST OF FIGURES viii

LIST OF SYMBOLS and ABBREVIATIONS ix

ABSTRACT xi

ÖZET xii

1. INTRODUCTION AND PURPOSE 1

2. GENERAL INFORMATION 3

2.1. Inflammation 3

2.1.1. Oxidative Stress, Free Radicals and Reactive Oxygen Species 4

2.1.2. NO's Function During Inflammation and In Inflammatory Disorders 6

2.2. Mutagenicity and Anti-Mutagenicity 10

2.3. Medicinal Plants 11

2.4. *Sideritis congesta* 12

2.5. Natural Products 18

2.5.1. Flavonoids 18

2.5.2. Cinnamic Acids 21

2.5.3. Terpenes and Essential Oils 24

2.6. Toxicological Evaluation of Bioactive Compounds via *In silico* Techniques 26

3. MATERIALS AND METHODS 28

3.1. Materials 28

3.1.1. Chemicals 28

3.1.2. Equipment 29
3.1.3. Plant Materials 30
3.2. Methods 30
3.2.1. Extract Preparation 30
3.2.2. Cell Culture 30
3.2.3. Cytotoxicity 30
3.2.4. Evaluation of Protective Effects Against Inflammation 31
3.2.5. Evaluation of Mutagenicity and Anti-Mutagenicity 31
3.2.6. *In silico* Evaluation 34
4. RESULTS 37
4.1. Cell Viability and Protective Effects Against Inflammation 37
4.2. Mutagenicity and Anti-Mutagenicity 38
4.3. *In silico* Analysis 41
5. DISCUSSION AND CONCLUSION 57
6. REFERENCES 64
7. APPENDICES 70
7.1. Curriculum Vitae 70

LIST OF TABLES

Table 2.1. List of natural components identified in *Sideritis congesta* extracts 17
Table 2.2. Medicinal use of some terpenes 25
Table 3.1. Preparation of solutions 33
Table 4.1. The cell viability and nitrite inhibition of controls and *Sideritis congesta* treated samples 37
Table 4.2. The number of revertant/plate, the standard deviation and the mutagenic index (MI) after the treatments with the compounds, in the two different strains of *Salmonella typhimurium*, with/ without S9 39
Table 4.3. Revertant colony numbers observed in the anti-mutagenicity, standard deviation and their inhibitory percentages of the *Sideritis congesta* extract with/ without S9 activation 40
Table 4.4. SMILES, CAS and EC numbers of active compounds found in *Sideritis congesta* 42
Table 4.5. *In silico* analysis results of Ames endpoints 49
Table 4.6. Results of other endpoints evaluated regarding genotoxicity in DanishQSAR database 51
Table 4.7. Activity prediction of compounds according to PASS Online platform 54

LIST OF FIGURES

Figure 2.1. Simplified illustration of NO production from L-arginine, NADPH and Oxygen 7
Figure 2.2. Representation of the potential adverse effects of prolonged inflammation and excessive NO production 8
Figure 2.3. Physical appearance of *Sideritis congesta* 13
Figure 2.4. Traditional use and biological processes for various *Sideritis* species 14
Figure 4.1. Effect of LPS stimulated RAW 264.7 cells treated with positive controls . 38
Figure 4.2. Anti-mutagenic activity (inhibitory rate %) of *Sideritis congesta* extract against positive control mutagens 40

LIST OF SYMBOLS and ABBREVIATIONS

#	Number
2-AF	(E)-2-(2-Furyl)-3-(5-nitro-2-furyl) Acrylamide
ABTS	2,2′-Azino-Bis (3-Ethylbenzthiazoline-6-Sulphonic Acid
BChE	Butyrylcholinesterase
COX	Cyclooxygenase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
eNOS	Endothelial NOS
FBS	Fetal Bovine Serum
FDA	Food And Drug Administration
GC/MS	Gas Chromatography Mass Spectrometry
GI	Gastrointestinal
Gpx	Glutathione Peroxidase
ILs	Interleukins
iNOS	Inducible NOS
JAK-STAT	Janus Kinase Signal Transducer and Activator of Transcription
K_2HPO_4	Potassium Phosphate
KCL	Potassium Chloride
L-NAME	N^G-Nitro-L-Arginine Methyl Ester
L-NMMA	N^G-Monomethyl- L-Arginine
MAPK	Mitogen-Activated Protein Kinase
MDA	Malondialdehyde
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulfate
MIP-2	Macrophage Inflammatory Protein-2
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate with Extra Hydrogen
$NaHN_H4PO_4$	Sodium Ammonium Phosphate
$NaNO_2$	Sodium Nitrite
NF-κB	Nuclear Factor Kappa-B

nNOS	Neuronal NOS
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NOX	NADPH Oxidase
Pa	Activity Probability
PAMPs	Pathogen-Associated Molecular Patterns
PBPK	Physiology-Based Pharmacokinetic
Pen-strep	Penicillin-Streptomycin
Pi	Inactivity Probability
PMNs	Polymorphonuclear Neutrophils
PRRs	Pattern-Recognition Receptors
QSAR	Quantitative Structure-Activity Relationship
RA	Rosmarinic Acid
ROS	Reactive Oxygen Species
RSV	Respiratory Syncytial Virus
SA	Sodium Azide
SOD	Superoxide Dismutase
TEAC	Trolox Equivalent Antioxidant Capacity
TNF-α	Tumor Necrosis Factor-A
VB	Vogel-Bonner
WHO	World Health Organization

ABSTRACT

Çokçeken Z. (2022). Evaluation of *Sideritis Congesta In Vitro* and *In Silico* for Protective Effects Against Inflammation And Anti-Mutagenic Activity. Yeditepe University, Institute of Health Sciences, Department of Pharmaceutical Toxicology, MSc Thesis, İstanbul.

The number of studies on the species of the genus *Sideritis* has increased significantly over the past 20 years. *Sideritis*, commonly known as "mountain tea" are widely used by traditional medicine for anti-inflammatory, anti-ulcer, antioxidant and anti-microbial activities. Previous studies have indicated that *Sideritis* has many medicinal applications. However, *Sideritis congesta*, especially for the endemic ones found in Turkey remains limited and not discovered, although local people extensively use this plant in our country. This paper is the first study in which protective effects of *Sideritis congesta* against inflammation is investigated by *in vitro* method, nitrite assay. Secondly, this paper is also the first study in which mutagenic and antimutagenic activity of *Sideritis congesta* has been investigated by Ames test. Moreover, our findings in here supported by *in silico* analysis in aspect of the same endpoints conducted *in vitro* assays. According to our results, *Sideritis congesta* exhibited protective effects against inflammation in LPS stimulated RAW264.7 murine macrophage cells by inhibiting NO production. Additionally, *Sideritis congesta* extracts showed anti-mutagenic activity at 1000 and 5000 µl/plate in TA98 and TA100 *Salmonella typhimurium* with S9 activation. *In silico* analysis revealed that some of the active compounds found in *Sideritis congesta* are potentially mutagenic, but none of the *in vitro* analysis in our study showed mutagenicity. As a conclusion, a proper formulation containing *Sideritis congesta* extract might be a potential anti-inflammatory and anti-mutagenic agent.

Key words: Anti-mutagenic Activity, Ames Test, *in silico* Evaluation, NO Assay, Protective Effects Against Inflammation, *Sideritis congesta*

ÖZET

Çokçeken, Z. (2022). *Sideritis congesta*'nın İnflamasyona Karşı Koruyucu Etkileri ve Anti-Mutajenik Aktivite Açısından *in vitro* ve *in silico* Değerlendirilmesi. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmasötik Toksikoloji Anabilim Dalı, Yüksek Lisans Tezi, İstanbul.

Sideritis cinsinin türleri üzerine yapılan çalışmaların sayısı son 20 yılda önemli ölçüde artmıştır. Yaygın olarak "dağ çayı" olarak bilinen *Sideritis*, anti-inflamatuar, anti-ülser, antioksidan ve anti-mikrobiyal aktiviteler için geleneksel tıp tarafından yaygın olarak kullanılmaktadır. Önceki çalışmalar *Sideritis*' in birçok tıbbi uygulamaya sahip olduğunu göstermiştir. *Sideritis congesta* yöre halkı tarafında ülkemizde yaygın olarak kullanmasına rağmen, bu bitki ile ilgili çalışmalar, özellikle de Türkiye'de bulunan endemik olanlar için çok sınırlıdır.. Bu çalışma, *Sideritis congesta*' nın inflamasyona karşı koruyucu etkilerinin *in vitro* yöntem olan nitrit testi ile araştırıldığı ilk çalışmadır. İkinci olarak, bu araştırma aynı zamanda *Sideritis congesta*' nın mutajenik ve antimutajenik aktivitesinin Ames testi ile araştırıldığı ilk çalışmadır. Ayrıca, buradaki bulgularımız*, in vitro* deneylerde yürütülen aynı son noktalar açısından *in silico* analizler ile desteklenmektedir. Sonuçlarımıza göre *Sideritis congesta*, LPS ile uyarılan RAW264.7 murin makrofaj hücrelerinde NO üretimini inhibe ederek inflamasyona karşı koruyucu etki göstermişti. Ek olarak *Sideritis congesta* ekstreleri, S9 aktivasyonlu TA98 ve TA100 *Salmonella typhimurium'da* 1000 ve 5000 µl/plakada anti-mutajenik aktivitesi olduğu saptandı. *In silico* analiz, *Sideritis congesta' da* bulunan bazı aktif bileşiklerin potansiyel olarak mutajenik olduğunu ortaya çıkardı, ancak çalışmamızdaki *in vitro* analizlerin hiçbiri mutajenite göstermedi. Sonuç olarak, *Sideritis congesta* ekstraktı içeren uygun bir formülasyon, potansiyel bir anti-inflamatuar ve anti-mutajenik ajan olabilir.

Anahtar Kelimeler: Anti-mutajenik Aktivite, Ames Testi, i*n silico* Değerlendirme, İnflamasyona Karşı Koruyucu Etki, NO Testi, *Sideritis congesta*

1. INTRODUCTION AND PURPOSE

Since the ancient times, many different medicinal and aromatic plants were traditionally used, and their importance and beneficial effects are becoming acknowledged for the contemporary life. Most of the scientific researches regarding ethnopharmacology is started from the traditional experience accumulated in the folk memory [1].

The origin of modern medicine is built by the use of plant for healing purposes in history. The majority of the few effective treatments from a century ago were plant-based, which explains why many conventional drugs now have plant origins. Examples include morphine, digoxin, quinine, and aspirin, all of which are derived from the *opium poppy, foxglove, cinchona bark* and *willow bark* respectively. However, just because a medicine is plant based it is not necessarily to be safe. Some herbs, such as comfrey and ephedra, can cause serious harm when unconsciously used. Some herbs can interact with prescribed or over-the-counter drugs. Herbal remedies have the potential to interact with prescription medications, and some of these interactions have been thoroughly studied. Unregulated herbal therapy undoubtedly poses a larger risk of side effects than other complementary therapies because many plants may have hazardous effects. However, they are easily accessible and have less adverse effects than synthetic drugs, especially for the hepatotoxicity, they are great candidate to be developed as drugs. To this end the development of drugs from plants still an ongoing popular process [1].

High plant diversity is a characteristic of southern regions of Europe. The family *Lamiaceae* and species of the genus *Sideritis* are among the most widely used herbs in South-Eastern Europe [2]. Several papers have been previously published about the ethnopharmacological uses of some *Sideritis* species. The majority of this diversified plant is utilized for therapeutic purposes. The ethnopharmacological uses of various *Sideritis* species have been covered in a number of published studies. The digestive, anti-inflammatory, and vulnerary qualities of *Sideritis* species are generally associated with the most frequently documented medical uses of these plants. Based on these qualities, aerial component infusions and decoctions have been used to treat gastrointestinal, respiratory, urogenital, and wound conditions as well as promote wound healing. Different *Sideritis* are used differently depending on where they grow. However, plant

characteristics are the basis for all of the uses. To alleviate abdominal pain, a poultice made from boiled *Sideritis psidica* leaves, barley flour, chopped onion, and pine tar is applied as a plaster to the abdomen in the Taurus Mountains of Turkey [3]. The aerial parts of plants in the genus *Sideritis*, also known as "mountain tea," are widely utilized in Mediterranean nations including Greece, Turkey, and Spain for anti-inflammatory [4], anti-ulcer [5], antioxidant [6] and anti-microbial [7] activities. The plant's numerous phytochemicals, including flavonoids, phenolic acids, and diterpenoids, have been associated to several of these functions [8]. All in all, *Sideritis* species have been demonstrated with many different activities included but not limited to flu and colds, respiratory, digestive and cardiovascular system diseases, anemia, anti-microbial and antivirus activities, disease of urinary system, anti-inflammatory activity, antioxidant activity, gastroprotective activity and pharmacological activities on the central nervous system diseases [9]. By far *Sideritis scardica* is the most studied species. However, studies regarding *Sideritis congesta*, especially for the endemic ones found in Turkey remains limited and obscure although indigenous people extensively use this plant [9].

The objective of the study is to identify the protective effect of *Sideritis congesta* against Lipopolysaccharide (LPS) induced inflammation. Furthermore, we aimed to evaluate mutagenic and anti-mutagenic activities of aqueous and ethanol extracts of *Sideritis congesta* by Ames Test. Additionally, our goal was to investigate toxicological endpoints of bioactive compound found in extracts of *Sideritis congesta* by using *in silico* modellings. Nowadays drug companies engaged in large-scale pharmacologic screening of herbs in order to develop new drugs. The outcome of this study may lead to development of new drugs based on the extract of *Sideritis congesta* and this plant may serve better and safer in mammalian species, than synthetic drugs. This study will help to evaluate safety profile of *Sideritis congesta* in manner of mutagenicity, anti-mutagenicity and efficacy of it as anti-inflammatory property with *in vitro* methods. Furthermore, *in silico* analysis will designate potential harms and benefits and pioneer to consider possible interactions of *Sideritis congesta*.

2. GENERAL INFORMATION

2.1. Inflammation

A biological reaction of the immune system's defense, inflammation can be brought on by a variety of things, such as pathogens, harmed cells, irritation, poisonous substances, etc. Any organ, including the heart, pancreas, liver, kidney, lung, brain, digestive tract, and reproductive system, may exhibit these variables, which could result in disease or tissue damage. Even thought, the main purpose of the inflammation is to trigger healing process via restoration of tissue haemostasis, long term and uncontrolled exposure of the harmful stimulus can lead to chronic inflammation and thus, chronic inflammatory diseases such as diabetes, arthritis, and cancer [10].

When examined at tissue level, inflammation appears as redness, swelling, heat, pain, and loss of tissue functions as a consequence of change in vascular permeability, recruitment and accumulation of leukocytes, and release of inflammatory mediators. However, at the cell level, inflammation process is more complicated and has various different cascades. First step of the inflammation at the cell level is pattern recognition and receptor activation. Patterns can be either endogenous signals activated by tissue or cell injury and are known as danger-associated molecular patterns (DAMPs), or they can be microbial structures called pathogen-associated molecular patterns (PAMPs). Pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and NOD-like receptors are responsible for identifying those patterns (NLRs). Signaling though each receptor can initiate a different pathway which will later on alter many intracellular activities including, transcription factors, interferon regulatory factors such as activator proteins, apoptosis, necrosis etc. The most prevalent and significant pathways that contribute to the inflammation process are nuclear factor kappa-B (NF-B), mitogen-activated protein kinase (MAPK), and Janus kinase signal transducer and activator of transcription (JAK-STAT) [10].

Not only receptors and pathways are responsible from inflammation process but also cellular molecules such as cytokines, enzymes and proteins are part of the process. Inflammatory cytokines, such as interleukin (IL)-1, IL-6, TNF (TNF), and inflammatory

proteins and enzymes are produced and released in response to harmful stimuli. These compounds are employed in clinical settings as biomarkers for the diagnosis, prognosis, and selection of treatment interventions. Monocytes, macrophages, and lymphocytes, which are immune cells, are the main sources of cytokines' secretion. ILs, also known as inflammatory cytokines, have the ability to either promote or suppress inflammation. Cells release cytokines largely to draw leukocytes to the area of injured tissue. They have a complex of network of interaction which helps them to modulate immune response and regulate inflammation. Uncontrolled production of cytokines can lead to tissue damage and change in hemodynamic of the tissue and finally to organ failure [10].

During trauma, stress, or infection, inflammatory proteins in the blood assist restore equilibrium and reduce microbial development independently from antibodies. C-reactive proteins, haptoglobin, serum amyloid A, fibrinogen, and alpha 1-acid glycoprotein are a few examples of proteins that can cause inflammation. The aberrant activation of inflammation enzymes such high-mobility group box 1, superoxide dismutase (SOD), glutathione peroxidase (GPx), NADPH oxidase (NOX), and cyclooxygenase-2 usually causes inflammation-related illnesses like cancer and cardiovascular disease (COX-2) [10].

2.1.1. Oxidative Stress, Free Radicals and Reactive Oxygen Species

Chemicals possessing an unpaired electron in the outer orbit are called free radicals. Free radical and non-free radical oxygen-containing molecules can both be found in Reactive Oxygen Species (ROS) [11]. Increased free radical generation and/or decreased physiological activity of antioxidant defenses against free radicals lead to oxidative stress.

During the inflammation, oxidative stress in highly elevated due to antioxidant defence systems such as polymorphonuclear neutrophils (PMNs) and antioxidant enzymes (SOD and GPx). The formation of ROS, malondialdehyde (MDA), 8-hydroxy-2-deoxyguanosine, and isoprostanes increases with increased oxidative stress. Additionally, there exist species of reactive nitrogen, iron, copper, and sulfur. Each of those can activate various transcription factors and alter expression of gene expression, cytokines and chemokines [10]. By itself, ROS can function during inflammation as a

mediator and a signaling molecule. Through the oxidation of essential cellular signaling proteins, such as tyrosine phosphatases, they encourage endothelial dysfunction. They are energetic compounds or partially reduced oxygen metabolites with powerful oxidizing properties. Even ROS serve complex signaling function in low concentrations, they are harmful to cells at high concentrations. They may oxidize every cellular constituent including proteins, lipids and even DNA, when they come across within the cell. ROS are produced as byproducts of cellular metabolism through the electron transport chain in mitochondria. In some cases, they are generated as a product of NOX that are present in phagocytes and endothelial cells, where inflammatory response centrally initiates. The mostly studied and revealed ROS family members are the superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), and hypochlorous acid. Other free radicals may be produced as a result of microbial infections, extensive exercise, pollutants, toxins such as cigarette smoke, alcohol, ionizing and UV radiations [11, 12].

When those free radicals are abnormally generated within the cell, normal redox balance is disrupted and cause a toxic effect for the cell and tissue. To avoid such cases, ROS scavenging system, prooxidant and antioxidant substances favour the mild oxidative stress. Biological antioxidants are compounds that can inhibit or delay oxidation of the substrate via free radicals. They are expected to be present at low concentrations compared to oxidizable substrates. An ideal antioxidant is expected to be readily absorbed, able to prevent or reduce free radical formation or scavenge radicals. Normally, antioxidant defence mechanism helps to repair oxidatively damaged nucleic acid, to remove oxidized proteins via proteolytic enzymes or to repair oxidized lipids via phospholipases, peroxidases, and acyl transferases. The initiation of failure of one or more of those systems contribute aging and age-related diseases. Age-related diseases are mostly tended to be occur in non-proliferating cells, such as neurons and cardiomyocytes (cardiac cells). Because they cannot divide, cell damages accumulate and even causes more stress for the cell. The use of exogenous antioxidants like vitamin C and E, carotenoids, and polyphenols (e.g., flavonoids) can help cells to fight better against free radicals and avoid or delay cell damage and thus tissue injury [11]. It is reported that even increased fruit and vegetable intake can help to reduce lower cell

damage, DNA damage, malignant transformation rate, thus the incidence of some types of diseases including cancer, degenerative diseases or hear disease.

The endogenous antioxidant can be sourced from fruits, vegetables, legumes. They can be synthetically produced and consumed as a nutritional supplement. Questionnaires showed that majority of the US adults uses food supplements that are antioxidants such as vitamin A (retinoids, carotenes), vitamins C and vitamin E (tocopherols), lycopene, lutein, ubiquinone, glutathione, and polyphenols (flavonoids). It is important that safety of the supplement is solid before it is marketed. They even need to be registered to Food and Drug Administration (FDA) in order to be marketed [11]. The use of antioxidant should be limited as well. Because inappropriate use of those supplement can even cause antioxidative stress which is another imbalance within the cell. Thus, the balance between oxidative state and antioxidant level should be well maintained.

2.1.2. NO's Function During Inflammation and In Inflammatory Disorders

The fact that one nitrogen atom is covalently bonded with one unpaired electron to an oxygen atom makes nitric oxide (NO) a member of the ROS family. When heme-iron and oxygen-containing groups are present, it becomes more reactive, converting NO into more stable nitrate molecules. Therefore, the site of synthesis affects the bioavailability of NO. Its availability and biological effects are quite weak at haemoglobin- and myoglobin-rich blood and muscle locations. In contrast, because NO is a small molecule in addition to being lipid soluble, it can diffuse over distances greater than a few microns and cross cell membranes quickly. Therefore, many of its effects, such as blood flow and pressure, platelet function, host defense, and neurotransmission in the central nervous system and peripheral nerves, can be explained by its ability to diffuse across cells and function as a paracrine-signaling molecule [13, 14].

Hibbs and colleagues initially identify NO as an effector chemical in macrophage-mediated cytotoxicity. Numerous studies investigating the role of NO in inflammatory reactions have been carried out since this discovery. High quantities of NO are created in response to inflammatory stimuli and mediate proinflammatory and destructive effects, according to the findings of those studies. Contrarily, NO, like the majority of other inflammatory mediators, has beneficial benefits in some inflammatory responses [13].

Nitric oxide synthase (NOS) produces NO in several organs. In macrophages taken from people with a variety of illnesses associated to infection and inflammation, such as tuberculosis, malaria, AIDS, and rheumatoid arthritis, NO, nitrogen oxide species, and NOS are easily identifiable [15]. Figure 2.1 shows the three isoforms of NOS are called after the regulation site and mechanism. These isoforms include endothelial constitutive NOS (eNOS or NOSIII), neuronal NOS (nNOS or NOSI), and inducible NOS (iNOS or NOSII). These enzymes convert arginine into L-citrulline and generate NO as a result. The formation of NO requires two co-factors: oxygen and NADPH (nicotinamide adenine dinucleotide phosphate with additional hydrogen) [14].

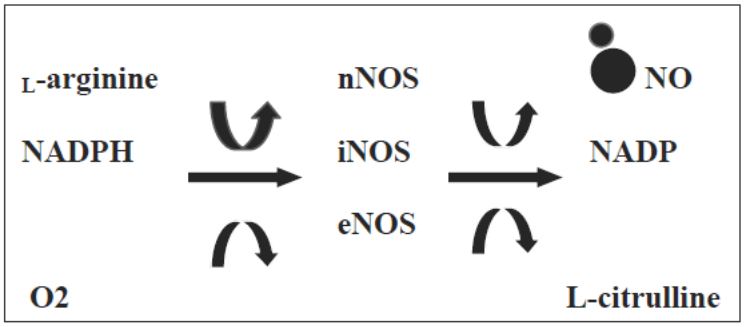

Figure 2.1. Simplified illustration of NO production from L-arginine, NADPH and O_2

Reprinted from: Sharma JN, Al-Omran A, Parvathy SS. Role of nitric oxide in inflammatory diseases. Inflammopharmacology. 2007

During inflammatory reactions such as bacterial infections, endotoxins etc., expression iNOS synthase in monocytes, neutrophil granulocytes, macrophages and many other cells is increased by the pro-inflammatory cytokines. As a result, excessive amounts of this, excessive amount of NO are synthesized (1000 times higher than the psychological condition). NO is secreted by neutrophiles and macrophages during the time phagocytosis of endogenous substances. During the initiation of inflammation, circulating neutrophiles, and endothelial cells migrates towards the site of the injury. This process is also called as chemotaxis. Many proteins, carbohydrates adhesion molecules and messenger molecules are released by both neutrophils and endothelial cells. Those messenger molecules help to recruitment of leukocytes and macrophages to the inflamed area. Recruited macrophages are than transformed to engulfing, killing phagocytes. The lysosomal enzymes break down the macromolecules of endogenous substances once the phagosome and lysosome membranes combine. These lysosomal enzymes are produced in addition to NO, hydrogen peroxide, and other oxygen derivatives. Furthermore, in

addition to releasing harmful chemicals within the phagosome, phagocytes can release these chemicals into the extracellular fluid, where they can fight and eliminate bacteria without first phagocytosing them. The inflammatory mediator NO is one of the chemicals released into the extracellular fluid. When phagocytes enter a region that is inflamed, they promote the recruitment of inflammatory mediators like chemokines and additional phagocytes, creating a positive feedback loop. When NO is produced excessively, it can cause tissue to be destroyed, just like in inflammatory autoimmune disorders. Therefore, it can process pro- or anti-inflammatory actions depending on the NO concentration. Therefore, NO is referred to as a "double-edged sword " [14]. Pacher et al., represented in Figure 2-2, consequences of increased iNOS concentration caused by infection and injury. Chronic inflammation can cause many intracellular activities including but not limited to damaged DNA, protein modification, apoptosis etc. [16].

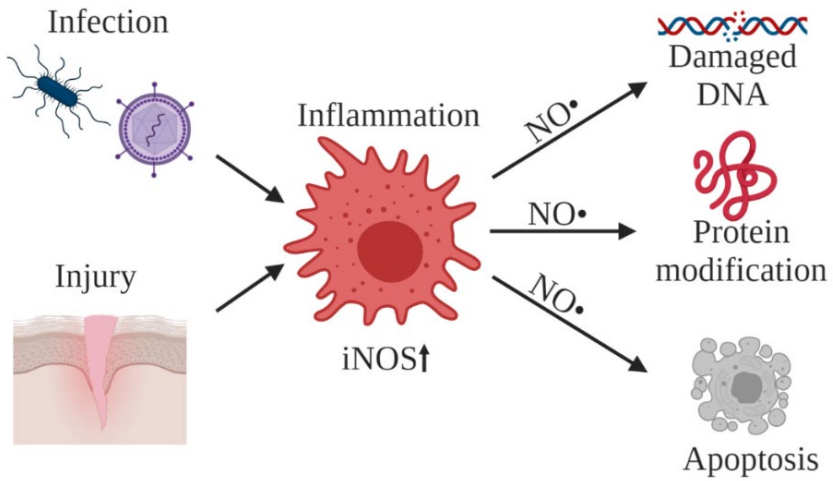

Figure 2.2. Representation of the potential adverse effects of prolonged inflammation and excessive NO production

Reprinted from: Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007

Numerous studies have shown a correlation between brain inflammation and the development of numerous degenerative disorders, including dementia, AIDS, Parkinson's disease, Alzheimer's disease, multiple sclerosis, and amyotrophic lateral sclerosis. The primary cause of brain inflammation is glial cell activation, which also stimulates the creation of several pro-inflammatory and neurotoxic substances, such as cytokines, fatty acid metabolites, and free radicals like NO and superoxide [14].

The primary NO synthase implicated in inflammatory reactions is iNOS. Due to cytokine activation, almost all inflammatory cells express the iNOS gene. Numerous inflammatory disorders, such as the bronchial epithelium of asthmatic patients, the mucosa of the colon in ulcerative colitis patients, and synoviocytes in inflammatory joint diseases are shown to have NOS. In addition, increasing levels of NO generation and NO-dependent oxidative stress are frequently linked to inflammatory disorders of the respiratory tract. Despite having antimicrobial, anti-inflammatory, and antioxidant capabilities, studies have shown that NO also causes lung damage in a number of disorders [14].

The discovery of the NO pathway in inflammatory disorders opened up the possibility of NO-related therapy. Since NO plays a dual role, it is critical to strike a balance between its excessive production and psychological conditions. In order to do this, the primary goal of NO-related therapy must be to decrease NO overproduction, which primarily results in inflammatory tissue damage. Such suppression has been prompted by two separate techniques. The first is a class of drugs called selective NO biosynthesis inhibitors, which only affect iNOs and not constitutive NOS. The synthetic arginine analogues, which would compete with arginine, are the second one of the category [14].

Most chronic inflammatory, non-infectious illnesses are treated by selectively inhibiting increased NO production. Some well-known medications used to treat various conditions impair iNOS expression or activity. While glucocorticoids reduce iNOS expression, they have no impact on iNOS and constitutive NOS activity. Its mechanism is intricate and includes decreased enzyme stability as well as transcriptional and translational inhibition. NOS expression is inhibited by derivatives of cyclosporin. IL secretion and its effects on gene transcription provide an explanation for its mechanism. Non-steroidal anti-inflammatory medicines have been shown to have similar effects on the expression of inducible NO synthase. Additionally, salicylates are referred to as NO scavengers. It has been discovered that 5-aminosalicylic acid reduces NO production and disease activity in inflammatory illnesses, particularly adjuvant arthritis [14].

Numerous mechanisms exist through which synthetic arginine analogues might suppress NOS or its activity. The competition between arginine mimics and NOS can

extend to the carrier that carries arginine into endothelial cells in some situations. Numerous substances, such as aminoguanidine, NG-nitro-L-arginine methyl ester (L-NAME), and NG-monomethyl-L-arginine (L-NMMA), have proven to be quite useful as experimental methods [17]. L-NMMA has been researched in the past for the treatment of neurodegenerative illnesses and inflammation brought on by an excess of NO. Sadly, the mortality rate in these individuals rose due to the use of L-NMMA [14].

2.2. Mutagenicity and Anti-Mutagenicity

Mutagenicity is an important toxicological endpoint required thorough evaluation of safety of a compound, molecule, product, etc. It refers to the induction of permanent transmissible changes in the amount or structure of the genetic material of cells or organisms. Traditionally used plants are become important screening and development and potential chemotherapeutic drugs. The increasing interest in medicinal plants led to investigation of their biological effects on human beings. Establishing their safety of their continuous use and supporting their cytotoxic, mutagenic and anti-mutagenic potential are now crucial [18].

It has been hypothesized that mutations are main responsible in carcinogenesis process and can also contribute to the pathogenesis of other chronic diseases, such as atherosclerosis, neurodegenerative diseases, degenerative hearth diseases, glaucoma etc. To this end, interest in research for anti-mutagens which strive against mutagens become important and popular. Plants are known as promising source of anti-mutagens thanks to their secondary metabolites such as flavonoids. Variety of anti-mutagenic and anti-genotoxic compounds of many different plant extracts and their constituent have been proved so far. These anti-mutagens may help in strengthening cellular defences against environmental mutagens, stress and thus preventing the development of several mutation-related diseases. Different mechanisms are proposed for their activity. They can destroy or block the DNA-damaging mutagens outside the cells, thus avoiding cell mutations [18, 19]. Today, the most well-known and dependable approach, the Ames Test, has been used to assess the mutagenic/ anti-mutagenic properties of extracts from diverse medicinal plants. According to the experiments, numerous plant extracts, including *Pteris multifida*, *Smilax china*, and *Prunella vulgaris* have anti-mutagenic activity [20].

2.3. Medicinal Plants

Since the ancient times, many different medicinal and aromatic plants were traditionally used, and their importance and beneficial effects are becoming acknowledged for the contemporary life. Nowadays medicinal plants are still representing an important inspiration for the identification of novel drug pioneers. Most of the scientific researches regarding ethnopharmacology is started from the traditional experience accumulated in the folk memory [1]. For instance, since the ancient times, in European countries peppermint and thyme have been used for medical purpose whereas, aromatic species like cinnamon or sandalwood have been used for traditional medicine in Chinese or Indian cultures. Currently many anti-spasmodic botanical remedies are used for symptomatic treatment of intestinal, colonic or ureteral spams [21]. Two successful example of plant natural products that are being currently used in hospitals are morphine isolated from *Papaver somniferum* also called opium poppy and Paclitaxel, a commonly used chemotherapeutic agent, isolated from *Taxus brevifolia* also called as pacific yew [22].

Currently, many different public and scientific interest are focused on natural products to cure contemporary diseases such as cardiovascular disease and inflammatory diseases such as diabetes and cancer [23]. Even in developed countries, England, Germany, France and China, medicinal plant extracts are sold under prescribed drugs [22]. According to the data given by World Health Organization (WHO), 80% of the population still uses and relies on plant-based drugs, because they are cheap and accessible, but meanwhile they have less severe side effects than sensitized drugs [24].

It is known that bioactive extracts of numerous medicinal plants are isolated/identified and shows pharmacological effects for many different acute and chronic diseases [25]. Based on a recent report published by State of Wold's Plants, at least 28 187 species have been recorded as being used in traditional medicine. However, there are no current data regarding how many of them have been examined for their pharmacological potential. It is estimated that only a few hundred of them have been deeply investigated for pharmacological, bioactivity and chemical composition analysis [26]. Preclinical tests such as cytotoxicity, mutagenicity and *in silico* analysis are needed to ensure that these compounds are safe for use in human.

2.4. Sideritis congesta

The most common and important family of herbal families is known as *Lamiaceae*. It has a wide variety of plants with biological and medical applications. The most famous members of this family are thyme, mint, oregano, basil, sage, savory, rosemary, etc. [24].

Sideritis, containing around 190 species of the family *Lamiaceae,* and commonly found in the Northern Hemisphere distributed across North Africa, the Iberian Peninsula, the Mediterranean countries and the Middle East [27, 28]. According to the most recent taxonomical classifications, the *Sideritis* genus contains about 150 species that are dispersed over the Western Palearctic zone. *Sideritis*, which means "iron" in Greek, got its name because it was traditionally used to treat wounds caused by metal weapons. Taxonomically speaking, *Sideritis* is a rather complex genus with two subgenera and seven sections. Even though the number of species largely depends on taxonomic concepts, Turkey is home to about 90% of all species, and about 80% of those species are indigenous to Turkey and the surrounding area. The majority of the species are traditionally utilized by the populace as herbal tea for medical purposes, according to the most recent statistics. Because they are primarily grown in mountainous regions, *Sideritis* is known as "mountain tea," particularly in the Balkans [9]. It is made up of short subshrubs that flourish at high elevations. A thin, fuzzy coating of small, interwoven hairs covers them. Verticillaster is a collection of flowers that are grouped on a main branch and grow.

The method preparing, applying and using medicinal plants mainly vary on geography. People in Western Europe used to use isolated biologically active substances, medications, extracts, and food supplements that had undergone clinical testing. In contrast, people used to gather, dry, and prepare the plants in Southeast Europe. For instance, making herbal teas at home is highly common and even preferred to taking medications in Albania, Bulgaria, Greece, Macedonia, and Turkey. In their ethnobotanical research, numerous authors have highlighted the usage of *Sideritis* species as herbal tea [9].

Figure 2.3. Physical appearance of *Sideritis congesta*

Herbarium no: (SÜTEB 60)
Sideritis congesta was cultivated in L. Selcuk University, Medical and Endemic Plants Training and Research Farm. Herbarium samples are kept in Selcuk University Medicinal and Endemic Plants Herbarium.

However, usage of the medicinal plant is mainly based on plant property and the general use of the folk. uses. To alleviate abdominal pain, a poultice made from boiled *Sideritis psidica* leaves, barley flour, chopped onion, and pine tar is applied as a plaster to the abdomen in the Taurus Mountains of Turkey [3]. The aerial parts of plants in the genus *Sideritis*, also known as "mountain tea," are widely utilized in Mediterranean nations including Greece, Turkey, and Spain for anti-inflammatory [4], anti-ulcer [5], antioxidant [6] and anti-microbial [7] activities. The plant's numerous phytochemicals, including flavonoids, phenolic acids, and diterpenoids, have been associated to several of these functions [8]. Overall, *Sideritis* species have been shown to have a wide range of activities, including but not limited to pharmacological activities on diseases of the central nervous system, respiratory, digestive, and cardiovascular system diseases, anemia, anti-microbial and anti-virus activities, disease of the urinary system, anti-inflammatory activity, antioxidant activity, and gastroprotective activity [9]. Traditional use and biological processes for various *Sideritis* species are illustrated in Figure 2.4.

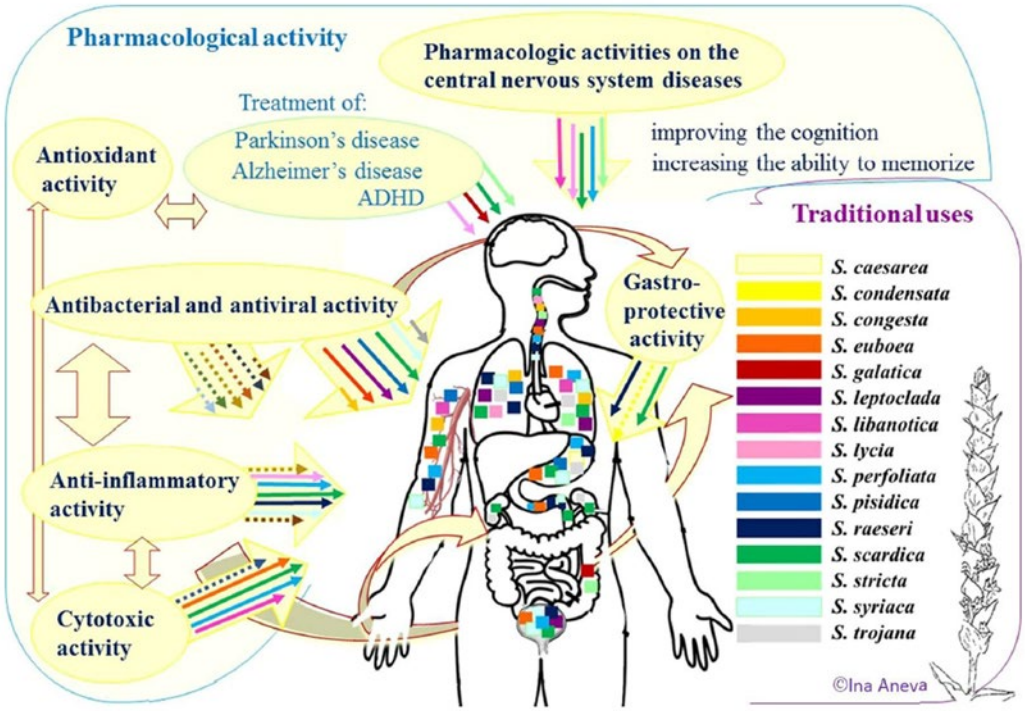

Figure 2.4. Traditional use and biological processes for various *Sideritis* species

Reprinted from: Genus Sideritis, section Empedoclia in southeastern Europe and Turkey – studies in ethnopharmacology and recent progress of biological activities

By far *Sideritis scardica* is the most studied species. However, studies regarding *Sideritis congesta*, especially for the endemic ones found in Turkey remains limited and obscure although indigenous people extensively use this plant.

Erkan et al., deeply examined the antioxidant activities of *Sideritis congesta* and *Sideritis arguta* and their components in pure form based on 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (also known as Trolox equivalent antioxidant capacity (TEAC) assay) assays. Both assays are used to predict and measure antioxidant scavenge capacities of the compounds. Their primary concern was searching for free flavonoids and derivatives of cinnamic acid in the extracts of these plants. Because the majority of the antioxidant activity of plants is derived from the groupings of chemicals known as flavonoids and cinnamic acid derivatives. These two categories of phenolic chemicals are primarily found in tea and herbal remedies in the human diet. By using the high-performance liquid chromatography with diode-array detection technique, they were able to identify the phenolic components in both the

acid-hydrolyzed *Sideritis congesta* MET extract and the untreated *Sideritis congesta*-MET extract. According to their results, *Sideritis congesta* were found to have high antioxidant capacity and contains, rosmarinic acid, ferulic acid, caffeic acid, p-coumaric acid, chlorogenic acid, apigenin, myricetin and kaempferol [29]. Within all these phenolic compounds, chlorogenic acid is the most famous one exhibiting anti-mutagenic, carcinogenic and antioxidant activities *in vitro*, mainly by scavenging reactive oxygen species [30]. Even though it is expected that flavonoids antioxidants would be more abundant, ferulic acid and chlorogenic acid from cinnamic acid derivatives family were abundant in majority of the extracts.

Topcu et al., evaluated ent-kaurene diterpenoids of *Sideritis congesta* which is endemic to Anatolia in Turkey "Ent-kaurane diterpenoids" are known as the integral parts of the tetracyclic natural products commonly spread in terrestrial parts of the plants [31]. They exhibit many different bioactivities from anti-tumor, anti-fungal and anti-bacterial to anti-inflammatory activities in plants. Topcu et al., elucidated its structure and 9 different ent-kraune diterpenoids are found to be present based on spectral, 1D and Two-Dimensional Nuclear Magnetic Resonance, and mass spectroscopic analysis. By using three different techniques—the b-carotene bleaching method, free radical scavenging activity, and superoxide anion scavenging activity—they have looked at the antioxidant potential of extracts and ent-kauranes. The study also assessed the anti-cholinesterase activity, and the majority of the diterpenes displayed a modest acetylcholinesterase inhibitory effect. Butyrylcholinesterase (BChE) was inhibited by practically all diterpenes; in particular, compounds 3 and 6 had stronger BChE inhibitory action than the common molecule galanthamine [32]. The diterpenes of *Sideritis congesta* may be advantageous for Alzheimer disease given that BChE activity gradually rises in people with the condition.

Krimmer et al., have been identified essential oils (EOs) via GC/MS (Gass Chromatography/ Mass Spectrometry) in *Sideritis congesta* collected from two different cities in Turkey (Kas and Alanya). They have characterized 39 components in each oil representing 85-90% of the total components detected with β-pinene (34-35%) and α-pinene (24-25%) [33].

One and only identified study conducted *in vivo* was published by Ozturk et al., in 1996. The analgesic activity of essential oil of *Sideritis congesta* has evaluated by using tail- flick method in mice and by comparing with standard analgesic drugs, morphine and fenoprofen. However, it did not show a significant analgesic action. According to their result, carvacrol content of essential oil is highly related to their analgesic activity [34].

Based on these the studies published by Kirimer et al., Erkan et al., and Topcu et al., natural components in *Sideritis congesta* has been identified and listed in Table 2.1 [29, 32, 33].

Table 2.1. List of natural components identified in *Sideritis congesta* extracts

Compound #	Compound Name	Compound #	Compound Name
Flavonoids		**Essential oils**	
1	Apigenin	**28**	P-cymene
2	Myricetin	**29**	Hexanol
3	Kaempferol	**30**	1-octen-3-ol
Cinnamic Acids		**31**	A-cubebene
4	Ferulic acid	**32**	A-copaene
5	Chlorogenic acid	**33**	B-bourbonene
6	Rosmarinic acid	**34**	A -gurjunene
7	Caffeic acid	**35**	B -cubebene
8	P-coumaric Acid	**36**	Linalool
Ent-kaurane diterpenoids		**37**	Pinocarvone
9	Ent-7a-acetoxy-16b,18-dihydroxy-kaurane (7-acetyldistanol)	**38**	Bornyl acetate
10	Ent-3b,7a-dihydroxy,18-acetoxy-15b,16b-epoxykaurane (epoxyisolinearol)	**39**	B -caryophyllene
11	Sideroxol	**40**	Myrtenal
12	Sideridiol	**41**	Trans-pinocarveol
13	Siderol	**42**	Calacorene
14	7-epicandicandiol	**43**	Trans-verbenol
15	Foliol	**44**	A-terpineol+borneol
16	Linearol	**45**	Germacrene D
17	Sidol	**46**	Δ-cadinene
Essential oils		**47**	Myrtenol
18	A-pinene	**48**	Calamenene
19	Camphene	**49**	Epi-cubebol
20	B-pinene	**50**	Cubebol
21	Δ-3-carene	**51**	Caryophyllene oxide
22	Myrcene	**52**	Ledol
23	A-phellandrene	**53**	(E)-nerolidol
24	Limonene	**54**	Globulol
25	B-phellandrene	**55**	Carvacrol
24	(E)-2-hexenal	**56**	Sabinene
27	Γ-terpinene		

2.5. Natural Products

Natural products, usually referred to as natural compounds, are complex chemical molecules that can be found in plants and microorganisms. Certain organic substances have pharmacological or biological properties that are therapeutic in the treatment of human diseases [35]. Among all components, some of them called as “bioactive components” and they initiate a specific biological response in humans and animals. In medicinal plants, these natural components can be terpenoids, steroids, flavonoids, cinnamic acids, alkaloids, etc.

2.5.1. Flavonoids

Flavonoids are derived from plants and are found throughout the entire plant. They are abundant throughout the plant world, especially in higher plants, and are low-molecular-weight phenolic chemicals. A class of organic compounds known as flavonoids, are mostly found in fruits, vegetables, roots, tea, and wine. They are abundant throughout the plant world, especially in higher plants, and are low-molecular-weight phenolic chemicals. Flavonoids are used by plants for both growth and defense against plaques. Additionally, they act as a screen against UV light and safeguard plants from various biotic and abiotic stresses. There are currently efforts on to separate those flavonoids from the plants because it has been determined that they have positive impacts on human health. They are essential for nutraceutical, pharmaceutical, medical, and cosmetic uses due to their antioxidative, anti-inflammatory, anti-mutagenic, and anti-carcinogenic characteristics. In relation to multiple disorders like cancer, Alzheimer's disease, atherosclerosis, etc., they have a variety of potential biochemical and antioxidant actions [36].

Flavonoids are subdivided into many different classes, such as flavones, isoflavones, flavanols, chalcones, and anthocyanins. The most significant subclass of flavonoids is flavones. They are mostly found in leaves, flowers, and fruits as glucosides. Celery, parsley, chamomile, mint, and other common plants are known as major producers of flavones. This subclass of flavonoids includes luteolin, apigenin, and tangeritin. Flavonoids with a ketone group are called flavonols. They have a lot of fruits and vegetables, like onions, kale, lettuce, and berries, among

others. The flavonols in this subclass that have been investigated the most include kaempferol, quercetin, myricetin, and fisetin. When compared to flavones, flavonols have a third-position C ring hydroxy group that may be glycosylated. This hydroxyl group acquires a variety of methylation and hydroxylation patterns in flovones. Another significant subclass of flavonoids is flavanones. The bitter flavor of citrus fruit juice and peel is brought on by flavanones. Examples of this class include hesperitin, naringenin, and eriodictyol. As a result of their capacity to scavenge free radicals, they are said to provide numerous health advantages. As antioxidant, anti-inflammatory, blood lipid-lowering, and cholesterol-lowering medicines, they exhibit potential pharmacological effects. Isoflavonoids are a large and recognized class of flavonoids that are mostly present in soy and other leguminous plants. The isoflavones family includes genistein and daidzein. A subclass of polyphenolic chemicals called neoflavanoids. Anthocyanins are pigments that give plants, flowers, and fruits their colors. The anthocyanins that are most frequently researched are cyanidin, delphinidin, malvidin, pelargonidin, and peonidin. Together, practically all flavonoids have the ability to function as antioxidants. By defending the body against ROS, catechins and flavones are discovered to be the most potent flavonoids (reactive oxygen species). The flavonoids' hydroxyl group makes them excellent free radical scavengers. Injury is avoided because radicals are rendered inert and stabilized after reacting with flavonoids. Additionally t is documented and confirmed by *in vivo* investigations, flavonoids' ability to reduce inflammation through a variety of methods [36].

Numerous studies have suggested that flavonoids exhibit a wide range of biological activities, including as anti-allergenic, anti-viral, anti-inflammatory, and vasodilating effects. The ability of antioxidants to scavenge free radicals and inhibit their production was still the focus of many investigations. Their antioxidant action has been the focus of numerous investigations in recent years, and it is now well established *in vitro* [36].

Furthermore, because of their ability to prevent lipid peroxidation, which is the root cause of many diseases, flavonoids have been discovered to be useful for a variety of illnesses, including but not limited to atherosclerosis, diabetes,

hepatotoxicity, and inflammation. According to studies, quercetin helps to reduce lipid peroxidation. In addition to quercetin, *Sideritis congesta* also contains myricetin, which inhibits the generation of superoxide radicals [36].

Many scientists isolated flavonoids determined their structural composition, and discovered about their antifungal, antiviral, and antibacterial effects. Flavonoids gained a reputation for having anti-microbial properties as a result, and they are now widely employed in nutrition, food safety, and health. Researchers discovered that flavonoids like quercetin, naringin, hesperetin, and catechin contain a varying degree of anti-viral action when they specifically researched flavonoids for the treatment of viral infections. They change how some RNA and DNA viruses replicate and spread. Quercetin and apigenin are the flavonoids with anti-bacterial effects that have been studied the most (present also in *Sideritis congesta*) [36].

Some flavonoids have hormone-like behaviors by resembling steroid hormones, particularly oestrogen. Fruits and vegetables, teas, red wine, and grains all contain these flavonoids. It is generally known that hormone-like steroids, particularly oestrogen, which has neuroprotective properties, can protect against a number of chronic disorders. Many different flavonoids, including genistein, daidzein, and equol, have demonstrated their effectiveness for treating a variety of ailments, including cancer, cardiovascular problems, and osteoporosis through their oestrogenic effects, which have been evaluated in clinical trials and studies. By preventing postmenopausal bone loss in women, genistein has been identified as the most promising flavonoid. Flavonoids in dietary supplements had a significant impact on atherosclerosis-related metrics such lipoprotein oxidation, blood platelet aggregation, and cardiovascular reactivity [36].

More importantly, Comalada et al., discovered that some flavonoids, in particular quercetin, help to reduce the severity of inflammation and boost the immune system [37]. Numerous researchers have also looked into flavonoids' potential anti-cancer and cancer-prevention properties. It has been discovered that certain flavonoids have chemo-preventive qualities and also aid in the production of apoptosis by stopping the cell cycle, controlling the metabolism of carcinogens, and controlling the expression of ontogenesis. The complementary and overlapping

mechanisms of antioxidant activity of flavonoids provide an explanation for the potential underlying mechanism. Second, the ability to neutralize free radicals, control carcinogenic metabolism, modulate gene expression by controlling oncogenes and tumor suppressor genes, start cell cycle arrest and apoptosis, control enzyme activities involved in detoxification, oxidation, and reduction, have anti-inflammatory effects, and act on other potential targets [36].

Flavonoids, like flavanols, are associated with lower incidence rates of dementia in the community. The effect of flavonoids on protecting the central nervous system has also been studied. Furthermore, it has been suggested that citrus flavanones such hesperidin, hesperetin, and naringenin may be able to cross the blood-brain barrier and be useful in the treatment of neurodegenerative illnesses. The anti-aging and anti-diabetic effects of flavonoids have also been documented. New flavonoids from nature's bounty must be discovered through further study in order to replace manufactured medications, which can have negative effects on the body [36].

2.5.2. Cinnamic Acids

Cinnamic acids are naturally present in all green plants. Despite the fact that they are also found in the reproductive organs of flowering plants, their biochemical pathway ultimately results in lignin, which gives the plant cell wall mechanical support. The spice cinnamon (*Cinnamomum zeilanicum*), which has been used historically as a flavoring and medical plant (stimulant, carminative, antiseptic, and pesticide), is where the name "cinnamic" originates. Coffee beans, tea, mate, cocoa, apples, pears, berries, citrus, grapes, brassica vegetables, spinach, beets, artichokes, potatoes, tomatoes, celery, fava beans, and cereals are other foods that contain cinnamic acids. Cinnamic acids typically manifest as chlorogenic acids, which are ester conjugates with quinic acid, but they can also form esters with other acids, such as those found in sugars or lipids, or they can combine with amines to produce amides and amide-containing compounds [38]. The term "chlorogenic acids" refers to a class of ester compounds formed when trans-cinnamic acid is combined with caffeic acid, p-coumaric acid, ferulic acid, and quinic acid [39].

The interest for cinnamic acids, especially for the medicinal application of cinnamic related molecules, has been significantly increased in the last several years. Many reviews and studies have focused on anti-cancer, anti-tuberculosis, anti-malarial, anti-fungal, anti-microbial, anti-atherogenic, and antioxidant activities of cinnamic acids. They have been also studied by pharmacology due to their potency to alter drug permeability, solubility or other parameters [38].

Many *in vitro* and *in vivo* studies showed that chlorogenic acids can exhibit many different physiological effects with/without their derivatives. An *in vivo* study conducted in rats focusing on caffeic acid and chlorogenic acids which are important members of cinnamic acid, showed that these natural compounds have cardio-protective properties [40] .It is also found that caffeic acid and its derivatives show anti-bacterial activity [41]. Kilani-Jazir et al., found that ferulic acid, caffeic acid and p-coumaric acid significantly affect the proliferation of splenocyte via lipopolysaccharide stimulation. This suggested that ferulic acid, caffeic acid and p-coumaric acid have potential effects in activation of B cells, and enhanced humoral immune response [42]. On the other hand caffeic acid by itself exhibited anti-viral, anti-carcinogenic, antioxidant and anti-inflammatory activities according to many in different studies [43], [44], [45], [46]. Recently anti-carcinogenic activity of caffeic acid against hepatocarcinoma is deeply examined. The anti-cancer activity of caffeic acid credited to its chemical structure that has free phenolic hydroxyls and thus is linked with its high antioxidant and pro-oxidant capacities [47].

Rosmarinic acid is another common ester derived from caffeic acid and lactic acid. Rosmarinic acid is commonly used in medicinal plant of the Lamiaceae family, such as rosemary. Rosmarinic acid has many different pharmacological and biological activities reported emerging from *in vivo, in vitro* and clinical studies. Rosmarinic acid exhibited a broad spectrum of activities *in vitro,* including antioxidant, anti-inflammatory, anti-mutagenic, anti-genotoxic, cytotoxic, anti-metastatic, anti-angiogenic, neuroprotective, anti-microbial, immunomodulatory, melanogenic, anti-diabetic and anti-venom effect. *In vivo* studies are commonly focused on anti-inflammatory, anti-tumoral, and tissue damage prevention activities. Like other cinnamic acids, antioxidant and radical scavenging activities are highlighted once again as a key function for anti-inflammatory and antioxidant activities for rosmarinic acid [48, 49].

P-coumaric acid is slightly unique among all cinnamic acids or chlorogenic acids. It is a precursor to other phenolic chemicals, first and foremost. Low amounts of it are found in free form in plants, while the majority of it is found in conjugated form. While its conjugates are less and more slowly absorbed in the GI tract than their free form, the free form can be swiftly and easily absorbed from the upper GI tract. P-coumaric acid has a wide range of biological effects, including the prevention of atherosclerosis, oxidative cardiac damage, UV-induced damage to eye tissues, neuronal injury, anxiety, gout, and diabetes. It also has antioxidant, anti-inflammatory, anti-mutagenic, anti-ulcer, anti-platelet, and anti-cancer properties. Unfortunately, with the production of its conjugates, the activities of p-coumaric acid significantly rise. Scientists are perplexed by their strong biological activity and benefits yet poor absorption rates [50].

The most prevalent hydroxycinnamic acid in the plant world, ferulic acid is also known as 4-hydroxy-3-methoxy cinnamic acid and is found in quite high amounts in the cell walls of many plants. In the study of Chinese medicine, ferulic acid received a lot of attention and was recognized as one of the potent elements found in medicinal herbs including *Angelica sinensis*, *Cimicifuga heracleifolia*, and *Lignsticum chuangxiong* [51].

In previous years, there have been numerous numbers of reports published on the physiological functions of ferulic acid, such as antioxidant activity, cholesterol- lowering activity, prevention against thrombosis and atherosclerosis, anti-microbial and anti-inflammatory activity, anti-cancer effect [52].

Over the past few years, researchers have begun to notice the bioactivity and possible health benefits of hydroxycinnamates such ferulic, caffeic, p-coumaric, and sinapic acid. A resonance-stabilized phenoxy radical that free ferulic acid produces makes it a potent antioxidant. Numerous investigations shown that ferulic acid has a strong capacity to neutralize free radicals such as those produced by hydrogen peroxide, superoxide, hydroxyl radicals, and nitrogen dioxide. In addition to neutralizing free radicals, ferulic acid boosts the activity of the enzymes that neutralize them and inhibits the enzymes that facilitate their formation [52].

It has been demonstrated that ferulic acid (a combination of ferulic acid esters of sterol and triterpene alcohols) has cholesterol-lowering properties [53]. According to one

theory, ferulic acid reduced the production of cholesterol by competitively reducing the liver's hydroxy-methyl-glutaryl CoA reductase activity and boosting acidic sterol excretion. For a very long time, ferulic acid-rich plants have been utilized in China to treat thrombosis. By breaking up the aggregation caused by ADP, ferulic acid can prevent platelet aggregation brought on by collagen. Furthermore, it is claimed that a substance produced from ferulic acid termed 4-0-[N-(carbobenzyloxy) aminoacyl] Ferulic acid can dissolve thrombi. Ferulic acid can be thought of as a potential chemopreventive drug for coronary heart disease due to its antioxidant, cholesterol-lowering, and preventative benefits against thrombosis and atherosclerosis properties [52].

The growth and reproduction of viruses like the influenza virus, respiratory syncytial virus, and AIDS have been demonstrated to be inhibited by ferulic acid. Ferulic acid and isoferulic acid are used as anti-inflammatory medications in Japanese Oriental medicine, particularly from the active ingredients of the rhizomes of *Cimicifuga* species. These research' findings indicated that ferulic acid might be used as an anti-inflammatory medication. Additionally, ferulic acid exhibits broad-spectrum antimicrobial action against both Gram-positive and Gram-negative bacteria. *Escherichia coli*, *Enterobacter aerogenes*, *Helicobacter pylori*, and other human gastrointestinal microflora demonstrated promising inhibitory effects on the proliferation of this substance. Ferulic acid's potential antioxidant properties have expedited research into its role in oncology. Studies, which were primarily *in vivo*, revealed that ferulic acid has chemopreventive action on colonic carcinomas and oral cancer [52].

2.5.3. Terpenes and Essential Oils

Terpenes or terpenoids are natural compounds that provide the unique smell and flavor of plants. Terpenes family is very diverse just-like other plant-based phytochemicals. They are derived from “isoprene” and their chemical structure is quite different from each other. Some of them can contain simple molecules whereas others can be highly complex. Terpenes are basically divided into 3 categories according to their isoprene units. First group is the “monoterpenes” which are constructed from two isoprene units. Second groups id the diterpenes which are made of two terpenes units. Third one is the “polyterpenes” which contain up to several hundred isoprene units and

make up natural materials like rubber. However, much more different terpenes classification is present according to their complexity [54].

Terpenes have many functions in plants such as a thermoprotectant, signaling functions, and not limited to, pigments, flavouring, and solvents but also have various medicinal uses. Cannabis is one of the most famous terpenes used for medicinal use. It contains many different properties including, anti-cancer, anti-microbial, antifungal, anti-viral, anti-hyperglycemic, analgesic, anti-inflammatory, and anti-parasitic. Medicinal properties of some terpenes are listed in Table 2.2 but their benefits are not only limited to this list. Anti-insect, anti-microbial, anti-plasmodial, anti-viral, anti-cancer, anti-diabetic and anti-depressant activity of terpenes have been also reported. Table 2.2 shows some examples of terpenes and their activity hoe they exert medicinal use [54].

Table 2.2. Medicinal use of some terpenes

Terpene	Medicinal Use
Tea Tree	Possesses many active ingredients to treat cutaneous infections
Thyme	Has powerful anti-bacterial and anti-fungal properties
Cannabis	Used for psychoactive activities and used for infectious diseases
Spanish Sage	Enhances memory and used in anti-dementia drugs
Citrus furis	Used in drugs against pediculosis
Citral	Used for its anti-bacterial and anti-fungal effects
Lemongrass	Used as an anti-insect (insect repellent)

One of the most significant natural products obtained from plants, essential oils are used for therapeutic and various biological purposes. Many different cultures around the world have been used essential oil domestically for different purposes without knowing exactly how they function. However, recent consideration into the traditional medicine has been enlighten effective use of essential oils in clinical procedures. Medicinal uses for human health, biological characterization and pharmacological aspects of essential oils, especially Mediterranean plants, have been revealed. Anti-inflammatory, antioxidant, anti-carcinogenic, anti-microbial, anti-fungal activities of essential oils are reported so far [55].

It might look like terpenes and essential oils are the same at the begging. They are similar, however, in fact; terpenes are much more pure components. In order to extract essential oils, plants are steam or cold pressed. Thus, essential oils contain all the volatile spectrum from the plants they're derived from, not just the terpenes [55].

Considering all active compounds such as flavonoids, cinnamic acids, ent-kraunes and essential oils such as terpenes, found in *Sideritis congesta,* it is expected to have many beneficial effects such as anti-inflammatory, antioxidant, anti-mutagenic, anti-ulcer etc. However, not all plant/natural products are safe. To this end, some safety endpoints such as cytotoxicity, mutagenicity etc. need to be evaluated to prove that plant is consumable safely. Then, later on, beneficial effects of the substance can be revealed by *in vitro, in vivo* and *in silico.*

2.6. Toxicological Evaluation of Bioactive Compounds via *In silico* Techniques

A computer simulation experiment is referred to as an *in silico* experiment. Computational toxicology uses large biological and chemical data sets to compute, manage, and identify patterns and relationships [56]. Regulatory agencies influence the use of computational toxicology. It is used by scientists in a variety of circumstances, such as the process of developing new drugs, determining the safety of cosmetic and food additives, estimating the toxicity of pesticides, etc. On the basis of computer assisted programs created by utilizing pre-existing experimental and chemical toxicity data, several toxicological endpoints are anticipated. The basic structure-activity paradigm, probabilistic reasoning, machine learning, data mining of existing toxicity data, and human expert knowledge are the key sources of these *in silico* methods [57].

Most industrial compounds' toxicological potential is currently assessed using in *vivo* animal model by standardized testing models. Any chemical that wants to be registered as a new product that may be released onto the market, must get first approval from those testing models Currently, there are many alternatives to animal testing, including *in vitro* procedures such using pieces of tissues, perfused organs, or cellular/ subcellular cultures, which can reduce the number of animals utilized for those studies. *In silico* approaches are currently being proposed for toxicological analyses, despite the fact that there exist numerous models based on *in vivo* and *in vitro* studies. First and

foremost, *in silico* methods aid in time, resource, and financial savings. They are capable of predicting the physical, chemical, or biological characteristics of molecules without necessarily synthesizing them chemically in a lab. However, industry is not yet favored at the regulatory level by computational methods [58].

Virtual screening is frequently used in the pharma industry to collect big structures since it is a high-performance, low-cost process. These studies are carried out in the preliminary phases since biological testing are not necessary. As a result, it facilitates the sorting of the compounds and enables quick evaluation of their efficacy and potential hazards. Additionally, computational toxicity contributes to our understanding of the operation of several biological processes. They can clarify why a substance is predicted to exhibit a particular form of toxicity [58].

In silico computational approaches come in a variety of forms. Based on the level of complexity of the type of toxicity research and the accessibility of the baseline data, the suitable methodologies are selected. The discovery and use of mathematical predictions led to the creation of the quantitative structure-activity relationship (QSAR) model, which is the most potent and useful instrument. Molecular coupling "docking," which analyzes how chemicals interact with biological receptors, "knowledge-based SAR systems," which are systematic studies of the structure-activity relationship based on prior knowledge and data, the "read-across" method based on extrapolating structural similarity, and the study of physiology-based pharmacokinetics (PBPK) are some other promising predictive approaches [58].

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

(E)-2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (2-AF)	Sigma Aldrich, Germany
3-[4,5-dimethylthiazol-2-yl]-strep2,5 diphenyl tetrazolium bromide (MTT)	Sigma-Aldrich, Germany
Agar	Sigma-Aldrich; Germany
Ammonium Sodium Phosphate Dibasic Tetrahydrate ($NaHNH_4PO_4$, $4H_2O$)	Aldrich; Germany
Broth	HiMedia; India
Citric Acid Monohydrate	Riedel de Haen; Germany
D-Biotin	Alfa Aesar; USA
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich; Germany
Dipotassium Phosphate (K_2HPO_4)	Fisher BioReagents; Germany
Fetal Bovine Serum (FBS)	Gibco; USA
Glucose-6-Phosphate	Sigma-Aldrich; Germany
Indomethacin	Sigma-Aldrich, Germany
Iso-propanol	Sigma Aldrich; USA
L-Histidine. HCl	Fluka; Germany
LPS from *E. coli*	Sigma Aldrich; USA
L-NAME	Sigma-Aldrich, Germany
Magnesium Sulfate ($MgSO_4$, $7H_2O$)	Sigma-Aldrich; Germany
Medium (Dulbecco's Modified Eagle Medium (DMEM))	Gibco; USA
N-(1-naphthyl) Ethylenediamine	Fluka, Germany
NaCl	Sigma-Aldrich; Germany
NADPH	Alfa Aesar; USA
O-Phosphoric Acid	Merck, Germany
Penicillin-Streptomycin (Penstrep)	Gibco; USA
RAW264.7 Murine Macrophage Cells	ATCC; USA
S9 Enzyme	Moltox, USA
Salmonella typhimurium TA98 and TA100	STDisc™ from MolTox Inc.
Sodium aside (SA)	Sigma Aldrich, Germany
Sodium Nitrite ($NaNO_2$)	Riedel de Haen; Germany
Sodium Nitrite (NaNO2)	Riedel de Haen; Germany
Sulfanilamide	Fluka, Germany

3.1.2. Equipment

48 Well-Plates	TPP; Germany
Autoclave	Tuttnauer; Germany
Beaker	IsoLab; Turkey
Centrifuge	Thermo Scientific; Germany
Danish QSAR Database	https://qsarmodels.food.dtu.dk/
EPA T.E.S.T Program	Version 2.1
Eppendorf Tubes	Eppendorf; Germany
Erlen	IsoLab; Turkey
Falcon Tube	Falcon; Germany
Glass Tubes	IsoLab; Turkey
Hemacytometer	IsoLab; Counting Chamber
Incubator	Binder CB-150; Germany
Incubator	IsoLab; Turkey
Inoculating Loop	IsoLab; Germany
Intel Core i7 3.20 GHz, 16 GB RAM 64-bit Computer	Acer; Turkey
Microplate Reader	Multiskan Ascent; Finland
Mixing incubator	Heidolph, Germany
OCHEM (The Online Chemical Modeling Environment)	https://ochem.eu/home/show.do
PASS Online	http://www.way2drug.com/passonline/predict.php
Petri Dishes	IsoLab, Germany
PkCSM (Predicting Small-Molecule Pharmacokinetic)	http://biosig.unimelb.edu.au/pkcsm/prediction
ProTox-II	https://tox-new.charite.de/protox_II
VEGA *in silico* Platform (version 1.2.0)	https://www.vegahub.eu/download/vega-qsar-download/
Vortex	Heidolph, Germany
Water Bath	BenchMark, England

3.1.3. Plant Materials

The flowering parts of mountain tea (*Sideritis congesta* Davis et Huber-Morath) were obtained from Medicinal and Endemic Plants Education and Research Farm, Faculty of Agriculture, Selcuk University, Konya, Turkey, where the plant species were cultivated. The herbarium specimens were deposited at Selcuk University Medicinal and Endemic Plants Herbarium (SÜTEB: 60). The Herbariums locality information is that *Sideritis congesta* Davis et Huber-Morath Medicinal and Endemic Plants Education and Research Farm, Selcuk University, 2019, 1020m, YK 1042 (SÜTEB 60).

3.2. Methods

3.2.1. Extract Preparation

The flowered parts of the dried and ground “mountain tea” were extracted with water and ethyl alcohol (50%). The extract obtained from this plant material was prepared with water and alcohol in the ratios given above at an average temperature of 60 °C for 48 hours. The alcohol was recovered in the resulting liquid extract and stored until dried. The liquid extract containing 5% dry matter was dried by spray dryer method and turned into powder.

3.2.2. Cell Culture

RAW264.7 murine macrophage cells were plated in 10^6 cells/ mL concentration in 48 well-plate and in DMEM, supplemented with 10% FBS and 1% streptomycin and penicillin at 37 °C in 5% CO_2 for 24 hours.

3.2.3. Cytotoxicity

For the evaluation of cell viability of cells treated with different concentrations of *Sideritis congesta* (0.03, 0.06, 0.125, 0.250, 0.5, 1 mg/ml), MTT test was conducted [59]. 100 µl of MTT was added to each well and incubated for an additional 2 hours at 37°C. After discarding all medium from plates, 200 µl of iso-propanol was added to each well. Absorbance of the dissolved blue formazan in iso-propanol was determined at 570 nm by a UV-spectrophotometric plate reader. All measurements were done in triplicates and

viability was defined as the ratio of absorbance of treated cells / absorbance of untreated cells.

3.2.4. Evaluation of Protective Effects Against Inflammation

The anti-inflammatory properties of *Sideritis congesta* extracts were assessed by quantifying the stable nitric oxide (NO) metabolite, nitrite, with Griess reagent [60].

Sideritis congesta was pre-treated with cultured cells at six different concentrations (0.03, 0.06, 0.125, 0.25, 0.5, and 1 mg/ml) for two hours before stimulation with 1 g/mL of LPS for an additional 22 hours. Griess reagent was combined with the culture supernatant (50 µL) and incubated at room temperature for 10 minutes. Using a microplate reader, the mixture's absorbance was measured at 540 nm. Calculating the concentration of nitrite in the test samples included using the $NaNO_2$ standard curve. Medium served as the negative control, while 100 µM of indomethacin and 100 µM of L-NAME were employed as positive controls. Using a nitrite standard curve, the nitrite concentrations were calculated.

3.2.5. Evaluation of Mutagenicity and Anti-Mutagenicity

For mutagenicity and anti-mutagenicity assays of *Sideritis congesta*, standard plate incorporation test was carried out by Ames test method [61]. Before all solutions are prepared and bacterial strains (Salmonella tester TA98 and TA100) were cultured. Preparation of all media and solutions used in mutagenicity and anti-mutagenicity assays are given and explained in Table 3-1.

Salmonella tester TA98 and TA100 were picked from frozen stock according to the aseptic techniques in the medium and incubated (in the dark) in the mixing incubator (168-170 speed) overnight.

For both mutagenicity and anti-mutagenicity testing, same *Sideritis congesta* extracts were used and dissolved in DMSO to obtain different concentrations (1, 10, 100, 1000, and 5000 microgram/plate). To test mutagenicity, plates were incubated in the inverted position at 37 °C for 48 hours, in the presence of bacterial strains, *Sideritis congesta* extract, histidine, phosphate buffer and with/ without S9 fractions. DMSO was

used as negative control. To test anti-mutagenicity, same assay was carried out in the absence and the presence of direct and indirect positive mutagens. All concentration and controls were carried out as triplicates. Following formula was used to calculate percentage of inhibition in antimutagenicity assay.

$$Precentage\ of\ inhibition = \left[\frac{(A-B)}{(A-C)}\right] x\ 100 \qquad (1)$$

where A = # of histidine revertants in the absence of sample

B = # of histidine revertants in the presence of sample

C = spontaneous revertants

Inhibitory effect lower than 25% was considered as weak and neglected. The inhibitory effect between 25-40% was considered as moderate and the inhibitory effect greater than 40% is expressed as strong.

In the mutagenicity assay, the number of reverse mutation colonies on sample-treated plates is divided by the number of reverse mutation colonies in the negative control group to determine the mutagenic ratio. A ratio less than two was regarded as non-mutagenic.

The mean and standard deviation of the experimental results were determined using Prism 9. Data on mutagenicity and anti-mutagenicity underwent Dunnett multiple comparisons. Statistics were deemed significant at $p<0.05$.

Table 3.1. Preparation of solutions

Chemical Name	Amount	Preparation
S9 fraction (rat liver microsomal enzymes + cofactors) per 50 ml of S9 mix		
Rat liver S9	2 ml	All of the chemicals are mixed.
MgCl2- KCl salts	1 ml	
1M glucose-6-phosphate	0.25 ml	
Preparation of 100 ml of Vogel-Bonner (VB) medium E (50X)		
Warm dH_2O (45 °C)	67 ml	All of the chemicals are mixed.
$MgSO_4$, $7H_2O$	1 g	
Citric Acid Monohydrate	10 g	
K_2HPO_4, dibasic (anhydrous)	50 g	
$NaHNH_4PO_4$, $4H_2O$	17. 5 g	
Preparation of 100 ml of 0.5mM histidine/biotin		
dH_2O	100 ml	All of the chemicals are mixed. The lid is covered by aluminium foil and incubated in autoclave at 120 °C for 15 min.
D-biotin (at -20°C freezer)	12.36 mg	
L-Histidine. HCl	9.6 mg	
Preparation of 100 ml of salt solution for S9 mix		
dH_2O	100 ml	All chemicals are mixed and autoclaved.
KCl	12.3 g	
$MgCl_2$, $6H_2O$	8.14 g	
Preparation of 1L of agar		
dH_2O	930 ml	dH_2O and agar is mixed in a beaker and autoclaved for 1 hour. Glucose and VB were added to beaker and mixed together. The mixture is poured in petri dishes, in 20-25 ml of volume in each.
Agar	15 g	
Glucose solution (+4 °C)	50 ml	
VB solution	20 ml	
Preparation of 100 ml of top agar		
dH_2O	100 ml	All of the chemicals are mixed in a beaker and autoclaved for ~ 1 hour
Agar	0.5 g	
NaCI	0.5 g	
Preparation of S9 mix		
dH_2O (Autoclaved)	5 ml	All of the chemicals are mixed and kept in iced water until use.
Buffer	6.25 ml	
Salt	250 µl	
Glucose 6 phosphate	63 µl	
NADPH	500 µl	
S9 Enzyme	500 µl	

3.2.6. *In silico* Evaluation

A computational prediction study was performed to estimate possible toxicity risks of *Sideritis congesta* active compounds. For this, five computer programs, DanishQSAR, PkCSM, OCHEM, Protox II, and EPA T.E.S.T. were employed.

Before starting *in silico* evaluation, active compounds of *Sideritis congesta* are identified and listed in Table 4.4 with their SMILE strings of molecules, CAS and EC numbers. The SMILES code of each molecule was obtained from official website of PubChem (https://pubchem.ncbi.nlm.nih.gov/). However, following molecules were not available in the database. Thus, endpoints for ent-7a-acetoxy-16b,18-dihydroxy-kaurane (7-acetyldistanol), ent-3b,7a-dihydroxy, 18-acetoxy-15b,16b-epoxykaurane (epoxyisolinearol), foliol and calacorene could not be evaluated and excluded from *the in silico* study.

3.2.6.1. DanishQSAR Database

In Danish QSAR freeware (https://qsarmodels.food.dtu.dk/), AMES Test, other *in vitro* endpoints and *in vivo* endpoint were selected in genotoxicity/ cancer tab. Danish QSAR models function based on QSAR technique. SMILES codes were given as input for each substance. Their results are given in Table 4.5 (only AMES Test) and Table 4.6. POS_OUT, NEG_OUT, INC_OUT results were excluded from the results and marked as N/A. Positive and negative results are listed. Experimental data are also taken into consideration, if available.

3.2.6.2. PkCSM

pkCSM (http://biosig.unimelb.edu.au/pkcsm/prediction) is used to predict small-molecule pharmacokinetic properties based on distance/pharmacophore patterns. In pkCSM freeware, SMILES codes were given as input and “Toxicity” endpoint was chosen for the prediction. Positive and negative results of each component was listed.

3.2.6.3. OCHEM

The Online Chemical Modelling Environment (https://ochem.eu/home/show.do), a web-based platform is used to implement Ames test result for selected compounds. It aims to automate and simplify the typical steps required for QSAR modelling [62]. In the freeware, SMILE strings are given as input and predictions were run. Active and inactive results are indicated as positive and negative accordingly.

3.2.6.4. ProTox II

ProTox-II contains computer-based models trained on real data. It incorporates molecular similarity, pharmacophores, fragment propensities, and machine-learning models for the prediction of various toxicity [63]. In ProTox II freeware (https://tox-new.charite.de/protox_II/index.php?site=compound_input), SMILE codes were used as an input and "mutagenicity" endpoint was chosen before the prediction. After molecule structure is loaded in the main page prediction was run. The values under the 0.7 of prediction were excluded from the table and marked as N/A. "Active" results are given as positive and "inactive" results are given as negative.

3.2.6.5. EPA T.E.S.T Program

EPA T.E.S.T program is another QSAR based tool and downloaded from its official website (https://www.epa.gov/chemical-research/toxicity-estimation-software-tool-test). All SMILE codes were loaded as "bulk" mode and "consensus" mode is chosen for mutagenicity endpoints. Experimental results are also taken into consideration, if they are available.

3.2.6.6. PASS Online Platform

PASS Online Platform (http://www.way2drug.com/passonline/predict.php)is a freeware used to quantify the activity probability (Pa) and inactivity (Pi) through experimental data of tests with animals deposited in the triage base to direct the biological activity and action mechanism of substance. SMILE code of each compound was given as input and anti-inflammatory, anti-mutagenic, antioxidant, nitric oxide antagonist, NOS

inhibitor and NO-forming inhibitor activity endpoints were evaluated. Pa activity under 0.5 is given as “N/A” and no activity for a compound is given as “- “.

3.2.6.7. V.E.G.A. *in silico* Platform

V.E.G.A. *in silico* is a platform based on QSAR and read- across tool and downloaded from its official website (https://www.vegahub.eu/download/vega-qsar-download/). This platform has been used to evaluate a consensus result for mutagenicity, Ames endpoint and search for structural alerts for the compound which gave positive result for any endpoints.

4. RESULTS

4.1. Cell Viability and Protective Effects Against Inflammation

Cytotoxicity of different doses (0.03, 0.06, 0.125, 0.250, 0.5 and 1 mg/ml) of *Sideritis congesta* has been investigated by MTT test by measuring cell viability of the cells. Table 4.1 shows the percentage of cell viability of each sample treated with different doses of extracts. The *Sideritis congesta* extracts affected the cell viability dose-dependently. However, they did not show any cytotoxic effect on RAW264.7 murine macrophage cells, since the percentage of cell viability of each sample is above 70%.

Table 4.1. The cell viability and nitrite inhibition of controls and *Sideritis congesta* treated samples

Groups	Cell Viability %	Nitrite Inhibition %
Medium	100 ± 1.0	No inhibition
Sideritis congesta Dose (mg/mL)		
0.03	95.20 ± 0.2	38.43 ± 2.5
0.06	90.63 ± 1.7	51.37 ± 2.0
0.125	87.98 ± 2.4	52.66 ± 1.5
0.250	85.34 ± 3.5	65.23 ± 3.8
0.5	78.27 ± 3.3	71.88 ± 2.0
1	73.87 ± 3.8	87.40 ± 1.5
L-Name 100 µM	-	44.90 ± 1.3
Indomethacin 100 µM	-	42.41± 1.5

The *in vitro* protective effects of the extracts against inflammation were assessed by nitrite assay in LPS-stimulated RAW 264.7 cells. As seen in Table 4.1, all *Sideritis congesta* extracts exerted protective activity against inflammation in a dose-dependent manner. *Sideritis congesta* extract even in lowest dose, 0.03 mg/ml, nearly showed the same activity as bioactive compounds L-Name and Indomethacin. All other doses of *Sideritis congesta* showed higher activity than the positive controls. Highest dose of extract, 1 mg/ml, almost showed twice (87.40% inhibition activity) the activity of L-Name (44.90% inhibition activity) and Indomethacin (41.41% inhibition activity).

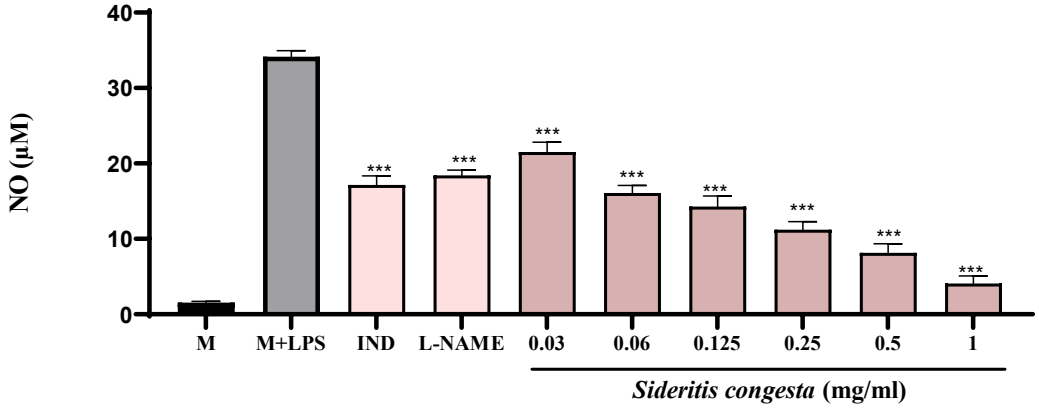

Figure 4.1. Effect of LPS stimulated RAW 264.7 cells treated with positive controls

(L-Name and Indomethacin) and different doses of *Sideritis congesta.* The significant differences between groups and M+LPS sample were defined with where ***p<0.0001.

Figure 4.1 shows the nitric oxide concentrations of LPS-stimulated RAW 264.7 cells treated with 100 µM of positive controls L-Name and Indomethacin and different doses of *Sideritis congesta.* Nitric oxide concentrations of samples treated with positive controls and different doses of *Sideritis congesta.* have been significantly and dose dependently decreased when compared to the sample treated with LPS (M+LPS).

4.2. Mutagenicity and Anti-Mutagenicity

The mutagenic and the anti-mutagenic activities of *Sideritis congesta* extracts with doses of 1, 10, 100, 1000, and 5000 µg/plate were investigated. The findings regarding mutagenic activity revealed that, in comparison to spontaneous mutation frequencies in the control group, the number of revertant colonies for the tested *Salmonella typhimurium* strains TA98 and TA100 did not change significantly in the presence of various concentrations of *Sideritis congesta* extracts. Additionally, all samples' mutagenic indices were calculated to be lower than 2. Table 4.2 shows the number of revertant/plate, the standard deviation and the mutagenic index (MI) after the treatments with the compounds, in the two different strains of *Salmonella typhimurium*, with or without metabolic activation. The outcomes also demonstrated that the viability of bacterial strains was unaffected by the various *Sideritis congesta* extract concentrations.

Table 4.2. The number of revertant/plate, the standard deviation and the mutagenic index (MI) after the treatments with the compounds, in the two different strains of Salmonella typhimurium, with/ without S9.

	TA98		TA100	
Without S9	Number of revertant/plate	Mutagenic Index (MI)	Number of revertant/plate	Mutagenic Index (MI)
Negative Control	30.0 ± 2.8		145.0 ± 25.4	
Dose (µg/plate)				
1	29.0 ± 4.9	0.9	146.4 ± 12.0	1.0
10	28.0 ± 2.8	0.9	128.0 ± 14.1	0.8
100	24.5 ± 3.53	0.8	133.5 ± 0.7	0.9
1000	20.5 ± 3.5	0.6	130.0 ± 2.8	0.8
5000	26.5 ± 0.7	0.8	147.0 ± 5.6	1.0
With S9				
Negative Control	39.0 ± 8.4		143.0 ± 15.5	
Dose (µg/plate)				
1	29.0 ± 0	0.7	133.5 ± 23.3	0.9
10	30.0 ± 8.4	0.7	150.5 ± 14.8	1.0
100	50.0 ± 7.0	1.2	132.0 ± 7.0	0.9
1000	48.0 ± 9.8	1.2	137.0 ± 7.0	0.9
5000	33.5 ± 3.5	0.8	134.5 ± 9.1	0.9

Table 4.3 shows the anti-mutagenicity of the *Sideritis congesta* extract's revertant colony numbers and their inhibitory percentages with and without S9 activation. A strong anti-mutagenic activity was seen in the samples treated with 1000 and 5000 µl/plate *Sideritis congesta* extracts against indirect (2-AF) mutagen in TA98 and TA100 strains, respectively.

On the other hand, all other extracts of the *Sideritis congesta* showed less than 20% inhibition ratio with and without S9. (Figure 4.2) The results showed that in the presence of the 1, 10 and 100 µl/plate concentrations of *Sideritis congesta* extract, the number of revertant colonies for the tested *Salmonella typhimurium* strains TA98 and TA100 did not change significantly when compared to number of revertant colonies.

Table 4.3. Revertant colony numbers observed in the anti-mutagenicity, standard deviation and their inhibitory percentages of the *Sideritis congesta* extract with/ without S9 activation

	TA98		TA100	
Without S9	Number of revertant/plate	Inhibition (%)	Number of revertant/plate	Inhibition (%)
Negative Control	20.0 ± 7.0		129.0 ± 29.7	
Positive Control	1018.0 ± 144.2		425.0 ± 12.7	
Dose (µg/plate)				
1	982.5 ± 3.5	3	362.0 ± 26.8	21
10	914.5 ± 85.5	10	371.5 ± 19.0	18
100	915.5 ± 70.0	10	366.0 ± 8.4	19
1000	927.0 ± 32.5	9	380.0 ± 24.0	15
5000	974.5 ± 7.7	4	381.0 ± 22.6	14
With S9				
Negative Control	29.5 ± 3.5		161.0 ± 19.8	
Positive Control	1029 ± 24.7		604.5 ± 61.5	
Dose (µg/plate)				
1	965.5 ± 68.5	6	551.0 ± 12.7	12
10	970.0 ± 49.5	5	587.5 ± 3.5	3
100	1002.0 ± 7.7	2	655.0 ± 162.6	11
1000	282.5 ± 17.6*	74	364.0 ± 19.8*	54
5000	35 ± 7.0*	99	176 ± 28.2*	96

The significant differences between groups and positive control were defined with where *p<0.05

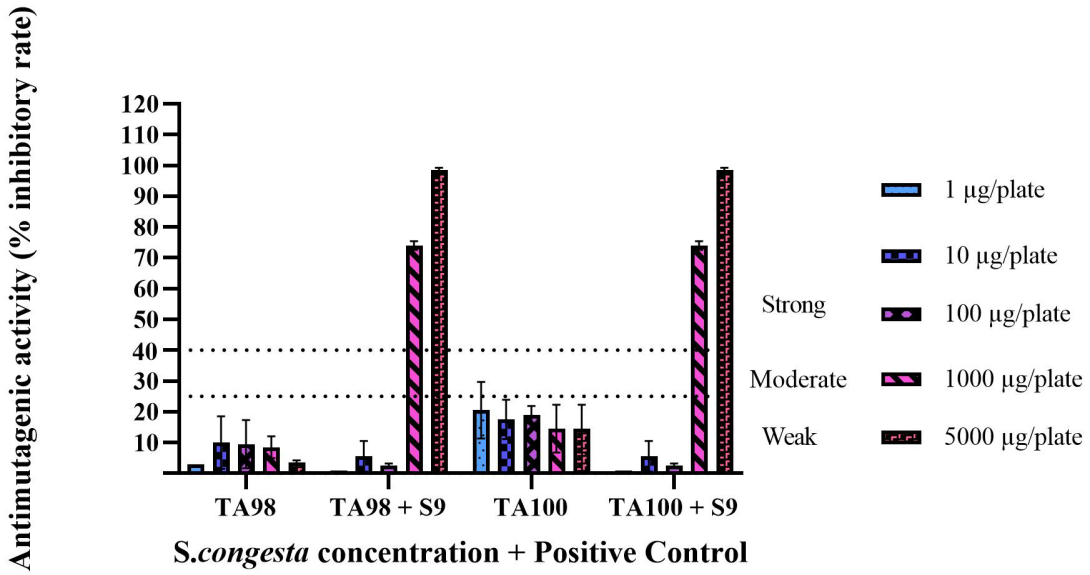

Figure 4.2. Anti-mutagenic activity (inhibitory rate %) of *Sideritis congesta* extract against positive control mutagens

In the experiments without the S9 activation SA was used a positive control. In the experiment with S9 activation 2-AF was used as positive control

4.3. *In silico* Analysis

The active compounds of *Sideritis congetsa* are identified and listed in Table 4.4 with their SMILE strings of molecules, CAS and EC numbers. The SMILES strings of each molecule were obtained from official website of PubChem. However, following molecules were not available in the database. Thus, endpoints for ent-7a-acetoxy-16b,18-dihydroxy-kaurane (7-acetyldistanol), ent-3b,7a-dihydroxy, 18-acetoxy-15b,16b-epoxykaurane (epoxyisolinearol), foliol, and calacorene could not be evaluated.

Table 4.4. SMILES, CAS and EC numbers of active compounds found in *Sideritis congesta*

Compound Number	Compound Name	SMILES	CAS Number	EC Number
1	Apigenin	C1=CC(=CC=C1C2=CC(=O)C3=C(C=C(C=C3O2)O)O)O	520-36-5	208-292-3
2	Myricetin	C1=C(C=C(C(=C1O)O)O)C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O	529-44-2	208-463-2
3	Kaempferol	C1=CC(=CC=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O	520-18-3	208-287-6
4	Ferulic acid	COC1=C(C=CC(=C1)C=CC(=O)O)O	537-98-4	214-490-0
5	Chlorogenic acid	C1C(C(C(CC1(C(=O)O)O)OC(=O)C=CC2=CC(=C(C=C2)O)O)O)O	202650-88-2	206-325-6
6	Rosmarinic acid	C1=CC(=C(C=C1CC(C(=O)O)OC(=O)C=CC2=CC(=C(C=C2)O)O)O)O	20283-92-5	606-487-1
7	Caffeic acid	C1=CC(=C(C=C1C=CC(=O)O)O)O	501-16-6	206-361-2
8	P-coumaric Acid	C1=CC(=CC=C1C=CC(=O)O)O	501-98-4	231-000-0
9	Sideroxol	CC1(CCCC2(C1CC(C34C2CCC(C3)C5(C4O5)C)O)C)CO	22338-60-9	-
10	Sideridiol	CC1=CC23CC1CCC2C4(CCCC(C4CC3O)(C)CO)C	-	-
11	Siderol	CC1=CC23CC1CCC2C4(CCCC(C4CC3OC(=O)C)(C)CO)C	19885-22-4	-
12	7-epicandicandiol	CC1(CCCC2(C1CC(C34C2CCC(C3)C(=C)C4)O)C)CO	-	-
13	Linearol	CC(=O)OCC1(C(CCC2(C1CC(C34C2CCC(C3)C(=C)C4)O)C)O)C	37720-82-4	-
14	Sidol	CC(=O)OCC1(C(CCC2(C1CC(C34C2CCC(C3)C(=C)C4)O)C)OC(=O)C)O	-	-
15	A-pinene	CC1=CCC2CC1C2(C)C	25766-18-1	201-291-9
16	Camphene	CC1(C2CCC(C2)C1=C)C	79-92-5	201-234-8
17	B-pinene	CC1(C2CCC(=C)C1C2)C	127-91-3	242-060-2
18	Δ-3-carene	CC1=CCC2C(C1)C2(C)C	13466-78-9	236-719-3
19	Myrcene	CC(=CCCC(=C)C=C)C	123-35-3	204-622-5
20	A-phellandrene	CC1=CCC(C=C1)C(C)C	99-83-2	202-792-5
21	Limonene	CC1=CCC(CC1)C(=C)C	138-86-3	205-341-0
22	B-phellandrene	CC(C)C1CCC(=C)C=C1	555-10-2	209-081-9
23	(E)-2-hexenal	CCCC=CC=O	6728-26-3	229-778-1
24	Γ-terpinene	CC1=CCC(=CC1)C(C)C	99-85-4	202-794-6
25	P-cymene	CC1=CC=C(C=C1)C(C)C	99-87-6	202-796-7
26	Hexanol	CCCCCCO	111-27-3	203-852-3
27	1-octen-3-ol	CCCCCC(C=C)O	3391-86-4	222-226-0
28	A-cubebene	CC1CCC(C2C13C2C(=CC3)C)C(C)C	17699-14-8	605-792-7

Compound Number	Compound Name	SMILES	CAS Number	EC Number
29	A-copaene	CC1=CCC2C3C1C2(CCC3C(C)C)C	3856-25-5	223-364-4
30	B-bourbonene	CC(C)C1CCC2(C1C3C2CCC3=C)C	5208-59-3	610-778-9
31	A -gurjunene	CC1CCC2C(C2(C)C)C3=C(CCC13)C	489-40-7	207-695-1
32	B -cubebene	CC1CCC(C2C13C2C(=C)CC3)C(C)C	13744-15-5	604-019-0
33	Linalool	CC(=CCCC(C)(C=C)O)C	78-70-6	201-134-4
34	Pinocarvone	CC1(C2CC1C(=C)C(=O)C2)C	30460-92-5	250-211-9
35	Bornyl acetate	CC(=O)OC1CC2CCC1(C2(C)C)C	76-49-3	204-727-6
36	B -caryophyllene	CC1=CCCC(=C)C2CC(C2CC1)(C)C	87-44-5	201-746-1
37	Myrtenal	CC1(C2CC=C(C1C2)C=O)C	564-94-3	209-274-8
38	Trans-pinocarveol	CC1(C2CC1C(=C)C(C2)O)C	3917-59-7	216-813-0
39	Trans-verbenol	CC1=CC(C2CC1C2(C)C)O	22339-08-8	244-920-2
40	A-terpineol	CC1=CCC(CC1)C(C)(C)O	98-55-5	202-680-6
41	Borneol	CC1(C2CCC1(C(C2)O)C)C	507-70-0	204-712-4
42	Germacrene D	CC1=CCCC(=C)C=CC(CC1)C(C)C	37839-63-7	-
43	Δ-cadinene	CC1=CC2C(CCC(=C2CC1)C)C(C)C	483-76-1	866-559-5
44	Myrtenol	CC1(C2CC=C(C1C2)CO)C	515-00-4	208-193-5
45	Calamenene	CC1CCC(C2=C1C=CC(=C2)C)C(C)C	483-77-2	610-417-5
46	Epi-cubebol	CC1C=CC(C2C13C2C(CC3)(C)O)C(C)C	38230-60-3	-
47	Cubebol	CC1CCC(C2C13C2C(CC3)(C)O)C(C)C	23445-02-5	-
48	Caryophyllene oxide	CC1(CC2C1CCC3(C(O3)CCC2=C)C)C	1139-30-6	214-519-7
49	Ledol	CC1CCC2C1C3C(C3(C)C)CCC2(C)O	577-27-5	-
50	(E)-nerolidol	CC(=CCCC(=CCCC(C)(C=C)O)C)C	40716-66-3	230-597-5
51	Globulol	CC1CCC2C1C3C(C3(C)C)CCC2(C)O	489-41-8	-
52	Carvacrol	CC1=C(C=C(C=C1)C(C)C)O	499-75-2	207-889-6
53	Sabinene	CC(C)C12CCC(=C)C1C2	3387-41-5	222-212-4

Overall results from five online computational program, DanishQSAR, PkCSM, OCHEM, Protox II, and EPA T.E.S.T. were listed in tables Table 4.5 (mutagenicity) and Table 4.6 (Other genotoxicity endpoints). According to our results, most of the compounds (46 of 53 compounds) evaluated according to the Ames endpoint did not show mutagenic potential. However, inconsistencies (both positive and negative results were present for Ames result) for 7 of the compounds have been observed. Moreover, genotoxicity endpoints of two of the thirds of compounds were negative or QSAR database was unable to predict a result. 17 of 53 of the compounds exerted inconsistent results based on the genotoxic endpoints. The compounds exerting negative results for all endpoints were concluded as non-mutagenic, non-genotoxic. However, the ones with inconsistent data are further evaluated by using V.E.G.A. platform. In this platform, consensus analysis for mutagenicity endpoint has been evaluated. In order to further evaluate genotoxicity endpoints, chromosomal aberration and micronucleus activity of compounds has been also investigated. Moreover, structural alerts which are believed to be responsible for adverse effects (such as genotoxicity, teratogenicity etc.) and can be used to predict the toxicity of similar compounds, was investigated.

The first compound, apigenin only showed positive result in OCHEM web-based platform with 58 % accuracy and negative results for all other databases and platforms for mutagenicity and there were available negative experimental results for Ames endpoint. Moreover, five endpoints for genotoxicity results were positive. Thus, possible structural alerts were investigated in VEGA platform and no structural alert was identified. However, it showed chromosomal aberration activity with moderate reliability. Thus, apigenin might be genotoxic for some cell lines but it is not mutagenic. According to the VEGA *in silico* platform as well, apigenin is non-mutagenic.

Myricetin showed positive result in OCHEM. OCHEM web-based platform with 78 % accuracy and negative results for all other databases and platforms for mutagenicity. However, there were available positive experimental results for mutagenicity, Ames endpoint. Moreover, five endpoints for genotoxicity results were positive. To this end, possible structural alerts were investigated in VEGA *in silico* platform and structural alerts were identified. Additionally, it showed chromosomal aberration activity and

mutagenic activity with moderate reliability and micronucleus activity with low reliability. Thus, myricetin might be genotoxic for some cell lines and it is mutagenic.

Kaempferol showed positive results in OCHEM web-based platform with 78% accuracy and EPA T.E.S.T program. Moreover, there are both positive and negative experimental values for mutagenicity, for Ames endpoint. Moreover, 5 endpoints for genotoxicity results were positive with one positive experimental result. To this end, possible structural alerts were investigated in VEGA *in silico* platform and no structural alert was identified. Additionally, it showed mutagenic activity with high reliability, chromosomal aberration activity with moderate reliability and micronucleus activity with low reliability. Thus, kaempferol is genotoxic for some cell lines and it is mutagenic.

Ferulic acid did not showed any mutagenic activity in none of the databases nor platforms but exerted three positive genotoxic endpoints. To this end, VEGA *in silico* platform in used to check structural alerts, possible chromosomal aberrations and micronucleus activity. There were no structural alerts for ferulic acid and there was no chromosomal aberration activity with moderate reliability, but micronucleus activity is expected with moderate reliability.

Chlorogenic acid did not showed any mutagenic activity in none of the databases nor platforms but showed positive results for three genotoxic endpoints and one of the positive results has positive experimental result. According to the VEGA *in silico* platform, chlorogenic acid has both chromosomal aberration and micronucleus activity with moderate reliability.

Rosmarinic acid did not showed any mutagenic activity in none of the databases nor platforms but showed two positive results and seven of the other results were N/A for genotoxic endpoints. According to the VEGA *in silico* platform, rosmarinic acid has chromosomal aberration activity with moderate reliability and it has no micronucleus activity with low reliability.

Caffeic acid did not showed any mutagenic activity in none of the databases nor platforms, but 1 positive result was observed for genotoxicity endpoints. According to the VEGA *in silico* platform, chromosomal aberration is active for the component with an

experimental data however, model prediction is inactive with low reliability Micronucleus activity is present with low reliability as well.

P-coumaric acid did not showed any mutagenic activity in none of the databases nor platforms but1 positive result was observed for genotoxicity endpoints whereas most of the results for other endpoints were N/A. In VEGA *in silico* platform, chromosomal aberration is inactive for the component with low reliability Micronucleus activity could not be predicted.

Sideroxol showed positive result in Danish QSAR database for mutagenicity endpoint. Moreover, it exerted 1 positive result for genotoxicity endpoints whereas most of the results for other endpoints were N/A. According to VEGA *in silico* platform predicted consensus mutagen activity was mutagen. However, chromosomal aberration and micronucleus activity was inactive with moderate reliability. So, sideroxol might be mutagenic.

Sidol did not show any mutagenic activity in none of the databases nor platforms but1 positive result was observed for genotoxicity endpoints whereas most of the results for other endpoints were N/A. According to the VEGA *in silico* platform, chromosomal aberration and micronucleus activity of the compound was inactive with moderate reliability.

(E)-2-hexenal showed mutagenic activity for three of five computational programs and one positive result was observed with experimental data. However, no positive genotoxic end point was observed. According to the VEGA *in silico* platform, predicted consensus mutagen activity was mutagenic with experimental values. Chromosomal aberration activity was concluded as inactive with moderate reliability and predicted micronucleus activity was active with low reliability. So, (E)-2-hexenal is more likely to be mutagenic thus genotoxic.

γ-terpinene did not show any mutagenic activity in none of the databases nor platforms but1 positive result was observed for genotoxicity endpoints whereas most of the results for other endpoints were N/A. According to the VEGA *in silico* platform, chromosomal aberration activity of the compound was inactive with good reliability but predicted micronucleus activity was active with low reliability.

P-cymene did not show any mutagenic activity in none of the databases nor platforms but1 positive result was observed for genotoxicity endpoints whereas most of the results for other endpoints were N/A. According to the VEGA *in silico* platform, chromosomal aberration activity of the compound was inactive with good reliability. The platform could not predict micronucleus activity.

Hexanol and 1-octen-3-ol, did not show any mutagenic activity in none of the databases nor platforms but one and positive results were observed for genotoxicity endpoints whereas most of the results for other endpoints were N/A. According to the VEGA *in silico* platform, chromosomal aberration and micronucleus activity of the compounds were inactive with good reliability. According to the results, hexanol and 1-octen-3-ol most probably non-genotoxic and non-mutagenic.

Calamenene exerted mutagenic activity for 2 of 5 computational programs and most of the genotoxic endpoint results were N/A. According to the VEGA *in silico* platform, predicted consensus mutagen activity was non-mutagenic with moderate reliability. Chromosomal aberration activity was concluded as inactive with moderate reliability and predicted micronucleus activity was active with moderate reliability. So, calamenene is most likely to be non-mutagenic and non-genotoxic.

Caryophyllene oxide, showed positive result in QSAR database for mutagenicity endpoint. Moreover, it has six positive results for genotoxicity endpoint. According to the VEGA *in silico* platform, predicted consensus mutagen activity was mutagenic with good reliability. However, chromosomal aberration and micronucleus activity was inactive with good reliability.

(E)-nerolidol, did not show any mutagenic activity in none of the databases nor platforms but1 positive result was observed for genotoxicity endpoints whereas most of the results for other endpoints were negative. According to the VEGA *in silico* platform, chromosomal aberration activity of the compound was inactive with good reliability. However predicted micronucleus activity was active with moderate reliability.

Carvacrol did not show any mutagenic activity in none of the databases nor platforms but1 positive result was observed for genotoxicity endpoints whereas most of the results for other endpoints were negative. According to the VEGA *in silico* platform, chromosomal aberration activity of the compound was inactive with low reliability whereas predicted micronucleus activity was active with good reliability.

Table 4.5. *In silico* analysis results of Ames endpoints

Compound Number	Compound Name	Danish QSAR	PkCSM	OCHEM	ProTOX II	EPA T.E.S.T.	Experimental
1	Apigenin	Negative	Negative	**Positive**	N/A	Negative	Negative a, b
2	Myricetin	Negative	Negative	**Positive**	N/A	N/A	**Positive** a
3	Kaempferol	Negative	Negative	**Positive**	N/A	**Positive**	Negative a **Positive** b
4	Ferulic acid	Negative	Negative	Negative	Negative	Negative	Negative b
5	Chlorogenic acid	Negative	Negative	Negative	Negative	Negative	Negative b
6	Rosmarinic acid	Negative	Negative	Negative	Negative	N/A	N/A
7	Caffeic acid	Negative	Negative	Negative	Negative	Negative	Negative b
8	P-coumaric Acid	Negative	Negative	Negative	Negative	N/A	N/A
9	Sideroxol	**Positive**	Negative	Negative	N/A	N/A	N/A
10	Sideridiol	Negative	Negative	Negative	Negative	N/A	N/A
11	Siderol	N/A	Negative	Negative	Negative	N/A	N/A
12	7-epicandicandiol	Negative	Negative	Negative	Negative	N/A	N/A
13	Linearol	Negative	Negative	Negative	Negative	N/A	N/A
14	Sidol	Negative	Negative	Negative	Negative	N/A	N/A
15	A-pinene	Negative	Negative	Negative	Negative	Negative	Negative a, b
16	Camphene	Negative	Negative	Negative	Negative	N/A	Negative b
17	B-pinene	Negative	Negative	Negative	Negative	Negative	Negative b
18	Δ-3-carene	Negative	Negative	Negative	N/A	N/A	N/A
19	Myrcene	Negative	Negative	Negative	Negative	N/A	Negative b
20	A-phellandrene	Negative	Negative	Negative	Negative	N/A	Negative a
21	Limonene	Negative	Negative	Negative	Negative	N/A	Negative (EPA)
22	B-phellandrene	Negative	Negative	Negative	Negative	N/A	N/A
23	(E)-2-hexenal	N/A	**Positive**	Negative	**Positive**	**Positive**	**Positive** a, b
24	Γ-terpinene	Negative	Negative	Negative	Negative	N/A	N/A
25	P-cymene	Negative	Negative	Negative	Negative	N/A	N/A
26	Hexanol	Negative	Negative	Negative	Negative	Negative	N/A
27	1-octen-3-ol	Negative	Negative	Negative	Negative	Negative	N/A
28	A-cubebene	Negative	Negative	Negative	Negative	N/A	N/A

Compound Number	Compound Name	Danish QSAR	PkCSM	OCHEM	ProTOX II	EPA T.E.S.T.	Experimental
29	A-copaene	Negative	Negative	Negative	Negative	Negative	N/A
30	B-bourbonene	Negative	Positive	Negative	Negative	Negative	N/A
31	A -gurjunene	N/A	Negative	Negative	Negative	N/A	N/A
32	B -cubebene	Negative	Negative	Negative	Negative	N/A	N/A
33	Linalool	Negative	Negative	Negative	Negative	Negative	Negative a, b
34	Pinocarvone	Negative	Negative	Negative	Negative	Negative	N/A
35	Bornyl acetate	Negative	Negative	Negative	Negative	Negative	N/A
36	B -caryophyllene	Negative	Negative	Negative	Negative	Negative	Negative a, b
37	Myrtenal	N/A	Negative	Negative	Negative	Negative	N/A
38	Trans-pinocarveol	Negative	Negative	Negative	Negative	Negative	N/A
39	Trans-verbenol	N/A	Negative	Negative	Negative	Negative	Negative a, b
40	A-terpineol	Negative	Negative	Negative	Negative	Negative	Negative a, b
41	Borneol	Negative	Negative	Negative	Negative	Negative	Negative a, b
42	Germacrene D	Negative	Negative	Negative	Negative	N/A	N/A
43	Δ-cadinene	Negative	Negative	Negative	N/A	N/A	N/A
44	Myrtenol	N/A	Negative	Negative	Negative	Negative	N/A
45	Calamenene	Negative	**Positive**	Negative	Negative	**Positive**	N/A
46	Epi-cubebol	Negative	Negative	Negative	N/A	Negative	N/A
47	Cubebol	Negative	Negative	Negative	Negative	Negative	N/A
48	Caryophyllene oxide	**Positive**	Negative	Negative	Negative	Negative	N/A
49	Ledol	N/A	Negative	Negative	Negative	Negative	N/A
50	(E)-nerolidol	Negative	Negative	Negative	Negative	Negative	N/A
51	Globulol	N/A	Negative	Negative	Negative	Negative	N/A
52	Carvacrol	Negative	Negative	Negative	Negative	Negative	Negative a, b
53	Sabinene	Negative	Negative	Negative	Negative	N/A	N/A

a: Danish QSARDB; b: EPA T.E.S.T.

Table 4.6. Results of other endpoints evaluated regarding genotoxicity in DanishQSAR database

Compound #	Chromosome Aberrations in Chinese Hamster Lung Cancer	Mutations in Thymidine Kinase Locus in Mouse Lymphoma Cells	Mutations in HGPRT Locus in Chinese Hamster Ovary Cells	Unscheduled DNA Synthesis in Rat Hepatocytes	Syrian Hamster Embryo Cell Transformation	Sex-Linked Recessive Lethal Test in Drosophila m	Micronucleus Test in Mouse Erythrocytes	Dominant Lethal Mutations in Rodents	Sister Chromatid Exchange in Mouse Bone Marrow Cells	Comet Assay in Mouse
1	**Positive**	**Positive**	**Positive**	Negative	N/A	N/A	**Positive**	N/A	**Positive**	N/A
2	**Positive**	**Positive**	**Positive**	Negative	N/A	Negative	**Positive**	N/A	**Positive**	N/A
3	**Positive**	**Positive**	**Positive**	Negative	N/A NEG*	Negative	**Positive** **POS***	N/A	**Positive**	N/A
4	Negative	**Positive**	N/A	Negative	**Positive**	Negative	N/A	N/A	**Positive**	N/A
5	**Positive**	**Positive** **POS***	N/A	Negative	Negative	Negative	Negative	**Positive**	Negative	Negative
6	**Positive**	**Positive**	N/A	N/A	N/A	Negative	N/A	N/A	N/A	N/A
7	Negative	**Positive**	Negative	N/A	N/A	Negative	N/A	N/A	N/A	N/A
8	Negative	**Positive**	Negative	N/A	N/A	Negative	N/A	Negative	N/A	N/A
9	N/A	N/A	**Positive**	Negative	N/A	N/A	N/A	Negative	N/A	N/A
10	Negative	Negative	N/A	Negative	N/A	Negative	N/A	N/A	Negative	Negative
11	Negative	N/A	Negative	Negative	Negative	Negative	N/A	N/A	N/A	N/A
12	Negative	N/A	N/A	Negative	N/A	Negative	N/A	N/A	Negative	Negative
13	Negative	Negative	Negative	Negative	N/A	Negative	Negative	N/A	N/A	Negative
14	Negative	Negative	N/A	N/A	N/A	Negative	N/A	**Positive**	N/A	N/A
15	Negative	N/A	Negative	Negative	N/A	N/A	N/A	N/A	N/A	Negative
16	Negative	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
17	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
18	Negative	N/A	Negative	N/A	N/A	N/A	N/A	N/A	N/A	Negative
19	Negative	Negative	N/A	Negative	N/A	N/A	Negative	N/A	N/A	Negative
20	Negative	Negative	Negative	Negative	N/A	N/A	N/A	N/A	N/A	Negative
21	Negative	N/A	N/A	N/A	N/A NEG*	N/A	N/A	N/A	Negative	Negative
22	Negative	N/A	Negative	N/A	N/A	N/A	N/A	N/A	N/A	N/A
23	Negative	N/A	Negative	Negative	N/A	N/A	Negative	N/A	N/A	N/A

Compound #	Chromosome Aberrations in Chinese Hamster Lung Cancer	Mutations in Thymidine Kinase Locus in Mouse Lymphoma Cells	Mutations in HGPRT Locus in Chinese Hamster Ovary Cells	Unscheduled DNA Synthesis in Rat Hepatocytes	Syrian Hamster Embryo Cell Transformation	Sex-Linked Recessive Lethal Test in Drosophila m	Micronucleus Test in Mouse Erythrocytes	Dominant Lethal Mutations in Rodents	Sister Chromatid Exchange in Mouse Bone Marrow Cells	Comet Assay in Mouse
24	Negative	N/A	Negative	N/A	N/A	N/A	N/A	N/A	**Positive**	N/A
25	N/A	N/A	N/A	N/A	N/A	Negative	N/A	N/A	**Positive**	N/A
26	Negative	N/A	Negative	Negative	N/A	N/A	Negative	**Positive**	Negative	N/A
27	N/A	N/A	Negative	N/A	N/A	Negative	Negative	**Positive**	**Positive**	N/A
28	Negative	N/A	Negative	N/A	N/A	N/A	N/A	N/A	N/A	Negative
29	Negative	N/A	Negative	Negative	N/A	N/A	N/A	N/A	N/A	Negative
30	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
31	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
32	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
33	Negative NEG*	N/A	Negative	N/A	N/A	Negative	Negative	N/A	N/A	Negative
34	N/A	N/A	Negative	N/A	N/A	Negative	N/A	N/A	N/A	N/A
35	Negative	N/A	Negative	Negative	N/A	Negative	Negative	**Positive**	N/A	N/A
36	Negative	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
37	Negative	N/A	N/A	Negative	N/A	N/A	N/A	N/A	N/A	Negative
38	Negative	N/A	Negative	N/A	N/A	Negative	Negative	N/A	Negative	Negative
39	Negative	N/A	Negative	N/A	N/A	Negative	Negative	N/A	Negative	Negative
40	Negative	N/A	Negative	N/A	N/A	N/A	Negative	N/A	Negative	Negative
41	Negative	N/A	Negative	Negative	N/A	Negative	Negative	N/A	Negative	Negative
42	Negative	Negative	N/A	Negative	N/A	N/A	N/A	N/A	Negative	Negative
43	Negative	N/A	Negative	N/A	N/A	N/A	N/A	N/A	N/A	Negative
44	Negative	N/A	Negative	N/A	N/A	Negative	Negative	N/A	Negative	Negative
45	Negative	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
46	Negative	N/A	N/A	N/A	N/A	Negative	N/A	N/A	Negative	Negative
47	Negative	N/A	Negative	N/A	N/A	Negative	N/A	N/A	Negative	Negative
48	**Positive**	**Positive**	**Positive**	N/A	N/A	**Positive**	N/A	Negative	**Positive**	**Positive**
49	N/A	N/A	Negative	N/A	N/A	N/A	N/A	N/A	N/A	N/A
50	Negative	N/A	Negative	N/A	N/A	Negative	Negative	**Positive**	N/A	Negative

Compound #	Chromosome Aberrations in Chinese Hamster Lung Cancer	Mutations in Thymidine Kinase Locus in Mouse Lymphoma Cells	Mutations in HGPRT Locus in Chinese Hamster Ovary Cells	Unscheduled DNA Synthesis in Rat Hepatocytes	Syrian Hamster Embryo Cell Transformation	Sex-Linked Recessive Lethal Test in Drosophila m	Micronucleus Test in Mouse Erythrocytes	Dominant Lethal Mutations in Rodents	Sister Chromatid Exchange in Mouse Bone Marrow Cells	Comet Assay in Mouse
51	N/A	N/A	Negative	N/A	N/A	N/A	N/A	N/A	N/A	N/A
52	N/A	**Positive**	N/A	N/A	N/A	Negative	Negative	Negative	N/A	N/A
53	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 4.7. Activity prediction of compounds according to PASS Online platform

Compound #	Compound Name	Anti-inflammatory	Anti-mutagenic	Antioxidant	Nitric oxide antagonist	Nitric oxide synthase inhibitor	Nitrite reductase (NO-forming) inhibitor
1	Apigenin	0.64	0.92	0.73	N/A	-	N/A
2	Myricetin	0.72	0.93	0.92	0.67	-	N/A
3	Kaempferol	0.67	0.94	0.85	0.59	-	N/A
4	Ferulic acid	0.64	0.90	0.54	N/A	-	N/A
5	Chlorogenic acid	0.59	N/A	0.75	N/A	-	N/A
6	Rosmarinic acid	N/A	0.72	0.53	N/A	-	N/A
7	Caffeic acid	0.65	0.84	0.60	N/A	N/A	0.65
8	P-coumaric Acid	0.64	0.88	0.55	N/A	-	0.68
9	Sideroxol	N/A	-	N/A	0.72	-	-
10	Sideridiol	N/A	-	N/A	0.69	-	-
11	Siderol	0.62	-	N/A	0.57	-	-
12	7-epicandicandiol	0.59	-	N/A	0.79	-	-
13	Linearol	0.75	-	N/A	0.65	-	-
14	Sidol	0.64	-	N/A	N/A	-	-
15	A-pinene	N/A	N/A	-	N/A	-	N/A
16	Camphene	-	N/A	-	N/A	-	N/A
17	B-pinene	0.61	-	-	N/A	-	N/A
18	Δ-3-carene	N/A	N/A	N/A	-	-	N/A
19	Myrcene	N/A	N/A	N/A	N/A	-	N/A
20	A-phellandrene	N/A	N/A	-	N/A	-	N/A
21	Limonene	0.61	N/A	N/A	N/A	-	N/A
22	B-phellandrene	N/A	-	-	N/A	-	N/A
23	(E)-2-hexenal	N/A	0.54	N/A	N/A	-	0.56
24	Γ-terpinene	N/A	N/A	-	N/A	-	N/A
25	P-cymene	0.64	N/A	N/A	-	-	0.60
26	Hexanol	N/A	0.64	N/A	N/A	-	0.81
27	1-octen-3-ol	0.60	0.54	N/A	N/A	-	0.52

Compound #	Compound Name	Anti-inflammatory	Anti-mutagenic	Antioxidant	Nitric oxide antagonist	Nitric oxide synthase inhibitor	Nitrite reductase (NO-forming) inhibitor
28	A-cubebene	0.88	-	-	-	-	N/A
29	A-copaene	N/A	-	-	N/A	-	N/A
30	B-bourbonene	0.66	-	-	N/A	-	N/A
31	A -gurjunene	N/A	-	-	N/A	N/A	N/A
32	B -cubebene	0.92	-	-	N/A	-	N/A
33	Linalool	0.53	N/A	N/A	N/A	-	N/A
34	Pinocarvone	N/A	N/A	-	0.57	-	N/A
35	Bornyl acetate	0.58	N/A	N/A	N/A	-	N/A
36	B -caryophyllene	0.74	N/A	N/A	N/A	-	N/A
37	Myrtenal	N/A	N/A	N/A	N/A	-	N/A
38	Trans-pinocarveol	N/A	N/A	N/A	N/A	-	N/A
39	Trans-verbenol	N/A	N/A	N/A	N/A	-	N/A
40	A-terpineol	0.65	N/A	N/A	N/A	-	N/A
41	Borneol	0.53	N/A	N/A	N/A	-	N/A
42	Germacrene D	N/A	-	-	N/A	-	N/A
43	Δ-cadinene	N/A	-	N/A	-	N/A	N/A
44	Myrtenol	0.74	N/A	N/A	N/A	-	N/A
45	Calamenene	0.71	N/A	-	-	-	N/A
46	Epi-cubebol	0.89	-	-	N/A	-	N/A
47	Cubebol	0.91	-	-	-	-	N/A
48	Caryophyllene oxide	0.75	-	N/A	N/A	-	-
49	Ledol	-	-	-	N/A	-	N/A
50	(E)-nerolidol	0.80	N/A	N/A	N/A	-	N/A
51	Globulol	-	-	-	N/A	-	N/A
52	Carvacrol	0.67	0.51	N/A	N/A	N/A	0.50
53	Sabinene	0.85	-	-	N/A	-	N/A

N/A* = Pa < 0.5

Table 4.7 summarizes the probability of activity of compounds found in *Sideritis congesta*, according to the PASS online platform. PASS online platform was used to quantify the activity probability (Pa) and inactivity (Pi) through experimental data of tests with active components available in the platform. Activity for anti-inflammatory, anti-mutagenic, antioxidant, NO antagonist, NO synthase inhibitor and NO-forming inhibitor endpoints were investigated. Very few numbers of components showed activity for NO antagonist and NO-forming inhibitor and none of the components showed NO-synthase inhibitory activity. 31 of the 53 components showed anti-inflammatory activity whereas only 8 of them showed antioxidant activity. 11 of the 53 components showed anti-mutagenic activity. According to the PASS online platform, cubebol (No 47) has the most probable anti-inflammatory activity with 0.92 probability. Apigenin, myricetin, kaempferol, and ferulic acid have high probability to act as an anti-mutagenic compound (Pa >0.80). Myricetin has the highest probability for the antioxidant activity.

5. DISCUSSION AND CONCLUSION

It is well known that many bioactive compounds from plants have possible beneficial health effects, based on their phytochemicals and specific activities. There has been a significant increase in studies on the species of the genus *Sideritis* over the past 20 years. Previous studies have indicated that *Sideritis* has many medicinal applications [9]. The aerial parts of plants in the genus *Sideritis*, also known as "mountain tea," are widely utilized in Mediterranean nations including Greece, Turkey, and Spain for their anti-inflammatory [4], anti-ulcer [5], antioxidant [6], and anti-microbial [7] activities. Numerous of these activities have been linked to a variety of phytochemicals found in *Sideritis,* including flavonoids, phenolic acids, diterpenoids, and essential oils [8]. Even the genus *Sideritis* has been widely investigated, *Sideritis congesta*, especially for the endemic ones found in Turkey remains limited and not discovered, although local people extensively use this plant in our country, Turkey. According to the current data, *Sideritis congesta* contains many beneficial phytochemicals including, flavonoids, phenolic acids, diterpenoids and essential oils. Even though abundance of phytochemical compounds within the plant is mainly based on the method of extraction and solvents used during the extraction, overall identified components of *Sideritis congesta* are examined deeply in the aspect of beneficial affects. It was expected that *Sideritis congesta* would exhibit the beneficial activities from each of those components. In this study, the protective effects of *Sideritis congesta* against inflammation has been investigated by NO assay on RAW264.7 murine macrophage cells. Furthermore, Ames test which is a screening tool for preclinical experiments to identify the mutagenic and antimutagenic capabilities of medicinal plants, has been conducted on *Salmonella typhimurium* TA98 and TA100. Furthermore, we used a series of *in silico* approaches to reveal the mutagenic basis and effects of *Sideritis congesta.*

First of all, before evaluating any activity, the cytotoxicity of the extracts was evaluated by MTT test on RAW264.7 murine macrophage cells. It is recommended to remain cautious when using the extracts in medicinal mixes because they may have potentially cytotoxic effects. Even though the *Sideritis congesta* extracts affected the cell viability dose-dependently, no cytotoxic effect on RAW264.7 murine macrophage

cells was observed. A sample is considered as cytotoxic if the if the percentage vitality value is lower than 70%. However, herein none of our samples showed cell viability lower than 70%. Even more, it is very expected that cell viability decreases with the increasing concentration. Secondly, bacterial culture preformed with *Salmonella typhimurium* showed that, bacterial growth was normal and *Sideritis congesta* extract was neither harmful nor mutagenic to the bacteria at the measured quantities.

Present studies conducted on *Sideritis congesta* suggested the antioxidant and antiinflammation activity by different methods including *in vivo* and *in vitro*, DPPH , scavenging analysis [30, 64]. However, none of them was conducted on a stabilized cell line and deeply examined the possible underlaying mechanism. This study is the first essay in which the possible anti-inflammatory activity was determined by measuring the nitric oxide levels in RAW264.7 murine macrophage cells stimulated by LPS. Endothelial cells, macrophages, and neurons all produce NO, a crucial chemical mediator that plays a role in the control of several physiological processes, including inflammation. Several disorders are linked to excessive NO generation and release. Specific nitric oxide synthases (NOSs) convert citrulline from arginine through a five-electron oxidative process to produce NO, which is then produced in biological tissues [65]. This is the reason why NO levels have a crucial role in inflammation.

The *in vitro* anti-inflammatory activities of the extracts and protective effects against inflammation were evaluated by using nitrite assay. The assay was employed both indomethacin (100 μM) and L-NAME (100 μM) as positive controls. L-NAME is a known synthetic arginine analogue that can inhibit NOS, thus, a preventive agent used against inflammation. Indomethacin is a non-steroidal, anti-inflammatory agent that inhibits COX activity, thereby blocking the production of prostaglandins [66]. We evaluated protective effects against inflammation of extracts in LPS-stimulated RAW 264.7 cells, by comparing the activity of L-NAME and Indomethacin. At normal conditions, LPS stimulates immune responses and induces the generation of cytokines and NO and as a result it induces inflammation. In here, we expected to see that concentration of NO of pre-treated samples with *Sideritis congesta* extracts would be lower than the negative control groups. According to our results, NO concentrations of samples treated with positive controls and different doses of *Sideritis congesta* have

been significantly and dose dependently decreased when compared to the sample treated with only LPS (Figure 4.1). Based on NO concentration of samples, we evaluated percentage of inhibition activity against inflammation. According to our results, *Sideritis congesta* extracts showed inhibition activity in a dose-dependent manner when compared to the only medium containing group. *Sideritis congesta* extract even in lowest dose, 0.03 mg/ml, nearly showed the same activity as bioactive compounds L-NAME and Indomethacin. All other doses of *Sideritis congesta* showed higher activity than the positive controls (Table 2.1). One of the most important findings of this study is that *Sideritis congesta* extracts have powerful inflammation inhibition activity as much as L-NAME and Indomethacin. These outcomes confirm previous findings and the knowledge of the local people, that *Sideritis congesta* extracts have anti-inflammatory and antioxidant properties.

Since medicinal plants contain a variety of biological functions and are thus utilized in both traditional medicine and as a source of raw materials for the pharmaceutical industry, the Ames test has emerged as one of the most popular methods for determining their mutagenic potential. Until now, limited number of species of *Sideritis* have been evaluated with Ames test in order to evaluate potential mutagenic effect of the plant.

European medicines agency has published a report on *Sideritis scardica*, *Sideritis clandestine*, *Sideritis raeseri* and *Sideritis syriaca*. According to their results, none of them showed mutagenic effect. Feistel et al., investigated acute and subchronic toxicity and mutagenicity of *Sideritis scardica* by *in vivo* and *in vitro* methods. The study revealed no toxicity of *Sideritis scardica* and no concerns for its mutagenic effect [67]. None of the existing articles, investigated potential mutagenic and anti-mutagenic effects of *Sideritis congesta*. This study is the first research which investigated mutagenic/ anti-mutagenic potential of the *Sideritis congesta.* In accordance with the previously published articles published about *Sideritis*, we observed that *Sideritis congesta* does not have mutagenic effect on TA98 and TA100 strains with and without metabolic activation.

In the anti-mutagenicity assay of our study, we wanted to highlight the importance of using the Ames test to identify direct and indirect mutagens in both the presence and absence of the exogenous metabolic system. To this end, the anti-mutagenic activity of the *Sideritis congesta* extract were evaluated via reverse mutation, Ames Test. First of all, we investigated anti-mutagenic potential of the *Sideritis congesta* by using *in silico* methods. According to our findings from PASS Online, active compounds found in *Sideritis congesta* have some anti-mutagenic activity. Based on those findings, we expected to see a decrease in the number of revertant colonies treated with, the known mutagenic compounds. According to our *in vitro* results, samples treated with 1000 and 5000 µl/plate *Sideritis congesta* extracts in TA98 and TA100 with S9 activation showed a considerable reduction in the number of revertant colonies when compared to only positive mutagen treated plates (Table 4.3). This result showed that the protective effect of *Sideritis congesta* against the SA and 2-AF in both TA98 and TA100 strains increased after metabolic activation with S9. Figure 4.2 shows apparent protective effect of *Sideritis congesta* metabolites against mutation in these strains. There are different pathways to explain this ant-mutagenic activity. First of all, anti-mutagenic activity of plan polyphenols after metabolic activation was already discussed by Edenharder et al,. [68]. Additionally, according to Buening et al., certain flavonoids have strong inhibitory effects on cytochrome P450 monooxygenases such CYP1A1 and CYP1A2. This inhibition stops some pro-carcinogenic substances from forming mutagenic or carcinogenic metabolites [69]. Overall, our findings show that *Sideritis congesta* extracts have considerable anti-mutagenic activity at high concentrations as seen by the decreased frequency of revertant colonies in both TA98 and TA100 with S9 activation. When combined with the *in silico* results, it was suggested that this anti-mutagenic activity comes more likely from flavonoids including, apigenin, myricetin, kaempferol and cinnamic acids including ferulic acid, Rosmarinic acid, caffeic acid and p-coumaric acid. Extraction method in favors of abundance of those components may even increase the anti-mutagenic activity in low doses as well.

In order to evaluate possible biological activity investigated throughout the study, each component found in *Sideritis congesta* extracts, Pass Online Platform freeware was used. The results revealed that, most of the flavonoids, cinnamic acids and some of the essential oils showed high activity for anti-inflammatory, anti-mutagenic and

antioxidant activities. 31 of the 53 components showed anti-inflammatory activity whereas only 8 of them showed antioxidant activity. 11 of the 53 components showed anti-mutagenic activity. Moreover, previous data from limited *in vitro* studies available showed that *Sideritis congesta* have antioxidant capacity via ROS scavenging capacity [28, 29].

Furthermore, we tried to construct a relationship between inflammation inhibition of *Sideritis congesta* and NO. To this end, we investigated NO antagonist, NO synthase inhibitor, and NO-forming inhibitor activities of each component present in *Sideritis congesta* using PASS online. However, very few numbers of components showed activity for NO antagonist and NO-forming inhibitor and none of the components showed NO-synthase inhibitory activity. Two different hypotheses may be asserted in this case. First one is that PASS online has low number invariant accuracy of prediction and low number of compound available in the platform for these endpoints and prediction of activity are insufficient or unreliable. It is well known that low number invariant reduces the consistency of the results. Second one is that components of *Sideritis congesta* exerts their anti-inflammatory activity via other pathways.

In the *in silico*, computational toxicology part of our study we tried to evaluate mutagenicity via Ames endpoint and genotoxicity via available endpoint on DanishQSAR database. For mutagenicity, we screened 53 compounds in 5 different programs for Ames test endpoint. The 46 of the 53 screened compounds did not show any mutagenic results. However, 7 of them exerted both negative and positive results in different databases and platforms. To this end, we conducted a consensus analysis via VEGA *in silico* platform. According to the consensus analysis five of the components (myricetin, kaempferol, sideroxol, E-2-hexanal, and caryophyllene oxide) more likely to be mutagenic. However, none of our results from our *in vitro* analysis, mutagenicity assay, showed mutagenic result (Table 4.3). Mutagenic Index was lower than two for all samples. There could be several reasons to explain this result. First of all, the concentration of the compounds in the extracted sample can be way too low to exert a mutagenic activity or when all compounds interact together, they simply do not show mutagenic activity.

In order to further investigate mutagenic activity for the ones which exerted positive result for mutagenic activity, we performed a literature search to find an experimental data. Apigenin gave positive result in OCHEM for mutagenicity. However, many different experimental studies showed that apigenin is not mutagenic [70, 71] .

Myricetin is another compound which gave positive mutagenicity result in OCHEM. According to the experimental data, myricetin tested positive for frameshift mutations in a bacterial reverse mutation assay under metabolic stimulation conditions, however myricitrin tested negative for mutagenic potential, especially when it is extracted with 90% aqueous methanol [72, 73].

For sideroxol which showed a positive result in Danish QSAR no experimental data found conducted *in vitro* neither *in vivo*. So, there are no evidence reporting the mutagenicity of the sideroxol.

(E)-2- hexenal exerted positive results for many of the platforms. According to the European Food Safety Authority review, many researches was conducted on hex-2(trans)-enal which is flavoring agent. Sokolowski et al., reported a positive mutagenic outcome for Ames test. However, the magnitude of the increase in revertant colony numbers is not substantial, their results do not exclude possible mutagenic potential in strain TA100. According to the Kato et al., hex-2(trans)- enal was “suspected to be positive”; however, no further details were provided and the validity of this study is limited [74].

Calamenene showed positive result for PkCSM and EPA T.E.S.T platforms however, no data found to be supporting its mutagenicity in experimental data.

The flavanol kaempferol is another potential mutagenic according to the OCHEM. There exist different data in favor and against the mutagenic potential of kaempferol. In several short-term experiments, such as the development of chromosomal abnormalities in eukaryotic cells, kaempferol is directly mutagenic [75].

Caryophyllene oxide showed positive results for OCHEM. However, according to the experimental data, both at the gene level (frameshift/ base-substitution mutations) and on the chromosome, β-caryophyllene was determined to have no mutagenic effects [76].

Furthermore, we tried to evaluate genotoxicity of the compounds via DanishQSAR database. We obtained 17 inconsistent results for different endpoints of genotoxicity. We further evaluated the results via VEGA *in silico* platform. However, VEGA *in silico* platform can only help to predict chromosomal aberrations and micronucleus activity of the compounds. To this end, not all endpoints for genotoxicity could be evaluated. Genotoxicity covers a broader spectrum of endpoint than the mutagenicity. All mutagens are genotoxic, however not all genotoxic substances are mutagenic. To this end, we can conclude that *Sideritis congesta* extracted by our method is non-mutagenic.

This is the first study of the anti-mutagenic and protective effects against inflammation of *Sideritis congesta* both *in vitro* and *in silico*. This study confirms that *Sideritis congesta* has protective effects against inflammation and anti-mutagenic activity. Moreover, the brewing of *Sideritis congesta* and use as an herbal tea by the folk, during cold, flu, illness etc. is satisfied with the outcome of this paper.

Unlike approved drugs, herbs are almost entirely unregulated for safety, uniformity of contents, contamination. Additional research with the standardized cultivation and extraction of *Sideritis congesta* may lead to regulated and controlled production of the extract. Each compound found in *Sideritis congesta* may be identified by high pressure liquid chromatography method and the correct dose of the compound and extraction may be established. By the help of this methods can validate the usage of *Sideritis congesta* as a supplement or one of its active ingredients in a drug.

6. REFERENCES

1. Atanasov AG, Waltenberger B, Pferschy-Wenzig EM, et al. Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnol Adv*. 2015
2. Weiss S, Ferrand N. Phylogeography of Southern European Refugia: Evolutionary Perspectives on the Origins and Conservation of European Biodiversity.; 2007
3. González-Burgos E, Carretero ME, Gómez-Serranillos MP. *Sideritis* spp.: Uses, chemical composition and pharmacological activities - A review. *J Ethnopharmacol*. 2011
4. Hernández-Pérez M, Rabanal RM. Evaluation of the antinflammatory and analgesic activity of *Sideritis canariensis* var. pannosa in mice. *J Ethnopharmacol*. 2002
5. Aboutabl EA, Nassar MI, Elsakhawy FM, Maklad YA, Osman AF, El-Khrisy EAM. Phytochemical and pharmacological studies on *Sideritis taurica* Stephan ex Wild. *J Ethnopharmacol*. 2002
6. Armata M, Gabrieli C, Termentzi A, Zervou M, Kokkalou E. Constituents of *Sideritis syriaca*. ssp. syriaca (Lamiaceae) and their antioxidant activity. *Food Chem*. 2008
7. Aligiannis N, Kalpoutzakis E, Chinou IB, Mitakou S, Gikas E, Tsarbopoulos A. Composition and antimicrobial activity of the essential oils of five taxa of *Sideritis* from Greece. *J Agric Food Chem*. 2001
8. Janeska B, Stefova M, Alipieva K. Assay of flavonoid aglycones from the species of genus *Sideritis* (Lamiaceae) from Macedonia with HPLC-UV DAD. *Acta Pharm*. 2007
9. Aneva I, Zhelev P, Kozuharova E, Danova K, Nabavi SF, Behzad S. Genus *Sideritis*, section Empedoclia in southeastern Europe and Turkey – studies in ethnopharmacology and recent progress of biological activities. *DARU, J Pharm Sci*. 2019
10. Chen L, Deng H, Cui H, et al. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*. 2018
11. Poljsak B, Šuput D, Milisav I. Achieving the balance between ROS and antioxidants: When to use the synthetic antioxidants. *Oxid Med Cell Longev*. 2013

12. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive oxygen species in inflammation and tissue injury. *Antioxidants Redox Signal*. 2014
13. Korhonen R, Lahti A, Kankaanranta H, Moilanen E. Nitric oxide production and signaling in inflammation. *Curr Drug Targets Inflamm Allergy*. 2005
14. Sharma JN, Al-Omran A, Parvathy SS. Role of nitric oxide in inflammatory diseases. *Inflammopharmacology*. 2007
15. Chi DS, Qui M, Krishnaswamy G, Li C, Stone W. Regulation of nitric oxide production from macrophages by lipopolysaccharide and catecholamines. *Nitric Oxide - Biol Chem*. 2003
16. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev*. 2007
17. Misko TP, Moore WM, Kasten TP, et al. Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. *Eur J Pharmacol*. 1993
18. Abdul-Hafeez EY, Karamova NS, Ilinskaya ON. Evaluation of mutagenic and antimutagenic potential of stem bark aqueous extracts of eight trees by the bacterial reverse mutation assay. *Ecol Genet*. 2018
19. Horn RC, Ferrão Vargas VM. Antimutagenic activity of extracts of natural substances in the Salmonella/microsome assay. *Mutagenesis*. 2003
20. Akram M, Riaz M, Wadood AWC, et al. Medicinal plants with anti-mutagenic potential. *Biotechnol Biotechnol Equip*. 2020
21. Heghes SC, Vostinaru O, Rus LM, Mogosan C, Iuga CA, Filip L. Antispasmodic effect of essential oils and their constituents: A review. *Molecules*. 2019
22. Thomford NE, Senthebane DA, Rowe A, et al. Natural products for drug discovery in the 21st century: Innovations for novel drug discovery. *Int J Mol Sci*. 2018
23. Yuan G, Wahlqvist ML, He G, Yang M, Li D. Natural products and anti-inflammatory activity. *Asia Pac J Clin Nutr*. 2006
24. Uritu CM, Mihai CT, Stanciu GD, et al. Medicinal plants of the family Lamiaceae in pain therapy: A review. *Pain Res Manag*. 2018
25. Arulselvan P, Fard MT, Tan WS, et al. Role of Antioxidants and Natural Products in Inflammation. *Oxid Med Cell Longev*. 2016
26. Salam AM, Quave CL. Opportunities for plant natural products in infection control. *Curr Opin Microbiol*. 2018

27. Fraga BM. Phytochemistry and chemotaxonomy of *Sideritis* species from the Mediterranean region. *Phytochemistry*. 2012
28. Aimond A, Calabro K, Audoin C, et al. Cytotoxic and Anti-Inflammatory Effects of Ent-Kaurane Derivatives Isolated from the Alpine Plant *Sideritis hyssopifolia. Molecules*. 2020
29. Erkan N, Cetin H, Ayranci E. Antioxidant activities of *Sideritis congesta* Davis et Huber-Morath and *Sideritis arguta* Boiss et Heldr: Identification of free flavonoids and cinnamic acid derivatives. *Food Res Int*. 2011
30. Gokbulut A, Yazgan AN, Duman H, Yilmaz BS. Evaluation of the antioxidant potential and chlorogenic acid contents of three endemic *Sideritis* Taxa from Turkey. *Fabad J Pharm Sci*. 2017
31. Zhao X, Cacherat B, Hu Q, Ma D. Recent advances in the synthesis of ent -kaurane diterpenoids. *Nat Prod Rep*. 2022
32. Topcu G, Erta A, Öztürk M, Dinel D, Kili T, Halfon B. Ent-kaurane diterpenoids isolated from *Sideritis congesta*. *Phytochem Lett*. 2011
33. Kirimer N, Tabanca N, Baser KHC, Tümen G. Composition of the essential oil of *Sideritis congesta* P.H.Davis et Hub.-Mor. *J Essent Oil Res*. 2001
34. Aydin S, Öztürk Y, Beis R, Hüsnü Can Bąer K. Investigation of Origanum onites, *Sideritis congesta* and Satureja cuneifolia essential oils for analgesic activity. *Phyther Res*. 1996
35. Watkins R, Wu L, Zhang C, Davis RM, Xu B. Natural product-based nanomedicine: Recent advances and issues. *Int J Nanomedicine*. 2015
36. Panche AN, Diwan AD, Chandra SR. Flavonoids: An overview. *J Nutr Sci*. 2016
37. Comalada M, Camuesco D, Sierra S, et al. In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-kappaB pathway. *Eur J Immunol*. 2005
38. Guzman JD. *Natural Cinnamic Acids, Synthetic Derivatives and Hybrids with Antimicrobial Activity*. Vol 19.; 2014
39. Lu H, Tian Z, Cui Y, Liu Z, Ma X. Chlorogenic acid: A comprehensive review of the dietary sources, processing effects, bioavailability, beneficial properties, mechanisms of action, and future directions. *Compr Rev Food Sci Food Saf*. 2020

40. Agunloye OM, Oboh G, Ademiluyi AO, et al. Cardio-protective and antioxidant properties of caffeic acid and chlorogenic acid: Mechanistic role of angiotensin converting enzyme, cholinesterase and arginase activities in cyclosporine induced hypertensive rats. *Biomed Pharmacother*. 2019
41. Verma RP, Hansch C. An approach towards the quantitative structure-activity relationships of caffeic acid and its derivatives. *ChemBioChem*. 2004
42. Kilani-Jaziri S, Mokdad-Bzeouich I, Krifa M, Nasr N, Ghedira K, Chekir-Ghedira L. Immunomodulatory and cellular antioxidant activities of caffeic, ferulic, and p-coumaric phenolic acids: a structure–activity relationship study. *Drug Chem Toxicol*. 2017
43. Huang Q, Lin Y, Yan Y. Caffeic acid production enhancement by engineering a phenylalanine over-producing Escherichia coli strain. *Biotechnol Bioeng*. 2013
44. Tosovic J. Spectroscopic features of caffeic acid: Theoretical study. *Kragujev J Sci*. 2017t
45. Lin Y, Yan Y. Biosynthesis of caffeic acid in Escherichia coli using its endogenous hydroxylase complex. *Microb Cell Fact*. 2014
46. Rodrigues JL, Araújo RG, Prather KLJ, Kluskens LD, Rodrigues LR. Heterologous production of caffeic acid from tyrosine in Escherichia coli. *Enzyme Microb Technol*. 2015
47. Monteiro Espíndola KM, Ferreira RG, Mosquera Narvaez LE, et al. Chemical and pharmacological aspects of caffeic acid and its activity in hepatocarcinoma. *Front Oncol*. 2019
48. Amoah SKS, Sandjo LP, Kratz JM, Biavatti MW. Rosmarinic Acid - Pharmaceutical and Clinical Aspects. *Planta Med*. 2016
49. Alagawany M, Abd El-Hack ME, Farag MR, et al. Rosmarinic acid: Modes of action, medicinal values and health benefits. *Anim Heal Res Rev*. 2017
50. Pei K, Ou J, Huang J, Ou S. p-Coumaric acid and its conjugates: Dietary sources, pharmacokinetic properties and biological activities. *J Sci Food Agric*. 2016
51. Mathew S, Abraham TE. Ferulic acid: An antioxidant found naturally in plant cell walls and feruloyl esterases involved in its release and their applications. *Crit Rev Biotechnol*. 2004
52. Ou S, Kwok KC. Ferulic acid: Pharmaceutical functions, preparation and applications in foods. *J Sci Food Agric*. 2004

53. Rong N, Ausman LM, Nicolosi RJ. Oryzanol decreases cholesterol absorption and aortic fatty streaks in hamsters. *Lipids*. 1997
54. Cox-Georgian D, Ramadoss N, Dona C, Basu C. Therapeutic and medicinal uses of terpenes. *Med Plants From Farm to Pharm*. 2019
55. Elshafie HS, Camele I. An overview of the biological effects of some mediterranean essential oils on human health. *Biomed Res Int*. 2017
56. Rusyn I, Daston GP. Computational toxicology: Realizing the promise of the toxicity testing in the 21st century. *Environ Health Perspect*. 2010
57. Arvidson KB, Valerio LG, Diaz M, Chanderbhan RF. *In silico* toxicological screening of natural products. *Toxicol Mech Methods*. 2008
58. Gozalbes R, Vicente de Julián-Ortiz J. Applications of Chemoinformatics in Predictive Toxicology for Regulatory Purposes, Especially in the Context of the EU REACH Legislation. *Int J Quant Struct Relationships*. 2018
59. Ferrari M, Fornasiero MC, Isetta AM. MTT colorimetric assay for testing macrophage cytotoxic activity *in vitro*. *J Immunol Methods*. 1990
60. Kiemer AK, Vollmar AM. Effects of different natriuretic peptides on nitric oxide synthesis in macrophages. *Endocrinology*. 1997
61. Maron DM, Ames BN. Revised methods for the Salmonella mutagenicity test. *Mutat Res Mutagen Relat Subj*. 1983
62. Sushko I, Novotarskyi S, Körner R, et al. Online chemical modeling environment (OCHEM): web platform for data storage, model development and publishing of chemical information. *J Comput Mol Des 2011 256*. 2011
63. Banerjee P, Eckert AO, Schrey AK, Preissner R. ProTox-II: A webserver for the prediction of toxicity of chemicals. *Nucleic Acids Res*. 2018
64. Yeşlada E, Ezer N. The antiinflammatory activity of some *Sideritis* species growing in Turkey. *Pharm Biol*. 1989
65. Knowles RG, Palacios M, Palmer RMJ, Moncada S. Formation of nitric oxide from L-arginine in the central nervous system: A transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc Natl Acad Sci U S A*. 1989
66. Mitchell JA, Akarasereenont P, Thiemermann C, Flower RJ, Vane JR. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc Natl Acad Sci U S A*. 1993

67. Feistel B, Wegener T, Rzymski P, Pischel I. Assessment of the acute and subchronic toxicity and mutagenicity of *Sideritis scardica* griseb. Extracts. *Toxins (Basel)*. 2018
68. Edenharder R, von Petersdorff I, Rauscher R. Antimutagenic effects of flavoniods, chalcones and structurally related compounds on the activity of 2-amino-3-methylinidazo[4,5-*f*]quinoline (IQ) and other heterocyclic amine mutagens from cooked food. *Mutat Res - Fundam Mol Mech Mutagen*. 1993
69. Buening MK, Fortner JG, Chang RL, Huang MT, Wood AW, Conney AH. Activation and inhibition of benzo(a)pyrene and aflatoxin b1 metabolism in human liver microsomes by naturally occurring flavonoids. *Cancer Res*. 1981.
70. Kang HK, Ecklund D, Liu M, Datta SK. Apigenin, a non-mutagenic dietary flavonoid, suppresses lupus by inhibiting autoantigen presentation for expansion of autoreactive Th1 and Th17 cells. *Arthritis Res Ther*. 2009
71. Anter J, Romero-Jiménez M, Fernández-Bedmar Z, et al. Antigenotoxicity, cytotoxicity, and apoptosis induction by apigenin, bisabolol, and protocatechuic acid. *J Med Food*. 2011
72. Hobbs CA, Swartz C, Maronpot R, et al. Genotoxicity evaluation of the flavonoid, myricitrin, and its aglycone, myricetin. *Food Chem Toxicol*. 2015
73. Elgorashi EE, Van Heerden FR, Van Staden J. Kaempferol, a mutagenic flavonol from Helichrysum simillimum. *Hum Exp Toxicol*. 2008
74. Younes M, Aquilina G, Castle L, et al. Scientific Opinion on Flavouring Group Evaluation 200, Revision 1 (FGE.200 Rev.1): 74 α,β-unsaturated aliphatic aldehydes and precursors from chemical subgroup 1.1.1 of FGE.19. *EFSA J*. 2018
75. Duarte Silva I, Rodrigues A, Gaspar J, Maia R, Laires A, Rueff J. Mutagenicity of kaempferol in V79 cells: The role of cytochromes P450. *Teratog Carcinog Mutagen*. 1996
76. Di Sotto A, Maffei F, Hrelia P, Castelli F, Sarpietro MG, Mazzanti G. Genotoxicity assessment of β-caryophyllene oxide. *Regul Toxicol Pharmacol*. 2013

7. APPENDICES

7.1. Curriculum Vitae

Personal Informations

Name	Zeynep	**Surname**	Çokçeken
Place of Birth		**Date of Birth**	
Nationality		**TR ID Number**	
E-mail		**Phone number**	

Education

Degree	Department	The name of the Institution Graduated From	Graduation year
Master	Pharmaceutical Toxicology	Yeditepe University	-
University	Genetics and Bioengineering	Yeditepe University	2019
High school	Saint Benoit Frenc High School	-	2015

Languages	Grades
English	B2
French	B2

Work Experience

Position	Institute	Duration
Sr. Site Management Coordinator	IQVIA	2022-still
Site Management Associate	ICON	2021-2022
Site Coordinator	ATLAS CRO	2021-2021
Medical Writer	DESIA Clinical Research	2019-2020

Computer Skills

Program	Level
Microsoft Package Programs	Excellent
Prism 9	Basic

Scientific works

The articles published in the journals indexed by SCI, SSCI, AHCI

Articles published in other journals

Proceedings presented in international scientific meetings and published in proceedings book.

Journals in the proceedings book of the refereed conference / symposium

Others (Projects / Certificates / Rewards)

Certificate of Animal Use in Experimental Research
Good Clinical Practices (ICH GCP)
Import/ Export of Dangerous Good Products (IATA)