

T.C.
YEDİTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF PHARMACEUTICAL TOXICOLOGY

***IN VITRO* AND *IN SILICO* EVALUATION OF
HEDERA HELIX EXTRACT FOR
MUTAGENIC/ANTIMUTAGENIC ACTIVITIES
AND PROTECTIVE EFFECTS AGAINST
INFLAMMATION**

MASTER THESIS

MAKBULE KÜBRA DOĞAN

İSTANBUL, 2022

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DECLARATION

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

Makbule Kübra
DOĞAN



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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.LITERATURE REVIEW	4
2.1. Botanical Characteristics of <i>Hedera helix</i>	4
2.2. Phytochemical Characteristics of <i>Hedera helix</i>	6
2.3. Clinical Characteristics of <i>Hedera helix</i>	10
2.3.1. Antimicrobial Activity of <i>Hedera helix</i>	11
2.3.2. Antileishmanial Activity of <i>Hedera helix</i>	12
2.3.3. Anthelmintic Activity of <i>Hedera helix</i>	13
2.3.4. Antispasmodic Activity of <i>Hedera helix</i>	13
2.3.5. Antioxidant Activity of <i>Hedera helix</i>	13
2.3.6. Effects of <i>Hedera helix</i> on Respiratory System	14
2.3.7. Anticancer/Antitumor Activity of <i>Hedera helix</i>	15
2.3.8. Anti-inflammatory Activity of <i>Hedera helix</i>	16
2.3.9. Antimutagenic Activity of <i>Hedera helix</i>	18
2.3.10.Clinical Studies of <i>Hedera helix</i>	18
2.4. Inflammation	27
2.4.1. Role of NO in Inflammation	30
3.MATERIALS AND METHODS	32
3.1. Materials	32
3.1.1. Evaluation of Protective Effects against Inflammation	32
3.1.2. Evaluation of Mutagenic and Antimutagenic Activities	32
3.1.3. <i>In silico</i> Evaluation	33
3.2. Methods	33

3.2.1. Preparation of <i>Hedera helix</i> Extract	33
3.2.2. Evaluation of Protective Effects against Inflammation	34
3.2.2.1. Preparation of Cell Culture	34
3.2.2.2. Measurement of Nitrite Levels	34
3.2.2.3. Measurement of Cell Viability	34
3.2.3. Evaluation of Mutagenic and Antimutagenic Activities	34
3.2.4. <i>In silico</i> Evaluation	37
4.RESULTS	44
4.1. Protective Effects against Inflammation Assay Results	44
4.2. Mutagenicity and Antimutagenicity Assay Results	45
4.3. <i>In Silico</i> Analysis Results	47
5.DISCUSSION and CONCLUSION	61
6.REFERENCES	68
7.APPENDICES	75
7.1. Curriculum Vitae	75

LIST OF TABLES

Table 2-1 Chemical compounds isolated from the leaves of <i>Hedera helix</i>	8
Table 2-3 Preparations of <i>Hedera helix</i> prescribed in Turkey	11
Table 2-4 Clinical studies of <i>Hedera helix</i>	20
Table 3-1 Preparation of VB medium E (50X)	36
Table 3-2 Preparation of top agar (100 ml)	36
Table 3-3 Preparation of 100 ml of 0.5 mM histidine/biotin	36
Table 3-4 Preparation of S9 mix	36
Table 3-5 Preparation of 1L of agar	36
Table 3-6 Compound list for <i>in silico</i> analysis	40
Table 4-1 The cell viability and nitrite inhibition percentages of the study groups	45
Table 4-2 Mutagenic index of <i>Hedera helix</i> extract in <i>Salmonella</i> strains TA98 and TA100 with and without S9	46
Table 4-3 Antimutagenic activity of <i>Hedera helix</i> extract in <i>Salmonella</i> strains TA98 and TA100 with and without S9 against a positive mutagen	46
Table 4-4 The Ames test predictions and experimental data (if available) from different platforms	48
Table 4-5 The predictions for other <i>in vitro</i> and <i>in vivo</i> genotoxicity/cancer endpoints in the Danish (Q)SAR database	53
Table 4-6 The predictions for selected activities in the PASS online freeware	58

LIST OF FIGURES

Figure 2-1 A general appearance of <i>Hedera helix</i>	6
Figure 2-2 The fruits of <i>Hedera helix</i>	6
Figure 2-3 The flowers of <i>Hedera helix</i>	6
Figure 2-4 The leaves of <i>Hedera helix</i>	6
Figure 4-1 The nitrite levels of different concentrations of <i>Hedera helix</i> extract, positive controls as indomethacin and L-NAME and negative control as medium	44
Figure 4-2 Antimutagenic activity (inhibition %) of <i>Hedera helix</i> extract against a positive mutagen	47



LIST OF SYMBOLS AND ABBREVIATIONS

#	Number
AnxA1	Annexin A1
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
cAMP	Cyclic Adenosine Monophosphate
CO ₂	Carbon dioxide
COX	Cyclooxygenase
CyPG	Cyclopentenone PG
dH ₂ O	Distilled Water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
DUSP-1	Dual Specificity Phosphatase 1
ED ₅₀	Median Effective Dose
eNOS	Endothelial NOS
EP	European Pharmacopoeia
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Fusion Protein
GILZ	Glucocorticoid-Induced Leucine Zipper
GRK	G Protein-Coupled Receptor Kinases
H ₂ O ₂	Hydrogen Peroxide
HCl	Hydrochloric acid
HEK	Human Embryonic Kidney
HPLC	High Performance Liquid Chromatography
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
JAK2	Janus Kinase 2
K ₂ HPO ₄	Potassium Phosphate
KCl	Potassium Chloride
LoVo cells	Human Colon Cancer Cells
LPS	Lipopolysaccharide
M	Medium
Mcl-1	Myeloid Leukemia Cell Differentiation Protein 1
MEF	Maximal Expiratory Flow
MgCl ₂	Magnesium Chloride
MgSO ₄	Magnesium Sulfate
MIC	Minimum Inhibitory Concentration
MKP-1	Mitogen-Activated Protein Kinase Phosphatase 1
Mres	Mresolution
MTT	3-[4,5-dimethylthiazol-2-yl]-strep2,5 diphenyl tetrazolium bromide
N ₂ O ₃	Dinitrogen Trioxide
N/A	Not Applicable
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaH ₂ PO ₄	Sodium Ammonium Phosphate
NaNO ₂	Sodium Nitrite
NF-κB	Nuclear Factor Kappa B

nNOS	Neuronal NOS
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
O ₂ ⁻	Superoxide Anion
OCHEM	Online Chemical Modeling Environment
(Q)SAR	(Quantitative) Structure–Activity Relationship
QSPR	Quantitative Structure Property Relationship
PASS online	Prediction of Activity Spectra for Substances
PENSTREP	Penicillin-Streptomycin
PG	Prostaglandin
PI3K	Phosphoinositide-3-Kinase
pkCSM	Small-Molecule Pharmacokinetics Prediction
ProTox-II	Prediction of Toxicity of Chemicals
ROS	Reactive Oxygen Species
SA	Sodium Azide
STAT3	Signal Transducer and Activator of Transcription 3
T.E.S.T.	Toxicity Estimation Software Tool
TGF- β	Transforming Growth Factor β
TLC	Thin Layer Chromatography
TLR	Toll-Like Receptor
TNF α	Tumor Necrosis Factor α
VB	Vogel-Bonner

ABSTRACT

Doğan, M. K. (2022). *In vitro* and *in silico* Evaluation of *Hedera helix* Extract for Mutagenic/Antimutagenic Activities and Protective Effects against Inflammation. Yeditepe University, Institute of Health Sciences, Department of Pharmaceutical Toxicology, MSc Thesis, İstanbul.

Hedera helix is a perennial woody plant with known medicinal properties for centuries. There have been several *in vitro/in vivo* studies examining the antibacterial, antifungal, antiviral, antileishmanial, anthelmintic, anti-inflammatory, antispasmodic, and anticancer/antitumor effects of this plant. In this study, it is aimed to evaluate the protective effects of 50% hydroalcoholic (50% EtOH) dry extract of *H. helix* leaves against LPS-induced inflammation, mutagenic/antimutagenic effects, and *in silico* predictions for genotoxicity/mutagenicity endpoints and anti-inflammatory properties. Stable nitrite levels were measured via a microplate reader and cell viability was examined via MTT assay using RAW 264.7 murine macrophage cell culture. Mutagenicity/antimutagenicity assays were conducted on *Salmonella* tester strain TA98 and TA100 with and without S9 in the Ames test. In addition, seven different platforms including Danish (Q)SAR Database, pkCSM web server, OCHEM web platform, ProTox-II web service, T.E.S.T. software, PASS online freeware, and VEGA (Q)SAR platform were used to conduct an *in silico* analysis of known compounds of *H. helix* leaves. The extract was found to be non-mutagenic on all of the strains tested. The predictions run in all of the *in silico* platforms supported this finding with negative predictions for Ames test and other *in vivo/in vitro* genotoxicity/cancer endpoints. A strong antimutagenic activity was only demonstrated at the concentration of 5000 µg/plate. The extract showed a similar effect when compared with indomethacin and L-NAME which was concentration-dependent. Anti-inflammatory activity predictions were positive for 36 compounds previously isolated from *H. helix* leaves. However, compounds with positive predictions for an activity like a nitric oxide antagonist, nitrite reductase (NO-forming) inhibitor and nitric-oxide synthase inhibitor were low. These findings suggested that the anti-inflammatory activity of the *H. helix* leaves extract was not likely seemed to be only dependent on NO inhibition. Further research may be warranted to address the other contributory anti-inflammatory mechanisms.

Key words: *Hedera helix*, Anti-inflammatory Activity, Mutagenicity, Antimutagenicity, *In silico*

ÖZET

Doğan, M. K. (2022). *Hedera helix* Ekstresinin Mutajenik, Antimutajenik ve İnflamasyona Karşı Koruyucu Etkilerinin *in vitro* ve *in silico* Değerlendirmesi. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmasötik Toksikoloji Anabilim Dalı, Yüksek Lisans Tezi, İstanbul.

Hedera helix, tıbbi özellikleri yüzyıllardır bilinen, otsu çok yıllık bir bitkidir. Antibakteriyel, antifungal, antiviral, antileishmanial, antihelmintik, anti-inflamatuvar, antispazmodik, antikanser/antitümör etkilerini değerlendiren pek çok *in vitro* ve *in vivo* çalışma mevcuttur. Bu çalışmada, *H. helix* yapraklarının %50 EtOH ekstresinin LPS ile indüklenmiş inflamasyona karşı koruyucu etkileri yanı sıra mutajenik ve antimutajenik etkileri araştırılmış ve genotoksisite/mutajenite ve anti-inflamatuvar özellikleri açısından *in silico* tahminler yürütülmüştür. RAW 264.7 fare makrofaj hücre kültüründe, bir mikropalak okuyucu kullanılarak stabil nitrit seviyeleri ve MTT analizi ile hücre canlılığı ölçülmüştür. Mutajenite ve antimutajenite analizleri, Ames testi ile *Salmonella* TA98 ve TA100 suşları üzerinde S9'lu ve S9'suz ortamda yapılmıştır. Ek olarak, *H. helix* yapraklarından izole edildiği bilinen bileşenlerin *in silico* analizini yapmak amacıyla, Danish (Q)SAR veritabanı, pkCSM web sunucusu, OCHEM web platformu, ProTox-II web hizmeti, T.E.S.T. yazılımı, PASS online yazılımı ve VEGA (Q)SAR platformu olmak üzere 7 farklı platform kullanılmıştır. *H. helix* ekstresi, test edilen suşların hiçbirinde mutajenik etki göstermemiştir. *In silico* platformlarda yapılan tahminler, Ames testi ve diğer *in vivo/in vitro* genotoksisite/kanser sonlanım noktalarında negatif tahminler vererek bu bulgumuzu desteklemiştir. Güçlü antimutajenik etki, yalnızca 5000 µg/plak konsantrasyonunda gözlenmiştir. *H. helix* ekstresi, konsantrasyona bağlı olarak indometazin ve L-NAME ile benzer etki göstermiştir. Anti-inflamatuvar aktiviteye dair tahminler, *H. helix* yapraklarından izole edilmiş 36 bileşen için pozitif bulunmuştur. Bununla beraber, nitrik oksit antagonisti, nitrit redüktaz (NO-yapıcı) inhibitörü ve nitrik oksit sentaz inhibitörü gibi aktiviteler için pozitif tahmin sayısının az olduğu görülmüştür. Bu bulgular, *H. helix* yaprak ekstresinin anti-inflamatuvar etkisinin, yalnızca NO inhibisyonuna bağlı olmadığını göstermiştir. Bu aktiviteye katkı sağlayan diğer anti-inflamatuvar mekanizmaların keşfedilmesi için ileri çalışmalar yapılabilir.

Anahtar kelimeler: *Hedera helix*, Anti-inflamatuvar aktivite, Mutajenite, Antimutajenite, *In silico*

1. INTRODUCTION and PURPOSE

Hedera helix, commonly known as ivy, is a plant that belongs to the family Araliaceae (1, 2). It is a root-climbing liana with evergreen leaves which is found in nature in different shapes like a climbing vine, herb, or tree (3, 4). It is a common plant with ornamental and medicinal uses (1). While the *H. helix* leaves have been used for medicinal purposes since ancient times, there are several formulations of its leaves extract-containing medicinal products (e.g. syrup, tablets, drops) for the treatment of common cold with cough, and acute and chronic inflammatory bronchial disorders today (2). There has been a rise in interest in the bioactive compounds found in *H. helix* leaves in recent years (5). The antifungal (1, 3, 6, 7), antibacterial (3, 5), anti-inflammatory (3, 8, 9, 10, 11, 12, 13, 14), antiviral (3), antispasmodic (15), antileishmanial (16, 17, 18), anthelmintic (19, 20), antioxidant (21), and anticancer/antitumor (22, 23, 24, 25) activities of active compounds of *H. helix* are investigated in many *in vitro* and/or *in vivo* studies. The potential of *H. helix* nanoparticles as a sunscreen protective agent is reported in another study (26).

The effects of *H. helix* on the respiratory system were examined and confirmed in several studies. A decrease in goblet cell counts and basement membrane thickness in the asthmatic airways, effect on interleukin (IL) 2 and IL-17 secretion pathways, and an increase in tracheal secretion and decrease in cough were shown in the studies conducted to examine the effects of *H. helix* extract or its compounds on lung histopathology and expectorant activities on mice or rats (27, 28, 29). Furthermore, possible β_2 -mimetic effects β -hederin and indirect G protein-coupled receptor kinases (GRK) -2 inhibitory activity of α -hederin were demonstrated in other studies carried out in human embryonic kidney HEK 293 cells (9, 30). There are many clinical studies carried out on efficacy and safety of *H. helix* in both adults and children. The dry *H. helix* leaves extracts are shown to be well tolerated and generally effective in adult patients and/or children with acute cough, contaminant cough, bronchitis, asthma, and upper respiratory tract infections (31, 32, 33, 34, 35, 36, 37, 38, 39). In addition, a special extract of *H. helix* leaves was found effective with a very good safety profile in a post-marketing surveillance study (40).

Inflammation is a physiological defense response occurring when pathogens invade the body or appear in the presence of a tissue injury. Inflammatory response is a well-organized process with limited duration aiming to maintain homeostasis via the

collaboration of cells and mediators to reduce and destroy the harmful stimuli (41). Inflammation can cause harm in life-threatening hypersensitive reactions to toxins, insect bites, and drugs as well as in chronic diseases like rheumatic arthritis, atherosclerosis, lung fibrosis, and cancer (13). Recently, there has been an increasing interest in herbal medicine, since adherence to conventional medical treatment for asthma is often poor and they may have fewer adverse effects than current therapy (27). Nitric oxide (NO) is a molecule in the gas form which plays role in modulating several physiological and pathophysiological processes in the human body such as neurotransmission in the nervous system, circulation and blood pressure, platelet function, and defense of the host. Several studies confirmed the role of NO in regulatory processes in inflammation such as regulation of vascular responses, signaling pathways, transcription factors, leukocyte migration and rolling, cytokine synthesis, apoptosis, and infection control (42). Furthermore, NO is an important inflammatory chemical in the respiratory tract with evidence for its modulatory effects on airway function and pro-inflammatory and immunomodulatory effects in pulmonary disease (43).

Chronic inflammation is thought to be an important element in the progression of neoplastic processes, as it has been shown to hasten cancer progression (13). A variety of natural antimutagenic compounds have been discovered in plant extracts until now. Mutagenic and antimutagenic activities of three saponins extracted from *H. helix* were examined in one study using a microsuspension technique of the Ames test. The saponins had no mutagenic activity on *Salmonella* tester strain TA98 with/without S9. Saponins exhibited antimutagenic activity against both benzo[a]pyrene and urine from a smoker on the strain TA98 with S9. Antimutagenic activity was dose-dependent in that study. (44). In another *in vitro* study, α -hederin which is a saponin isolated from *H. helix* and a promising anticancer agent did not show any clastogenic or aneugenic effects in human lymphocytes (45). Moreover, the antimutagenic activity of α -hederin against doxorubicin and carbendazim was also shown (3).

H. helix is a plant that is known for its anti-inflammatory and antimutagenic activities. To the best of our knowledge, its mutagenic properties were investigated only on *Salmonella* tester strain TA 98 using the Ames test. In addition, there was no study investigating its effects on NO inhibition (3, 8, 9, 10, 11, 12, 13, 14, 44, 45). The aim of this study was to evaluate the protective effects of 50% hydroalcoholic (50% EtOH) dry extract of *H. helix* leaves against lipopolysaccharide (LPS) induced inflammation in RAW 264.7 macrophage cells via measuring nitrite levels and investigation of

mutagenic/antimutagenic effects of the extract on *Salmonella* tester strains TA98 and TA100 using the Ames test. Both mutagenicity and antimutagenicity assays were performed with *Salmonella* tester strain TA98 and TA100 with and without S9. Additionally, *in silico* analysis was conducted using Danish (Q)SAR Database, pkCSM web server, OCHEM web platform, ProTox-II web service, T.E.S.T. software, PASS online freeware, and VEGA (Q)SAR platform to analyze genotoxicity/mutagenicity endpoints and anti-inflammatory properties of *H. helix* secondary metabolites.



2. LITERATURE REVIEW

2.1. Botanical Characteristics of *Hedera helix*

Hedera L. is a genus classified under the plant family of Araliaceae and the plant order Apiales Nakai (46). Evidence addresses 5.8 million years of existence of the *Hedera* L. genus (4). There are 17 species discovered so far in the genus *Hedera* L., one of which is *Hedera helix* L. (AKA English ivy, Common ivy) (3, 46). *H. helix* is a root-climbing liana with evergreen leaves and has been used as an ornamental plant in numerous countries for ages (3, 47). It exists in nature in different shapes like a climbing vine, herb, or tree. It is also known as a forest weed and fertile humid soil is a good source for its aggressive growth (4).

H. helix grows naturally throughout the world, predominantly in humid regions of temperate climates (4). It is found in, Libya, Morocco, Algeria, and Tunisia in Africa; Russia, Armenia, Georgia, Palestine, Lebanon, Syria, Iran, Iraq, and Turkey in Asia; Ukraine, Belarus, Germany, Hungary, Netherlands, Poland, Switzerland, Belgium, Norway, Denmark, Sweden, Austria, Ireland, United Kingdom, Albania, Bulgaria, France, Italy, Spain, and Portugal in Europe; Canada and United States in Northern America (48). In Turkey, it is found in Çanakkale, Balıkesir, İstanbul, Bolu, Ankara, Sinop, Ordu, Giresun, Rize, İzmir, Denizli, Antalya, Hatay (49).

H. helix has woody stems with a diameter of up to 25 cm and thin branches covered with short soft hair. Recruited seedlings form a thin and delicate primary root which produces several thin, small height branches by rising vertically. Some of these short branches evolve into distinct horizontal secondaries having lateral branches. Greater juveniles form as adventitious roots growing from the nodes along the ground. Thin roots have a diameter of 300–500 µm. The roots are remarkably dissected (50).

It has climbing ability on several materials such as rocks, barks, and mortar since its interesting root characteristics enable it to have an adjustable attachment system. Functional unbranched adventitious roots grow at the side of their shoots towards material while nutritional branched roots are produced under the ground if the shoots are in connection with the soil. *H. helix* is capable of adhering to the materials and climbing up to 30 m through its unbranched roots. The steps of this attachment system are outlined as generation of contact, root closure with the material, adhesion via releasing a chemical from the root hairs for gluing, and change in the form of root hairs. In one study investigating the biomechanics of its attachment system, it has been shown

that it has an effective attachment system with a fail-safe mechanism and its attachment roots specialize as anchorage organs which differ from subterranean roots (47).

H. helix consists of glossy, radiate-veined dark green leaves at the upper surface and lighter green-greyish leaves with pale, distinctly raised veins at the lower surface (3, 4). It has coriaceous and broad leaves which are 4-10 cm in length and cordate at the base and a lamina formed as a palmate with 3-5 lobes (3). Physiologically adult reproductive shoots are only produced by vertical stems (50). The appearance varies between the adult and juvenile leaves. While the juveniles have arranged, palmate-shaped lobes, the adults are large and symmetric around a central axis and ovate leaves (4). The leaf thickness is between 230 μm in juveniles to 330 μm in adults.

The stems producing flowers are formed from ovate to lanceolate leaves with a height of 3-8 cm (3). The flowering period is from summer to late autumn or to January in Southern Europe (3, 50). The flowers of the *H. helix* are actinomorphic. There are approximately 20 flowers in a terminal umbel which has a globular form. They have 5 tiny, triangular-shaped sepals and 5-6 triangular or ovate, yellowish green petals 3-4 mm long. The flowers are hermaphrodite with an ovary inferior and 5 stamens with bright yellow anthers. Nectar production begins 1-2 days after anthesis and stamens fall from the plant after approximately 7 days since the beginning of anthesis. Nectar production on the floral disc lasts for 1-3 days (50).

The fruits of the *H. helix* ripen in winter (3). Generally, one umbel in each panicle evolves into a fruit which is a small purple blackberry with a size of 5.5-7.5 x 5.5-8.7 mm. The berries have 1-5 seeds which are usually spread across by birds and start growing after 7-10 days (50).

A general appearance, fruits, flowers and leaves of *H. helix* are shown in Figure 2-1, Figure 2-2, Figure 2-3, and Figure 2-4, respectively.



Figure 2-1: A general appearance of *Hedera helix*



Figure 2-2: The fruits of *Hedera helix*



Figure 2-3: The flowers of *Hedera helix* (49)



Figure 2-4: The leaves of *Hedera helix*

The figures 2-1, 2-2, and 2-4 were taken from the Medicinal and Endemic Plants Education and Research Farm in the Faculty of Agriculture at Selçuk University in Konya, Turkey.

2.2. Phytochemical Characteristics of *Hedera helix*

There are several studies that investigated the isolation of chemical compounds contained in the extracts of *H. helix*. Triterpene saponins (2.5–6%) are the main

compounds with biological activity representing the medicinal use of *H. helix* (hederacoside C (1.7–4.8%), hederacoside D (0.4–0.8%), hederacoside B (0.1–0.2%) and α -hederin (0.1–0.3%)). *H. helix* extract also comprises flavonoids, anthocyanins, steroids, coumarins, volatile compounds, phenolic acids, amino acids, vitamins, fatty acids, carbohydrates, β -lectins, and polyacetylenes (3). Compounds that have been isolated from the leaves and fruits of *H. helix* so far are listed in Table 2-1 and Table 2-2, respectively.

β_2 -adrenergic effects of α -hederin have been identified which led to mucolytic, expectorant, bronchodilatory, and spasmolytic effects (2). The bronchodilatory and the secretolytic activities of α -hederin can be the result of its indirect β_2 -mimetic mechanism of action. Increased β_2 -adrenergic response in human airway smooth muscle cells and alveolar type II cells by α -hederin was shown in the preclinical studies. It also has an inhibitory effect on the internalization of β_2 -adrenergic receptors when even stimulating conditions occur. Indeed, stimulation of β_2 -adrenergic receptor was described in alveolar type II cells pretreated with α -hederin which leads to elevation of intracellular cyclic adenosine monophosphate (cAMP) levels resulting in increased surfactant production and secretion in these cells. Moreover, pretreatment with α -hederin provided an increase in isoprenaline mediated relaxation in airway smooth muscles precontracted with methacholine which was an indicator of bronchospasmolytic effect of *H. helix* dry extract (30). Hederacoside C is also an important compound isolated from the *H. helix* extract. It can be considered as the inactive form of α -hederin as it is converted to α -hederin after passing into the blood and metabolism. Thus, hederacoside C also exerts the same effects (2, 30). According to European Pharmacopoeia (EP), an identification test carried out using thin layer chromatography (TLC) is necessary to identify these substances in the *H. helix* extract as α -hederin and hederacoside C are the most recognized compounds known for the therapeutic effects of this plant. Moreover, the quantity of hederacoside C in the *H. helix* extract can also be estimated using an identified test method carried out with high-performance liquid chromatography (HPLC). Additionally, a new determination method using HPLC with a diode array detector for a few compounds including α -hederin and hederacoside C presented in one study which provided further information about the contents of *H. helix* extracts (2).

The polyacetylenes found in the leaves of *H. helix*, falcarinol, and dihydrofalcarinol, are allergens with powerful irritating and sensitizing properties.

They are alkylating substances and have the ability for easy reaction with protein or amino groups. This feature leads to binding covalently easier to surface markers on the macrophages and Langerhans cells present in the skin. Concentrations of these compounds and the ratio of one compound to another vary to a significant extent during the year and the lowest concentration of *H. helix* extract may cause skin irritation in August. Falcarinol is also found in the celery family, carrots, parsnips, ginseng, and Schefflera species within the same family as *H. helix*. There are several plants like *Brassaia actinophylla*, *Dendropanax trifidus*, *Fatsia japonica*, and *Fatshedera lizei* with a reported cross-reaction to *H. helix*. Falcarinol has been thought to be the main cause of these cross-reactions (4).

It has not been clarified whether other compounds in the *H. helix* have β_2 -adrenergic effects (30). Moreover, the inadequacy of detection of a few pharmacologically active compounds to characterize the clinical effects of a plant extract has been confirmed by most scientists. Even though several active compounds have been determined as active compounds in *H. helix* extract, the possible efficacy and toxicity of other compounds should also be considered (2).

Table 2-1: Chemical compounds isolated from the leaves of *Hedera helix* (1, 2, 3, 16, 51, 52, 53, 54)

<i>Triterpene saponins (as derivatives of hederagenin, oleanolic acid, and bayogenin (2β-OH-hederagenin))</i>
Bidesmosides:
Hederasaponin (hederacoside) C and hederasaponins A, B, D, E, F, G, H, I
3-sulfates of oleanolic acid and echinocystic acid (α -16-OH-oleanic acid)
3-sulfate of 28-O- β -gentiobiosyloleanate (Helicoside L-8a)
Monodesmosides:
α -hederin
Hederagenin 3-O- β -glucoside
α -amyrin
β -amyrin
<i>Flavonoids</i>
Aglycones:
Quercetin
Kaempferol
Glycosides:
Rutin (quercetin 3-O-rutinoside)
Isoquercitrin (quercetin 3-O-glucoside)
Astragalin (kaempferol 3-O-glucoside)
Nicotiflorin (Kaempferol 3-O-rutinoside)
<i>Coumarins</i>

Scopolin (scopoletin 7-O-glycoside)
<i>Polyacetylenes</i>
Falcarinon
Falcarinol
11,12-dehydrofalcarinol
<i>Phenolic acids</i>
Caffeic acid
Chlorogenic acid (5-O-caffeoylquinic)
Neochlorogenic acid (3-O-caffeoylquinic)
3,5-O-dicaffeoyl-quinic acid
4,5-O-dicaffeoyl-quinic acid
Rosmarinic [(R)-(+)] enantiomer] acid
Protocatechuic acid
<i>p</i> -coumaric
Palmitic acid
<i>Anthocyanin</i>
Cyanidin 3-monoside
<i>Sterols</i>
Cholesterol
Campesterol
Stigmasterol
Sitosterol
α -spinasterol
5 α -stigma-7-en-3 β -ol
<i>Alkaloid</i>
Emetin
<i>Volatile compounds</i>
Germacrene B
β -elemene
γ -elemene (elixen)
Methylethyl ketone
Methylisobutyl ketone
Trans-2-hexanal
Trans-2-hexanol
Germacrene D
β -caryophyllene
Sabinene
α -pinene
β -pinene
Limonene
Furfurol
<i>Amino acids</i>
Asparagine
Glycine
Proline
Tyrosine
Valine
Leucine

Phenilalanine
<i>Vitamins</i>
Vitamin E
Vitamin C
Pro-vitamin A
<i>Carbohydrates</i>
Hamamelitol (2-C-hydroxy-methyl-D-ribitol)

2.3. Clinical Characteristics of *Hedera helix*

H. helix is a therapeutical plant that has been used since ancient times with different indications of use expressed in the books of herbal medicine in the 16th century (1). Aqueous extraction of juvenile leaves of *H. helix* has been used as a traditional medication therapy in the treatment of respiratory diseases since the 19th century due to its expectorant and bronchodilatory effects (2). In the mid of 20th century, it is highly considered that the administration of safe doses of leaves and fruits have beneficial therapeutical effects, however, higher doses could cause respiratory paralysis which was a sign of its obvious poisonous effect (1).

A commission monograph published by the regulatory authority in Germany has allowed to use *H. helix* extract since 1988 as described. Moreover, several formulations containing *H. helix* extract (in tablet, syrup, or suppository forms) have been marketed since then with the indications of common cold with cough and acute/chronic inflammatory disorders in bronchial airways. A great increase in prescriptions has been noted in several countries in Europe as its efficacy and safety have been proved with empirical data as well as experimental findings. *H. helix* extract-containing medications in Germany comprised 80% of herbal expectorants prescribed in 2007 which equaled 2,000,000 prescriptions with a sales volume of more than 13,000,000 €. It is also sold in larger quantities as an herbal remedy, especially for children in the treatment of cough in Korea. There are many prescribed or non-prescribed formulations containing *H. helix* extract with an obligation with testing the hederacoside C content using the HPLC method described in Pharmaceutical Codex of Korea (2).

According to the publicly available “List of Summary of Product Characteristics / Package leaflet” obtained from the official website of the Turkish Ministry of Health, there are currently five licensed drug preparations of *H. helix* prescribed in Turkey (55). There are also several preparations of the extract registered as a dietary supplement which can be queried via the website of the Turkish Ministry of Agriculture and Forestry.

Table 2-2 Preparations of *Hedera helix* prescribed in Turkey

Product Name	Active Ingredients	Therapeutic Indications	References
Aerolet [®] Syrup, Proxar [®] Syrup, Tuspamin [®] Syrup	7 mg of <i>H. helix</i> dried leaves extract in 1 ml syrup (4-8:1) with a minimum 10% of hederacoside C in 24-30% (w/w) ethanol	• Adjunctive therapy for the symptomatic treatment of acute respiratory inflammation and chronic inflammatory bronchial diseases accompanied by coughing • Expectorant in cough with phlegm	56, 57, 58
Bronchipret [®] Syrup	In 100 ml syrup; 16.8 g of liquid extract of <i>Thymus vulgaris</i> L. and/or <i>Thymus zygis</i> L., thyme plant (1:2 – 2.5) with extracting agents of 10% ammonia solution (m/m): 85% glycerol (m/m): 90% ethanol (v/v): water (1:20:70:109) 1.68 g of liquid extract of <i>H. helix</i> L. leaves (1:1) with extracting agent of 70% ethanol [v/v]	• In the treatment of acute bronchitis accompanied by cough characterized by excessive mucus accumulation	59
Lornia [®] Syrup	40 mg of <i>H. helix</i> dried leaves extract in 5 ml syrup (4-8:1) with a minimum 10% of Hederacoside C	• Adjunctive therapy for the symptomatic treatment of acute respiratory inflammation and chronic inflammatory bronchial diseases accompanied by coughing • Expectorant in cough with phlegm	60

2.3.1. Antimicrobial Activity of *Hedera helix*

There are several studies demonstrating the effect of *H. helix* extract on different microorganisms. It was demonstrated that monodesmosidic hederagenin derivatives exhibited an extended spectrum of activity against yeast and dermatophyte species in an *in vitro* study in which the agar dilution method was used with concentrations of saponins up to 200 µg/ml. The most effective compound was α -hederin (7). Moreover, fungicidal activity of α -hederin (25 µg/ml) was also confirmed in another study which was carried out to evaluate the effects of the compound isolated from *Hedera helix* on

Candida albicans ultrastructure using a transmission electron microscopy (6). The antifungal activity of the ethanol extract of *Hedera helix* on phytopathogenic fungi (*Aspergillus niger*, *Botrytis cinerea*, *Botrytis tulipae*, *Fusarium oxysporum* f. sp. *tulipae*, *Penicillium gladioli*, *Sclerotinia sclerotiorum*) with minimum inhibitory concentrations (MIC) of 10% to 14% was also shown in one study. The effect was dose-dependent and comparable to fluconazole. The polyphenols (rutin, quercetin, and kaempferol) and the sterol (stigmasterol) were suggested as the compounds which exerted an antifungal effect (1). It was also shown that orally administered 50 mg/kg/bw saponin mixture with 60% of hederacoside C extracted from *H. helix* was effective on infections caused by *Candida albicans* in mice after 10 days. 90% and 40% of the animals had no infections after the administration of α -hederin and hederacoside C at the same dose and duration, respectively.

The effect of saponins with a large amount of hederacoside C extracted from *H. helix* on 23 strains (22 of them were gram-positive and -negative bacteria and one of them was yeast) was investigated. The MICs were 0.3–1.25 mg/ml, 1.25–5 mg/ml and 2.5 mg/ml for gram-positive bacteria, gram-negative bacteria and *Candida albicans*, respectively.

The efficacy of hederacoside C on the influenza virus A2/Japan-305 has also been revealed at a concentration of 100 μ g/ml (3).

2.3.2. Antileishmanial Activity of *Hedera helix*

The saponins of *H. helix* were tested *in vitro* on promastigote and amastigote forms of *Leishmania infantum* and *Leishmania tropica* to evaluate their leishmanicidal activity. It was concluded that no effect was shown with 60% saponic complex and hederasaponin B, C, and D. α -, β - and δ -hederin and hederagenin had exerted activity on promastigote forms and had harmful effects on macrophages at low concentrations. Hederagenin also showed a significant effect on amastigote forms (16).

A different *in vitro* study was carried out to examine antileishmanial activities of α -hederin, β -hederin, and hederacolchiside A₁ on *Leishmania infantum* promastigotes and human monocytes. The results showed a potent antileishmanial activity and a strong antiproliferative activity of the compounds on *Leishmania* and human monocytes (17).

On the contrary, the antileishmanial effect of an alcoholic *H. helix* extract was not supported by an *in vivo* study performed on the experimental ulcer of zoonotic cutaneous leishmaniasis in Balb/c mice (18).

2.3.3. Anthelmintic Activity of *Hedera helix*

Anthelmintic activities of saponic complex 60%, purified saponic complex 90%, and α -hederin were studied both *in vitro* on *Fasciola hepatica* and *Dicrocoelium* spp. and *in vivo* on a sheep infected by *Dicrocoelium* spp. After 24 hours, α -hederin eliminated *Fasciola hepatica* and *Dicrocoelium* spp. at concentrations of 0.005 and 0.001 mg/ml, respectively. Although administration of α -hederin caused a noticeable reduction in the egg number, 500 mg/kg of saponic complex 60% and 800 mg/kg of purified saponic complex 90% which were orally administered once and twice respectively were enough to remove the eggs in the sheep's feces (19).

In another study investigating the anthelmintic effect of the aqueous and hydro-alcoholic extracts of ripe fruits of *H. helix* on *Haemonchus contortus* *in vitro* and *in vivo*, both aqueous and hydro-alcoholic extracts of *H. helix* led to inhibition on egg hatch with similar median effective doses (ED₅₀). On the 2nd day after treatment, a significant reduction in faecal egg count in the sheep was shown with the aqueous extract at doses of 1.13 and 2.25 g/kg but did not continue the following days (20).

2.3.4. Antispasmodic Activity of *Hedera helix*

One study investigated the antispasmodic activity of the *H. helix* and the constituents contributing to this activity. Five fractions obtained from the dry extract of *H. helix* leaves by fractionation using polyamide column chromatography with subsequent isolation of phenolic substances and saponins and antispasmodic activity were determined in guinea pig ileum. Both compound categories showed significant activity with approximate papaverine equivalent values of 143, 55, 54, 49, and 22 for kaempferol, α -hederin, quercetin, hederagenin, and 3,5-dicaffeoylquinic acid, respectively. Saponins made the highest contribution to antispasmodic activity because of the higher concentration, followed by dicaffeoylquinic acids and flavonol derivatives (15).

2.3.5. Antioxidant Activity of *Hedera helix*

In one study, antioxidant properties of α -hederin and hederasaponin C at a concentration of 75 μ g/ml extracted from *H. helix* are tested using various endpoints to measure the antioxidant efficacy. The used tests are free radical scavenging activities on 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide (H₂O₂), and superoxide anion (O₂⁻), total antioxidant effect measured by inhibition of linoleic acid system, iron chelating activity and reducing power. Their antioxidant activities were compared with

the known antioxidants such as α -tocopherol, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT).

All tested compounds had an effect in reducing DPPH with scavenging activities of 79%, 78%, 76%, 54%, and 46% for BHA, α -tocopherol, BHT, α -hederin, and hederacoside C, respectively. Moreover, they strongly inhibited superoxide production by 82%, 82%, 75%, 74% and 70% for BHT, hederacoside C, α -tocopherol, α -hederin, and BHA, respectively. Regarding the H_2O_2 scavenging effects, the inhibition values were determined as 97%, 93%, 88%, 83%, and 63% for BHT, α -tocopherol, BHA, α -hederin, and hederasaponin C, respectively. Total antioxidant capacities were measured as 95%, 94%, 90%, 86%, and 67% for BHT, α -hederin, BHA, hederasaponin C, and α -tocopherol, respectively. The percentages of iron chelating activities of α -tocopherol, α -hederin, hederasaponin C, BHA, and BHT were shown to be 75%, 73%, 71%, 69%, and 66%, respectively. Finally, the reducing powers of BHA, BHT, α -tocopherol, α -hederin, and hederasaponin C were calculated as 3.204, 2.311, 1.929, 1.412, and 0.696, respectively (21).

2.3.6. Effects of *Hedera helix* on Respiratory System

The effects of the *H. helix* on the respiratory system were examined and confirmed in several studies. In order to detect the activity of *H. helix* (per oral) on lung histopathology in BALB/c mice of chronic asthma for which ovalbumin was used for sensitization, 100 mg/kg of *H. helix* was administered once daily for one week and the effects were compared with 1 mg/kg of dexamethasone. *H. helix* caused a decrease in goblet cell counts and basement membrane thickness in the asthmatic airways. However, no statistically significant effect on mast cell counts and epithelium and subepithelial smooth muscle layers thickness were shown compared to the control group. A better activity was seen with dexamethasone except for basement membrane thickness (27).

In order to explore whether there was an additive effect of a plant mixture containing *H. helix* and *Rhizoma coptidis* as antitussive and expectorant agents, a study was conducted in mice. The plant extracts had a significant increase in tracheal secretion and a decrease in cough. The extract in 1:1 concentration at a dose of 200 mg/kg led to a more effective activity on the parameters tested compared to individual use of each plant extract. In addition, an optimal expectorant effect was shown with the extract in 3:1 concentration at a dose of 200 mg/kg (28).

The activity of some of the main compounds isolated from *H. helix* leaves dry extract (EA 575) on β_2 -adrenergic receptor- green fluorescent fusion proteins (GFP) was studied in transfected HEK293 cells. The tested compounds were protocatechuic acid, neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, rutin, kaempferol-3-O-rutinoside, 3,4-, 3,5- and 4,5-dicaffeoylquinic acid, hederacoside B, and β -hederin. The results showed that only β -hederin had an inhibitory effect on the internalization of GFP-tagged β_2 -adrenergic receptors in the cells. According to the further analysis for receptor binding, a statistically significant increase in the β_2 -adrenergic receptor binding was demonstrated in human airway smooth muscle cells pretreated with β -hederin (1 mM, 24 h) (30).

In order to evaluate the effect of α -hederin on protein kinase A and GRK-2 mediated phosphorylation of GFP-tagged β_2 -adrenergic receptors, a further study was conducted in HEK293 cells. α -hederin showed significant repression on GRK2 mediated phosphorylation at Ser355/356 under stimulating conditions at a dose of 1 μ M (12 h) with no effect on GRK2 and protein kinase A in Förster resonance energy transfer experiments suggesting an indirect GRK2 inhibitory activity of the compound (61).

In another study investigating the effectiveness of α -hederin on lung tissue pathology and inflammatory mediators (IL-2 mRNA, IL-17 mRNA, and MicroRNAs-133a) in an ovalbumin-sensitized asthmatic model of rats, a decrease in IL-17 mRNA levels and an increase in MicroRNAs-133a gene expression were suggested in the group pretreated with α -hederin (29).

2.3.7. Anticancer/Antitumor Activity of *Hedera helix*

Sixteen saponins were tested on mouse B16 melanoma cells, mouse 313 non-cancer fibroblasts, Flow 2002 non-cancer human cells, and human HeLa tumor cells for any possible cytotoxic activity. Saponins were less active than strychnopentamine. α - and β -hederin were the most active compounds showing low cytotoxicity at concentrations of 10 μ g/ml and higher. No activity was seen at concentrations up to 200 μ g/ml of bisdesmosides (24).

The effects of α -hederin extracted from the leaves of *H. helix* in mouse B16 melanoma cells from a tumor in a C57BL/6 mouse and in non-cancer 3T3 fibroblasts from a mouse were analyzed. The cytotoxicity of α -hederin in a medium without serum and inhibition of proliferation in both cell lines at concentrations lower than 5 μ g/ml

after 8 hours of treatment were demonstrated. Moreover, the compound stimulated cytoplasm vacuolization and membrane changes leading to cell death (23).

In a study conducted to examine the effects of hederagenin in terms of apoptosis in human colon cancer (LoVo) cells, 3-[4,5-dimethylthiazol-2-yl]-strep2,5 diphenyl tetrazolium bromide (MTT) assay demonstrated significant inhibition of viability of LoVo cells and the effect was concentration and time-dependent. 1 and 2 μ M of hederagenin caused a significant increase to 32.46% and 81.78% in the apoptosis ratio, respectively. It could also stimulate nuclear changes representing apoptosis, reactive oxygen species (ROS) generation, up-regulation of Bax and down-regulation of Bcl-2, Bcl-xL, and surviving and release of cytochrome c (25).

α -hederin had a significant inhibitory effect on cell viability in human SW620 colon cancer cells stimulated with IL-6 at a concentration of 6.25 ng/ml to mimic the inflammatory microenvironment of tumor cells. This effect was both concentration and time-dependent. α -hederin could have a suppressive effect on malignant biological activities of colon cancer cells as it also inhibited migration and invasion in these cells. Taken together, the compound disrupted Janus kinase 2/Signal transducer and activator of transcription 3 (JAK2/STAT3) signaling in these cells and repressed IL-6 induced epithelial-to-mesenchymal transition which could make it a promising candidate in the treatment of colon cancer and metastasis (22).

2.3.8. Anti-inflammatory Activity of *Hedera helix*

The anti-inflammatory activity of *H. helix* was studied in an *in vivo* rat model of acute and chronic inflammation induced by carrageenan and cotton pellet. There was a decrease in inflammation by crude saponin and saponin's purified extracts of the plant. It was shown that the extracts inhibited serotonin and/or histamine release more efficiently than bradykinin and/or prostaglandin (PG) as they showed better efficacy in the first phase compared to the second phase in the acute inflammation model. The crude saponin extract of 100 and 200 mg/kg of body weight was the most effective extract with less activity compared to indomethacin. In the chronic inflammation model, saponin's purified extracts demonstrated better activity than crude saponin extract with a decreased potency compared with indomethacin. An inhibitory effect on macrophages and fibrosis was likely the cause of the observed anti-inflammatory activity in this model. It was concluded that the block of some inflammatory mediators could be the focus of *H. helix*'s mode of action (3, 8).

In another study, anti-inflammatory activities of two saponins (α -hederin and hederasaponin C) extracted from *H. helix* at a dose of 0.02 mg/kg (dissolved in 1 ml of water) was studied in a rat model of acute paw edema induced by carrageenan. In the first phase (on the 1st hour), no anti-inflammatory effect was observed statistically. In the second phase, there was a slight anti-inflammatory effect by hederasaponin C which was not potent as indomethacin. Hederasaponin C most likely inhibited bradykinin as well as other inflammatory mediators whereas indomethacin was considered to have an inhibitory action on both phases with an additional effect on histamine and serotonin release. It was also suggested that the glucose molecule in the sugar residue connected to the C3 position of the genin had a significant role, particularly in the late phase of inflammation (12).

The ethanol extract of *H. helix* applied intraperitoneally to a mice model of formalin-induced paw oedema was tested to examine its potential against inflammation and arthritis compared to diclofenac in a study. The paw thickness returned almost to its normal size in the mice treated with diclofenac and plant extract of 75 μ l within 3h. In the third hour, the percentage of inhibition was highest with 75 μ l plant extract (88.89%) followed by 77.7% of inhibition with 50 μ l plant extract and 61.11% of inhibition with 25 μ l plant extract. However, the highest peritoneal injection of ethanol extract of *H. helix* that could be tolerated by the mice was 75 μ l (13).

In a study, the effect of *H. helix* dry extract (EA 575[®]) on IL-6 release from LPS-induced murine macrophages was investigated with different concentrations (40 - 400 μ g/ml) and 1.7 μ g/ml corticosterone was used as the positive control. A dose-dependent reduction in IL-6 release was shown with *H. helix* dry extract and this reduction was statistically significant with concentrations of 80, 160, and 400 μ g/ml (9).

In another study conducted with the same dry extract of *H. helix* (EA 575[®]), it was examined whether the extract showed its anti-inflammatory effect via the nuclear factor kappa B (NF- κ B) pathway in various epithelial and immune system-derived cell lines. Cell cultures were stimulated with tumor necrosis factor α (TNF α). The nuclear translocation of NF- κ B, the phosphorylation of I κ B α , and the stability of the NF- κ B:I κ B α complex were tested using immunofluorescence microscopy, western blot, and a protein fragment complementation assay, respectively. It was shown that the extract had a regulatory effect on the NF- κ B pathway and the possible reason was the specificity switch of I κ B kinase β (11).

In two different studies, the effect of hederacoside C on *Staphylococcus aureus* induced inflammation was investigated both *in vivo* (on mammary gland tissues and lung tissues) and *in vitro* (RAW 264.7 cells). In one of them, reduction in pulmonary edema in the mice, inhibition of pro-inflammatory cytokine production, and suppression of Toll-like receptors (TLR) 2 and 4 were shown with the hederacoside C treatment. In another study, an improved mammary gland was demonstrated with hederacoside C by reducing the myeloperoxidase level and mammary gland tissue pathology and modulating pro-inflammatory and anti-inflammatory cytokines. Moreover, suppression of TLR 2 and 4 was also shown in this study (10, 14).

2.3.9. Antimutagenic Activity of *Hedera helix*

Mutagenic and antimutagenic activities of several saponins were examined in one study using a microsuspension technique of the Ames test. *Salmonella* tester strain TA98 with/without S9 was used for the mutagenicity test and TA98 with S9 was used for the antimutagenicity test. Thirteen saponins were isolated from the dried leaves of *H. helix* L., *Calendula officinalis* L. and *Calendula arvensis* L., three of which were extracted from *H. helix* L. Antimutagenic activity was screened against benzo[a]pyrene and urine concentrate of a smoker and compared with chlorophyllin. It was shown that all of the saponins from *H. helix* had no mutagenic activity. Moreover, a significant antimutagenic activity against benzo[a]pyrene and a dose-dependent reduction of mutagenicity of the smoker's urine were also demonstrated (44).

In a cytokinesis-block micronucleus assay, inhibitory activities of a few compounds including α -hederin extracted from *H. helix* on the clastogenic effect of doxorubicin were investigated *in vitro* in human lymphocytes. All concentrations of α -hederin (1.3×10^{-2} , 0.13, 1.3, and 13 nmol/ml) exerted an antimutagenic effect on clastogenicity and no clastogenic or aneugenic effects and cytotoxicity were seen in human lymphocytes (45).

Antimutagenic activity of α -hederin against doxorubicin and carbendazim was also confirmed in a further study (3).

2.3.10. Clinical Studies of *Hedera helix*

There are many studies carried out to evaluate the efficacy and safety of *H. helix* preparations in both adults and children. Specifically, the activities of *H. helix* extract preparations on cough stand out from the rest of the studies. As antibiotics use is not a convenient treatment method in viral respiratory infections leading to pathogen

resistance with no effect on cough resolution, alternative therapies including *H. helix* extract preparations are widely prescribed. *H. helix* extracts were one of the herbal expectorants with approximately 2 million prescriptions in Germany in 2007 (62). These studies are summarized in Table 2-4.



Table 2-3 Clinical studies of *Hedera helix*

Aim of Study	Study Design	Patient Characteristics	Dosage and Administration	Results	Reference
Evaluating the efficacy and safety of a liquid containing <i>H. helix</i> leaves (EA 575 [®]) in the treatment of patients with acute cough	Randomized, placebo-controlled, double-blind, multi-center	181 adults	Liquid (Prospan [®]) contained 35 mg <i>H. helix</i> extract in 5 ml (ethanol 30%), Applied three times a day for one week	Statistically significant and clinically relevant decrease in severity of cough and symptoms associated with cough and bronchitis (vs. placebo group). A remarkable early onset of efficacy (within 2 days) and consistent superior efficacy. Adverse events: non-serious, mildly and moderately severe and non-related with drug.	31
Comparison of the efficacy and tolerability of two different extracts of <i>H. helix</i> leaves in patients with acute bronchitis	Randomized, double-blind, two-arm	590 patients (≥ 2 years)	Hedelix [®] : 50% ethanol Prospan [®] : 30% ethanol Applied three times a day for one week (24, 16, or 12 drops per dose depending on age scale)	A gradual decrease in Bronchitis Severity Score at a similar rate in both groups. 2.7% of patients had a non-serious adverse event.	32

Aim of Study	Study Design	Patient Characteristics	Dosage and Administration	Results	Reference
Assessing the efficacy and safety of <i>H. helix</i> cough syrup compared with acetylcysteine in patients with acute bronchitis	Prospective, open, non-interventional	139 patients (6 - 95 years)	Prospanex [®] : 35 mg <i>H. helix</i> extract in 5 ml, Applied according to leaflet information for one week	A comparable reduction in both groups for all assessments except for the ones below. A higher reduction in dyspnoea, cough attack number and cough-associated sleeping disorders in the <i>H. helix</i> group. Comparable tolerability between the groups.	33
Assessing the efficacy and safety of the combination of <i>H. helix</i> and Thyme herb in the treatment of acute bronchitis with productive cough	Double-blind, placebo-controlled, multi-centre, phase IV	361 patients (≥18 years)	(Bronchipret [®] Saft): 100 g syrup contained 15.00 g of thyme herb and 1.50 g of <i>H. helix</i> , Applied 5.4 ml three times daily for 11 days	A significant reduction in coughing fits on days 7 to 9 vs. placebo with 2 days earlier onset of efficacy. Faster symptom regression and higher responder rate at 2 nd and 3 rd visits vs. placebo. Adverse events: Well tolerated, non-severe and -serious.	34

Aim of Study	Study Design	Patient Characteristics	Dosage and Administration	Results	Reference
Reviewing the efficacy of an extract from dried leaves of <i>H. helix</i> in the treatment of chronic airway obstruction in bronchial asthma	Systematic review of 3 randomized controlled trials	75 patients (4 - 16 years)	Prospan [®] drops: 35 mg/d for 3 days Prospan [®] drops: 42 mg/d for 10 days Prospan [®] suppositories: 160 mg/d for 3 days Prospan [®] syrup: 105 mg/d for 10 days	Higher decrease in airway resistance and superiority in all other objective measures with the drops vs. placebo. Preservation of at least 54% and 35% of effect against placebo for syrup and suppositories, respectively.	35

Aim of Study	Study Design	Patient Characteristics	Dosage and Administration	Results	Reference
Assessing the efficacy and tolerability of <i>H. helix</i> in the treatment of acute upper respiratory tract infections	Systematic review of six randomized controlled, one controlled clinical and four observational studies	3592 patients (0 - 86 years)	(Prospan®) syrup, drops, effervescent tablets, lozenges: 105 mg/d. Applied dose: Various for about 7-8 days Cough (EMA) granules: <i>A. officinalis</i> 1000 mg/10 mL, <i>S. irio</i> 100 mg/10 mL, <i>H. helix</i> 70 mg/10 mL. Applied dose: 1 sachet three times daily for 7 days Cofnovex plus (EMA) syrup: Same as above. Applied dose: Not reported Bronchipret® syrup: <i>T. vulgaris</i> 1.5 g LE/10 g; <i>H. helix</i> 0.15 g/10 g. Applied dose: Age-dependent for 7-10 days Hedelix® drops: 40 mg/5 ml. Applied dose: Age-dependent for 7±1 and 10 days Mukalbion tablets: <i>A. officinalis</i>, <i>S. irio</i>, <i>H. helix</i>. Applied dose: 2 tablets twice daily for 15 days Hedussin ® syrup: 33 mg DE/4 mL. Applied dose: Age-dependent for 17±13 days	An effective and safe option in the treatment of cough in upper respiratory tract infections and bronchitis. A more rapid decrease in severity/frequency of cough in three randomized trials. Minimal clinical significance of its efficacy. Adverse events: non-serious, but reporting had low quality and high risk of bias.	36

Aim of Study	Study Design	Patient Characteristics	Dosage and Administration	Results	Reference
Examining the tolerability and safety of film-coated tablets of dried <i>H. helix</i> in the treatment of cold with coughs	A non-interventional and open post-marketing surveillance study	330 patients (11–85 years)	Prospan [®] Cough Tablets: 25 mg in ethanol 30%. Applied dose: ≥ 2 tablets twice daily for at least 7 days	According to the global assessment results, good to very good tolerability by practitioners (98.5%) and patients (96.4%).	37
Testing of efficacy and safety of <i>H. helix</i> extracts in the treatment of cough and bronchitis	Independent, open, non-interventional two studies with same design	268 patients (0 - 12 years)	Hedelix [®] Syrup and cough drops: drug:extract ratio 2.2–2.9:1	Only mildly expressed major symptoms (rhinitis, cough and viscous mucus) and absence of them in 93, 94.2 and 97.7% of patients. In 96.5% of patients, ‘good’ or ‘very good’ global effect. Adverse events: Mild and non-serious in 1.9% of patients. ‘Good’ to ‘very good’ tolerability and compliance in 99% (syrup) and 100% (drops) of patients.	38

Aim of Study	Study Design	Patient Characteristics	Dosage and Administration	Results	Reference
Investigating for potential efficacy of <i>H. helix</i> use in acute viral infections like COVID-19	Review of five observational, four randomised controlled (single-blind and double-blind), two open-label and one controlled trials with one retrospective analysis of case notes	210,481 patients (both children and adults)	Oral administration in several forms in a single-herbal or multi-herbal formulation for 7 to 20 days (median duration of 8.5 days)	A possible therapeutic agent for early symptoms of respiratory tract infections with a best confirmed effectiveness for coughing. Limited finding for its potential for night coughing and related sleep disturbance. Weak evidence for effectiveness in cough expectoration and other viral symptoms.	63
Determining the effectiveness of <i>H. helix</i> extract on nasal secretion and local infections after post-septoplasty nasal packing removal	Open-label, prospective and randomized study	70 patients (18-56 years)	Syrup containing 105 mg of dry <i>H. helix</i> extract: 5 mL three times a day for 5 days	Significant reduction in nasal secretion according to patients' assessment and nasal endoscopic examination. No occurrence of local infections in the post-op follow up.	64

Aim of Study	Study Design	Patient Characteristics	Dosage and Administration	Results	Reference
Assessing of efficacy and tolerability of <i>H. helix</i> extract as an add-on treatment in patients with bronchial asthma	Double blind, placebo-controlled, randomized, cross-over study	30 patients (6 - 11 years)	Prospan® Cough Syrup: 5–7, 5:1 in ethanol 30%. Applied dose: 5 ml twice daily for 4 weeks in addition to 400 µg budesonide equivalent therapy.	A partial increase in lung function vs. placebo. No significant change in Forced Expiratory Volume 1. A significant difference in maximal expiratory flow (MEF) ₇₅₋₂₅ , MEF ₂₅ and vital capacity vs. placebo	39
Reviewing the efficacy and safety of <i>H. helix</i> extract in the treatment of bronchitis	Prospective, open, multicentre, postmarketing study	9657 patients (0 - 98 years)	A syrup containing 700 mg of dried <i>H. helix</i> leaves extract with a drug-to-extract ratio: 5–7.5:1 in ethanol 30% Applied dose: Age-dependent for 7 days	Improvement/healing of symptoms in 95% of patients. Adverse events: in 2.1% of patients with a very good safety profile.	40

2.4. Inflammation

Inflammation is a physiological defense response which occurs when pathogens invade the body or there is a tissue injury. Inflammatory response is a well-organized process with limited duration in which the main purpose is to maintain homeostasis via collaboration of cells and mediators to reduce and destroy the harmful stimuli. Even though it is a beneficial event for an organism, non-resolved inflammatory responses may lead to onset and progression of several inflammatory diseases such as rheumatoid arthritis, asthma, multiple sclerosis and ischemia.

Cornelius Celsus described the acute inflammatory reaction macroscopically as calor (heat), rubor (redness), tumor (swelling), and dolor (pain). Tissue injury causes the release of several pro-inflammatory mediators by the resident cells such as tissue macrophages, epithelial cells, and dendritic cells. Thus, the inflammatory cascade starts with a vascular response that includes hyperemia and increased vessel wall permeability caused by these mediators. Firstly, transient vasoconstriction in the arteriole occurs and is aided by vascular smooth muscle contraction. As a result of the increased blood flow into the injured area caused by arteriolar vasodilation, local hyperemia develops. Changes in the vascular endothelium are easily observable at this early stage, resulting in the exudation of plasma proteins and fluid from the blood into the tissue. Leukocyte migration from the circulation into tissue follows. Circulating leukocytes react with post-capillary venule endothelial cells upon a suitable stimulus such as mechanical damage, chemicals, infection, poisons, and ischemia. Selectins are glycoprotein adhesion molecules found on leukocytes such as L-selectin and endothelial cells such as P and E-selectin. Selectins and their carbohydrate ligands (P-selectin glycoprotein ligand 1, E-selectin-ligand 1, and CD34) play a major role in this process. Leukocytes rolling on the endothelium have now the ability to interact with chemoattractant molecules located on the endothelial luminal surface (e.g., platelet-activating factor, complement factor 5a, IL-8/chemokine ligand 8, and leukotriene B₄). When relevant chemoattractant receptors located on rolling leukocytes are activated, increased expression and affinity of integrins. Integrins bind their ligands such as vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 and induce a well-fixed adhesion of activated leukocytes to endothelial cells. Then, leukocytes migrate into interstitium. Leukocyte transfer into the tissue can lead to further activation resulting in the release of several molecules such as cytokines, colony-stimulating factors,

chemokines, and ROS. This process initiates the removal of harmful stimuli. But it may also cause adjacent tissue damage resulting in increased severity of symptoms.

The objective of the initial inflammatory response is to protect the host. This response is self-limiting and continues with the resolution of inflammation. Any dysregulation of an inflammatory response to resolution may lead to a chronic condition resulting in scar and fibrosis. Resolution of inflammation is a dynamic process with the contribution of signaling pathways and biochemical mediators. These pathways and mediators control the discontinuation of the inflammatory response primarily by reducing granulocyte recruitment and reversal of vasodilatation and permeability of vessels, changing from production of pro-inflammatory mediators to pro-resolution mediators, stopping the activity of signaling pathways which lead to the survival of leukocytes and cytokine production, recruited inflammatory cells apoptosis, clearance of apoptotic cells by phagocytes (mainly by macrophages), change in phenotypes from pro-inflammatory cell to pro-resolution. Apoptosis and phagocytic clearance of granulocytes (which is called efferocytosis) have significant importance in the resolution process of acute inflammation. During efferocytosis, a change in macrophage phenotypes from M1 to M2 phenotype occurs. Macrophages with the M2 phenotype have profound efferocytic properties and release anti-inflammatory chemicals (e.g., IL-10 and transforming growth factor (TGF) β) and pro-resolving mediators. The latter events are associated with limiting tissue injury at the inflammation site. In these events, the phenotype of M2 macrophages switches to Mresolution (Mres) resulting in a reduction in phagocytosis and an increase in antifibrotic and antioxidant proteins. Hence, the promotion of granulocyte apoptosis may be beneficial to the control of acute inflammatory diseases. Leukocytes may undergo cell death via necrosis, NETosis, and autophagy. Studies have revealed that NF κ B, cAMP, phosphoinositide-3-kinase (PI3K), and myeloid leukemia cell differentiation protein 1 (Mcl-1) are the key molecules functioning in leukocyte survival and apoptosis maintenance.

Several mediators have been detected in the natural resolution process. Resolvins, lipoxins, maresins, and protectins/neuroprotectins are some of the lipid mediators which are defined by their anti-inflammatory and pro-resolution properties. Their modulatory role in the animal models of both acute and chronic inflammation such as asthma, arthritis, inflammatory pain, peritonitis, and ischemia-reperfusion injury has been shown in many studies. Glucocorticoid-induced proteins consist of another group of these mediators. The production of endogenous glucocorticoids by the adrenal

glands has an essential function in the resolution of inflammation. They have effects on signaling pathways, such as NF- κ B and activating protein 1 and inducing anti-inflammatory regulatory proteins which generate their broad-spectrum anti-inflammatory and immunosuppressive properties (41). Glucocorticoids also inhibit NOS and phospholipase A2 and cyclooxygenase (COX) 2 leading to suppression of inflammatory mediator secretion such as NO and PGs (65). Glucocorticoid-induced leucine zipper (GILZ), mitogen-activated protein kinase phosphatase 1/dual specificity phosphatase 1 (MKP-1/DUSP-1) and Annexin A1 (AnxA1) are the glucocorticoid-induced proteins which has recently aroused much interest. Significant anti-inflammatory effects of GILZ and MKP-1/DUSP-1 have been shown in one *in vivo* study and anti-inflammatory and pro-resolving effects of AnxA1 in different experimental inflammation models. One other important mediator group is reactive oxygen species (ROS) like H_2O_2 , O_2^- and hydroxyl radical. Under normal conditions, they are generated during energy production in aerobic cells and eliminated by antioxidant enzymes including catalase, superoxide dismutase, glutathione peroxidase, and peroxiredoxins. Even though ROS is associated with a sufficient antimicrobial response, it also has effect on hormone biosynthesis, host defense against pathogens, and intracellular signaling with harmful effects on the host's DNA, proteins, and lipids resulting in cell injury and death. The possible role of ROS in the development of various inflammatory diseases (e.g., cancer, diabetes, arthritis, and atherosclerosis) has been demonstrated in a few studies. ROS activates various signaling pathways and transcription factors essential for pro-inflammatory stages (e.g., PI3K, NF- κ B, activator protein 1, and mitogen-activated protein kinases) via increasing gene expression of pro-inflammatory chemicals. In addition, ROS release in injured tissue leads to the gathering of leukocytes in the injury site. Moreover, different *in vitro* and *in vivo* studies have shown the role of ROS in the regulation of leukocyte survival. The fourth mediator group involved in the inflammatory resolution is COX-2 derived PGs. Studies suggest that PGD_2 and its cyclopentenone PG (CyPG) metabolites may have pro-resolution and anti-inflammatory properties. CyPG metabolites may show a significant effect on platelet aggregation, inhibition of cell cycle progression, and cytokine release in immune cells. Furthermore, 15d-PGJ2 which is an endogenous CyPG, has strong immunomodulatory and anti-inflammatory activity as well as regulatory effects on apoptosis of granulocyte, macrophage, and myofibroblast cells and redox-sensitive transcription. It also activates intracellular peroxisome proliferator-activated receptors γ

stimulation of which coordinate activities on inflammatory signaling pathways resulting in suppression of pro-inflammatory cytokine expression and inducible nitric oxide synthase (iNOS). The cytokine IL-10 is the fifth mediator secreted by macrophages, monocytes, mast cells, regulatory T cells, B lymphocytes, dendritic cells, epithelial cells, keratinocytes, and granulocytes. It regulates inflammatory biochemical secretion by macrophages, monocytes, and neutrophils and also restricts the production of pro-inflammatory cytokines such as TNF- α , IL-6, IL-1, IL-8, and IL-12. The potential of IL-10 on limiting the inflammatory process in virus infection of the respiratory system, ischemia, acute myocarditis, colitis, and endotoxic shock has been demonstrated in *in vivo* studies. It is suggested that IL-10 may be helpful in the resolution of inflammation by decreasing pro-inflammatory cytokine secretion and increasing anti-inflammatory cytokine secretion and phagocytic ability of macrophages (41).

2.4.1. Role of NO in Inflammation

NO is a signaling molecule in the gas form which modulates several physiological and pathophysiological processes in the human body such as neurotransmission in the central and peripheral nervous system, circulation and blood pressure, platelet function, and host defense.

It is derived from L-arginine and its synthesis reaction is catalyzed by nitric oxide synthase (NOS) enzymes. There are 3 different NOS isoforms which are neuronal NOS (nNOS), endothelial NOS (eNOS), and iNOS. nNOS and eNOS are predominantly expressed in neurons in the central and peripheral nervous system and endothelial cells, respectively, and inactive in resting cells. Both of the enzymes are activated with an increase in free calcium concentration in cells to produce NO. iNOS is known as calcium-independent NOS. Most of the resting cells do not have the expression of iNOS. The expression of iNOS in tissue and inflammatory cells is stimulated by exposure to LPS and pro-inflammatory cytokines. While nNOS and eNOS make transient NO production, iNOS produces a higher level of NO for a longer period.

NO is a reactive molecule with direct effects exerted by itself such as the production of cGMP as a result of interaction with soluble guanylate cyclase and indirect effects via ROS generated by the interaction between NO and oxygen or O₂⁻. The molecular mode of action of NO can be summarized in three categories. The first one is the regulation of enzyme activity as a result of its reaction with transition metals like iron, zinc, and copper which are found in the prosthetic groups of enzymes and other proteins. The chemical structure of NO serves it as an electron donor leading to

metal-nitrosyl complex generation. The presence of NO activates soluble guanylate cyclase by binding to the heme in this enzyme. The activation of soluble guanylate cyclase leads to the conversion of guanosine triphosphate to cGMP which is an essential signaling molecule within the cells. NO also suppresses mitochondrial respiration by inhibiting cytochrome c oxidase which is the last enzyme in the electron transport chain and plays role in adenosine triphosphate production in mitochondria. The second one is the consequence of its ability to involve in the S-nitrosylation reaction which modifies some proteins serving a function in regulatory mechanisms in the cells. NO interacts with oxygen in aqueous solutions and forms dinitrogen trioxide (N_2O_3). Nitrosonium ion and nitrite are readily generated from N_2O_3 . Nitrosonium ion plays role in nitrosylation of phenolics, thiols, and secondary amines. It has been discovered that several transcription factors, kinases, caspases, ion channels, and metabolic proteins are activated, inhibited, or desensitized via this reaction such as activation of $\text{p}21^{\text{ras}}$ by S-nitrosylation of Cys118 or inhibition of deoxyribonucleic acid (DNA) binding of NF- κ B by S-nitrosylation of p50 (Cys62). The final one is that NO causes the formation of strong oxidant agent peroxynitrite via the interaction with O_2^- leading to modification of nucleic acids, lipids, and proteins. This very rapid interaction between NO and O_2^- has a three times higher reaction rate constant than superoxide reduction by superoxide dismutase. Nitration of proteins, suppression of mitochondrial respiration, DNA damage, apoptosis and necrosis, and cell energy depletion are the consequences of higher levels of peroxynitrite production with a final result of cell or tissue damage. Increased levels of synthesis of both NO and superoxide have been observed in inflammatory conditions.

The role of NO in regulatory processes in inflammation such as regulation of vascular responses, signaling pathways, transcription factors, leukocyte migration and rolling, cytokine synthesis, apoptosis, and infection control has been revealed in several studies. Anti-inflammatory activities of NO synthesis inhibitors (predominantly selective iNOS inhibitors) have been demonstrated in a number of experimental models of inflammation like colitis and arthritis (42). Furthermore, NO is an essential inflammatory chemical in the respiratory tract. There is evidence for its modulatory effects on airway function and pro-inflammatory and immunomodulatory effects on pulmonary disease. In asthma, a rise in exhaled NO is frequently associated with eosinophilic inflammation of the airways and increased expression of corticosteroid-sensitive iNOS (43).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Evaluation of Protective Effects against Inflammation

- Griess reagent [1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 5% phosphoric acid]
- Sodium Nitrite (NaNO₂)
- 10% MTT
- Indomethacin
- LPS from *E. coli* (0111:B4, Sigma, USA)
- Centrifuge
- Medium (Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) 10%, 1% Penicillin-Streptomycin (Penstrep))
- Hemocytometer
- RAW 264.7 murine macrophage cells (ATCC, USA)
- 48 well-plate
- L-NAME
- Isopropanol
- 5% CO₂
- Incubator
- 50% hydroalcoholic (50% EtOH) dry extract of *H. helix* leaves (0.03, 0.06, 0.125, 0.25, 0.5, 1 mg/ml)
- Microplate reader (Multiskan Ascent, Finland)

3.1.2. Evaluation of Mutagenic and Antimutagenic Activities

- *Salmonella* tester TA98 and TA100 strains (STDisc™, Molttox®)
- S9 enzyme (Molttox®)
- 50% hydroalcoholic (50% EtOH) dry extract of *H. helix* leaves (1, 10, 100, 1000, 5000 µg/ml)
- Autoclave
- Petri dishes
- Glass tubes
- Erlen
- Falcon tube

- Water bath
- Vortex
- Broth
- Inoculating loop
- Beaker
- Aluminum foil
- Top Agar (Agar, dH₂O, NaCl)
- Agar (Agar, glucose 40%, dH₂O, Vogel-Bonner (VB) medium E)
- VB medium E (50X) (Warm dH₂O, magnesium sulfate, citric acid monohydrate, potassium phosphate (dibasic), sodium ammonium phosphate)
- Histidine/Biotin 0.5 mM (dH₂O, D-Biotin, L-Histidine.HCl)
- Dimethyl Sulfoxide (DMSO)
- Incubator
- Mixing incubator
- Phosphate Buffer
- Sodium azide (SA)
- 2-aminofluorene

3.1.3. *In silico* Evaluation

- Computer (Intel core i7 3.20 GHz, 16 GB RAM 64-bit)
- Danish (Q)SAR Database (66)
- pkCSM web server (67)
- OCHEM web platform (68)
- ProTox-II web service (69)
- T.E.S.T. software (70)
- PASS online freeware (71)
- VEGA (Q)SAR platform (72)

3.2. Methods

3.2.1. Preparation of *Hedera helix* Extract

H. helix was cultivated on Medicinal and Endemic Plants Education and Research Farm in the Faculty of Agriculture at Selçuk University in Konya, Turkey at an altitude of around 1020 m and the leaves were collected in June 2019. The specimen of *H. helix* L. was stored at Medicinal and Endemic Plants Herbarium of Selçuk

University with the herbarium number of SÜTEB 201 after the identification of the plant by a scientific expert Prof. Yüksel Kan.

3.2.2. Evaluation of Protective Effects against Inflammation

The protective effects of *H. helix* extracts against LPS-induced inflammation were evaluated by two different methods. At first, RAW 264.7 murine macrophage cell culture was prepared. Then, nitrite (a stable NO metabolite) levels were measured. Moreover, cell viability was examined via MTT assay.

3.2.2.1. Preparation of Cell Culture

For the preparation of the cell culture, RAW 264.7 murine macrophage cells were plated with a density of 10^6 cells per mL in a 48 well-plate in DMEM, 10% FBS, and 1% streptomycin and penicillin and incubated at 37 °C in 5% CO₂ for 24 hours. Then, plated cells were treated with six different concentrations of *H. helix* extracts (0.03, 0.06, 0.125, 0.25, 0.5, 1 mg/ml) for 2 hours and then supplemented with 1 µg/mL of LPS from *E. coli* for additional 22 hours.

3.2.2.2. Measurement of Nitrite Levels

To measure nitrite levels, Griess reagent was added to the culture supernatant (50 µL) and incubated at room temperature for 10 minutes. The absorbance of the mixture was measured at 540 nm via a microplate reader and a NaNO₂ standard curve was generated to calculate the amount of nitrite in the test samples. Positive controls were 100 µM of indomethacin and 100 µM of L-NAME and medium was used as the negative control.

3.2.2.3. Measurement of Cell Viability

For the MTT assay, each well was supplemented with 100 µL of MTT and incubated for 2 hours at 37 °C. After the incubation period, all medium was discarded from plates and 200 µl of isopropanol was added to all wells. An ultraviolet-visible spectrophotometric plate reader was used to measure the absorbance of the blue formazan at 570 nm. Viability was defined as absorbance of treated cells/absorbance of untreated cells and expressed as a percentage. All measurements were done in triplicates.

3.2.3. Evaluation of Mutagenic and Antimutagenic Activities

To examine mutagenic and antimutagenic activities of different concentrations of *H. helix* extract, three steps approach was used which started with the preparation of

all of the media and solutions accordingly, followed by creating a bacterial culture of *Salmonella* tester TA98 and TA100 strains and carrying out Ames test at the final step.

The preparation method and the content of the media and solutions used in mutagenic and antimutagenic activity assay are shown in Table 3-1, Table 3-2, Table 3-3, Table 3-4, and Table 3-5.

Salmonella tester TA98 and TA100 strains both with and without S9 fraction were used for mutagenicity and antimutagenicity tests. Mutagenicity testing was carried out according to Maron and Ames (73). *H. helix* extract was dissolved in DMSO with concentrations of 1, 10, 100, 1000, and 5000 µg/plate and used for both assays.

To give a short summary of the assays, top agar with histidine, phosphate buffer, *H. helix* extract, and bacterial strains are mixed in the glass tubes for each concentration of *H. helix*, then poured into the petri dishes and incubated at 37 °C for 48 h in dark. DMSO was used as the negative control. To conduct the experiments with S9, the S9 was also added without additional phosphate buffer. In antimutagenicity experiment, a positive mutagen was used using the same process with mutagenicity assays. SA and 2-aminofluorene were positive mutagens for experiments with S9 and without S9, respectively. Three petri dishes were used for each concentration for each set of experiments. After incubation, bacterial colonies in the petri dishes were counted.

The mutagenic activity was evaluated according to mutagenic ratios which were calculated by dividing the number of revertant colonies on sample treated plates by the number of revertant colonies in negative control plates. A mutagenic ratio lower than 2 represented the non-mutagenic activity of the tested compound. To interpret results for antimutagenic activity, the number of revertant colonies (containing the mutagen without *H. helix* extract) was defined as 100% which represents inhibition of 0% and the following formula (Eq. 1) was used to calculate the percentage of inhibition:

$$\frac{\# \text{ of histidine revertants (without sample)} - \# \text{ of histidine revertants (with sample)}}{\# \text{ of histidine revertants (without sample)} - \text{Spontaneous revertants}} \times 100 \quad (1)$$

The antimutagenic effect was classified as weak, moderate, and strong when the inhibitory effect was less than 25%, 25–40%, and more than 40%, respectively. A weak effect was not interpreted as a positive result.

Table 3-1 Preparation of VB medium E (50X)

Chemical Name	Amount	Preparation
Warm dH ₂ O (45 °C)	67 ml	All of the chemicals are mixed
MgSO ₄ , 7H ₂ O	1 g	
Citric Acid Monohydrate	10 g	
K ₂ HPO ₄ , dibasic (anhydrous)	50 g	
NaH ₂ PO ₄ , 4H ₂ O	17.5 g	

Table 3-2 Preparation of top agar (100 ml)

Chemical Name	Amount	Preparation
dH ₂ O	100 ml	All of the chemicals are mixed in a beaker and autoclaved for ~ 1 hour
Agar	0.5 g	
NaCl	0.5 g	

Table 3-3 Preparation of 100 ml of 0.5 mM histidine/biotin

Chemical Name	Amount	Preparation
dH ₂ O	100 ml	All of the chemicals are mixed. The lid is covered by aluminum 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	

Table 3-4 Preparation of S9 mix

Chemical Name	Amount	Preparation
dH ₂ O (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	

Table 3-5 Preparation of 1L of agar

Chemical Name	Amount	Preparation
dH ₂ O	930 ml	dH ₂ O 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	

3.2.4. *In silico* Evaluation

Compounds identified in *H. helix* extract and listed in Table 2-1 were evaluated in this study in terms of genotoxicity or mutagenicity endpoints and anti-inflammatory properties. All compounds isolated from the leaves of the plant so far were included for evaluation in this study. The prediction was carried out using SMILES strings of the compounds. Hederasaponin A, E, G, H, and I, 3-sulfates of oleanolic acid and echinocystic acid, 3-sulfate of 28-O- β -gentiobiosyloleanate, 11,12-dehydrofalcarinol and cyanidin 3-monoside were excluded from the analysis because of non-identified molecular structure. The included compounds are listed in Table 3-8 including compound number, compound name, CAS and EC number, and SMILES. The SMILES strings were obtained via PubChem (<https://pubchem.ncbi.nlm.nih.gov/>).

The used *in silico* freeware/databases in this study were Danish (Q)SAR Database, pkCSM and OCHEM web platform, ProTox-II web service and PASS online freeware. All endpoints under genotoxicity were selected as models in Danish (Q)SAR Database, mutagenicity in ProTox-II, the Ames test in OCHEM and pkCSM, and PASS online were also used to evaluate anti-inflammatory and antimutagenic effects as biological activity of *Hedera helix*. When there was inconsistency between the predictions obtained for the Ames test, genotoxicity, and mutagenicity, an additional computational platform, VEGA (Q)SAR, was used to provide additional data for the relevant compound.

The Danish (Q)SAR Database provides predictions for (Q)SAR screening profiles with >30 Technical University of Denmark models developed for more than 600,000 chemical compounds covering many endpoints of toxicity, environmental toxicity and fate, physicochemical properties, and pharmacokinetics which have been developed by the National Food Institute, Technical University of Denmark with the Danish Environmental Protection Agency and the Nordic Council of Ministers (74). The database was accessed via [https://\(Q\)SARmodels.food.dtu.dk/runmodel/index.html](https://(Q)SARmodels.food.dtu.dk/runmodel/index.html). The endpoints of the Ames test, other *in vitro* endpoints, and other *in vivo* endpoints were selected in the genotoxicity/cancer tab. The SMILES strings of molecules were used to predict results. The Ames test predictions and other *in vitro/in vivo* endpoints were included in Table 4-1 and Table 4-2, respectively. The predictions of Inc_Out, Pos_Out, and Neg_Out were excluded and presented as N/A. Experimental results were also recorded if available. The predictions with the symbol * showed the experimental results in Table 4-2.

The pkCSM is a web server using graph-based signatures encoding repeated distance motifs between atoms to provide a platform for the evaluation of toxicity and pharmacokinetics of molecules (75). This web server was accessed via <http://biosig.unimelb.edu.au/pkcsml/prediction>. The SMILES strings of molecules were provided and “toxicity” was selected as the prediction mode to predict Ames test. Predicted values were given as Yes or No in the web server representing a positive or negative result in the Ames test, respectively. The predictions were included in Table 4-1.

The OCHEM is a web-based platform for researching a full (Q)SAR/QSPR cycle containing experimentally estimated biological and physicochemical aspects of molecules and operates on the basis of the wiki principle (76). It was accessed via <https://ochem.eu/home/show.do>. The SMILES strings of molecules were loaded and the Ames test was chosen as the prediction model. Results were provided as Active or Inactive in this freeware representing a positive or negative result in the Ames test, respectively. The “out of ad (this prediction is out of the model’s applicability domain)” results were indicated as N/A. All predictions were included in Table 4-1.

ProTox-II is a virtual lab providing predictions for 33 different toxicity endpoints via a free web service incorporating similarities between molecules and machine-learning models for many toxicity-related endpoints (77). To provide the Ames test prediction results from the ProTox-II web service, it was accessed via https://tox-new.charite.de/prottox_II/index.php?site=compound_input. The SMILES strings of molecules were submitted and mutagenicity was selected as the prediction model. Prediction results were given as Active or Inactive in this web service representing a positive or negative result in the Ames test, respectively. The results with a probability value below 0.7 were excluded and represented as N/A. All predictions were included in Table 4-1.

The T.E.S.T. software is developed by The United States Environmental Protection Agency to estimate toxicity using one of the advanced (Q)SAR methodologies (78). For prediction of the Ames test results in the T.E.S.T. software, the program was downloaded via the website of <https://www.epa.gov/chemical-research/toxicity-estimation-software-tool-test>. The program was used in the batch mode and the SMILES strings of molecules were loaded. In the calculation options tab, mutagenicity and consensus were selected as the endpoint and method, respectively.

Experimental results were also recorded if available. All predictions were included in Table 4-1.

The PASS online freeware allows the users to obtain predictions of over 4000 different biological activities such as mode of action, toxic/adverse effects, interaction with enzymes and transporters, effect on gene expression, etc. The predictions are created based on the structural formula of compounds (79). It was accessed via <http://www.way2drug.com/passonline/>. The SMILES strings of the compounds were used for prediction. Predictions for “Antiinflammatory”, “Antimutagenic”, “Antioxidant”, “Nitric oxide antagonist”, “Nitric-oxide synthase inhibitor” and “Nitrite reductase (NO-forming) inhibitor” activities were taken into account. These predicted activities were based on the evaluation of 5983, 34, 843, 146, 1075, and 3 active compounds, respectively. All predictions were included in Table 4-3. Only Pa (probability "to be active") levels higher than 0.5 were recorded and the levels lower than 0.5 were presented as N/A. If no prediction result was found for a specific activity, it was represented as "-" in the table.

VEGA (Q)SAR *in silico* platform is used for making predictions for toxicity, ecotoxicity, environmental toxicity, and physical and chemical properties of compounds providing many (Q)SAR models (72). It was downloaded from the website [https://www.vegahub.eu/download/vega-\(Q\)SAR-download/](https://www.vegahub.eu/download/vega-(Q)SAR-download/). The SMILES strings of the compounds were used for prediction. Mutagenicity (Ames test), chromosomal aberration, and micronucleus assay were chosen as endpoints for prediction. The results were provided in a .pdf report for each compound.

Table 3-6 Compound list for *in silico* analysis

Compound Number	Compound Name	CAS Number	EC Number	SMILES
1	Hederasaponin B	36284-77-2	-	<chem>CC1C(C(C(C(O1)OC2C(OC(C(C2O)O)OCC3C(C(C(C(O3)OC(=O)C45CCC(CC4C6=CCC7C8(CCC(C(C8CCC7(C6(CC5)C)C)(C)C)OC9C(C(C(CO9)O)O)OC1C(C(C(C(O1)C)O)O)O)C)(C)C)O)O)O)CO)O)O</chem>
2	Hederacoside C	14216-03-6	238-072-2	<chem>C[C@H]1[C@@H]([C@H]([C@H]([C@@H](O1)O[C@@H]2[C@H](O[C@H]([C@@H]([C@H]2O)O)OC[C@@H]3[C@H]([C@@H]([C@H]([C@@H](O3)OC(=O)[C@@]45CC[C@@]6(C(=CC[C@H]7[C@]6(CC[C@@H]8[C@@]7(CC[C@@H]([C@@]8(C)C)O)[C@H]9[C@@H]([C@H]([C@H](CO9)O)O)O[C@H]1[C@@H]([C@@H]([C@H]([C@@H](O1)C)O)O)O)C)[C@@H]4CC(CC5)(C)C)O)O)O)CO)O)O</chem>
3	Hederasaponin D	760961-03-3	-	<chem>CC1C(C(C(C(O1)OC2C(OC(C(C2O)O)OCC3C(C(C(C(O3)OC(=O)C45CCC(CC4C6=CCC7C8(CCC(C(C8CCC7(C6(CC5)C)C)(C)C)O)OC9C(C(C(CO9)O)O)O)C)(C)C)O)O)O)CO)O)O</chem>
4	Hederasaponin F	-	-	<chem>CC1C(C(C(C(O1)OC2C(OC(C(C2O)O)OCC3C(C(C(C(O3)OC(=O)C45CCC(CC4C6=CCC7C8(CCC(C(C8CCC7(C6(CC5)C)C)(C)C)OS(=O)(=O)O)C)(C)C)O)O)O)CO)O)O</chem>
5	α -hederin	27013-91-8	248-166-5	<chem>CC1C(C(C(C(O1)OC2C(C(COC2OC3CCC4(C(C3(C)CO)CCC5(C4CC=C6C5(CCC7(C6CC(CC7)(C)C)C(=O)O)C)C)O)O)O)O)O</chem>
6	Hederagenin 3-O- β -glucoside	39776-12-0	-	<chem>CC1(CCC2(CCC3(C(=CCC4C3(CCC5C4(CCC(C5(C)CO)OC6C(C(C(C(O6)CO)O)O)O)C)C)C2C1)C)C(=O)O)C</chem>
7	α -amyrin	638-95-9	211-352-1	<chem>CC1CCC2(CCC3(C(=CCC4C3(CCC5C4(CCC(C5(C)C)O)C)C)C2C1)C)C</chem>
8	β -amyrin	559-70-6	209-204-6	<chem>CC1(CCC2(CCC3(C(=CCC4C3(CCC5C4(CCC(C5(C)C)O)C)C)C2C1)C)C</chem>
9	Quercetin	117-39-5	204-187-1	<chem>C1=CC(=C(C=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O)O</chem>
10	Kaempferol	520-18-3	208-287-6	<chem>C1=CC(=CC=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O</chem>

Compound Number	Compound Name	CAS Number	EC Number	SMILES
11	Rutin	153-18-4	215-672-2	<chem>CC1C(C(C(C(O1)OCC2C(C(C(C(O2)OC3=C(OC4=CC(=CC(=C4C3=O)O)O)C5=CC(=C(C=C5)O)O)O)O)O)O)O</chem>
12	Isoquercitrin	482-35-9	640-533-1	<chem>C1=CC(=C(C=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)OC4C(C(C(C(O4)CO)O)O)O)O)O</chem>
13	Astragalin	480-10-4	610-400-2	<chem>C1=CC(=CC=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)OC4C(C(C(C(O4)CO)O)O)O)O</chem>
14	Nicotiflorin	17650-84-9	685-716-7	<chem>CC1C(C(C(C(O1)OCC2C(C(C(C(O2)OC3=C(OC4=CC(=CC(=C4C3=O)O)O)C5=CC=C(C=C5)O)O)O)O)O)O</chem>
15	Scopolin	531-44-2	-	<chem>COC1=C(C=C2C(=C1)C=CC(=O)O2)O[C@H]3[C@@H]([C@H]([C@@H]([C@H](O3)CO)O)O)O</chem>
16	Falcarinon	4117-11-7	-	<chem>CCCCCCCCC=CCC#CC#CC(=O)C=C</chem>
17	Falcarinol	21852-80-2	-	<chem>CCCCCCCCC=CCC#CC#CC(C=C)O</chem>
18	Caffeic acid	331-39-5	206-361-2	<chem>C1=CC(=C(C=C1C=CC(=O)O)O)O</chem>
19	Chlorogenic acid	202650-88-2	206-325-6	<chem>C1C(C(C(CC1(C(=O)O)O)OC(=O)C=CC2=CC(=C(C=C2)O)O)O)O</chem>
20	Neochlorogenic acid	906-33-2	212-997-1	<chem>C1C(C(C(CC1(C(=O)O)O)OC(=O)C=CC2=CC(=C(C=C2)O)O)O)O</chem>
21	3,5-O-dicaffeoyl-quinic acid	89919-62-0	815-082-0	<chem>C1C(C(C(CC1(C(=O)O)O)OC(=O)C=CC2=CC(=C(C=C2)O)O)O)OC(=O)C=CC3=CC(=C(C=C3)O)O</chem>
22	4,5-O-dicaffeoyl-quinic acid	57378-72-0	-	<chem>C1C(C(C(CC1(C(=O)O)O)OC(=O)C=CC2=CC(=C(C=C2)O)O)OC(=O)C=CC3=CC(=C(C=C3)O)O)O</chem>
23	Rosmarinic [(R)-(+)-enantiomer] acid	20283-92-5	606-487-1	<chem>C1=CC(=C(C=C1CC(C(=O)O)OC(=O)C=CC2=CC(=C(C=C2)O)O)O)O</chem>
24	Protocatechuic acid	99-50-3	202-760-0	<chem>C1=CC(=C(C=C1C(=O)O)O)O</chem>
25	<i>p</i> -coumaric acid	501-98-4	231-000-0	<chem>C1=CC(=CC=C1C=CC(=O)O)O</chem>
26	Palmitic acid	57-10-3	200-312-9	<chem>CCCCCCCCCCCCCCCC(=O)O</chem>
27	Cholesterol	57-88-5	200-353-2	<chem>CC(C)CCCC(C)C1CCC2C1(CCC3C2CC=C4C3(CCC(C4)O)C)C</chem>
28	Campesterol	474-62-4	207-484-4	<chem>CC(C)C(C)CCC(C)C1CCC2C1(CCC3C2CC=C4C3(CCC(C4)O)C)C</chem>

Compound Number	Compound Name	CAS Number	EC Number	SMILES
29	Stigmasterol	83-48-7	201-482-7	<chem>CCC(C=CC(C)C1CCC2C1(CCC3C2CC=C4C3(CCC(C4)O)C)C)C(C)C</chem>
30	β -Sitosterol	83-46-5	201-480-6	<chem>CCC(CCC(C)C1CCC2C1(CCC3C2CC=C4C3(CCC(C4)O)C)C)C(C)C</chem>
31	α -spinasterol	481-18-5	-	<chem>CCC(C=CC(C)C1CCC2C1(CCC3C2=CCC4C3(CCC(C4)O)C)C)C(C)C</chem>
32	5 α -stigma-7-en-3 β -ol	521-03-9	-	<chem>CCC(CCC(C)C1CCC2C1(CCC3C2=CCC4C3(CCC(C4)O)C)C)C(C)C</chem>
33	Emetin	483-18-1	207-592-1	<chem>CCC1CN2CCC3=CC(=C(C=C3C2CC1CC4C5=CC(=C(C=C5CCN4)OC)OC)OC)OC</chem>
34	Germacrene B	15423-57-1	-	<chem>CC1=CCCC(=CCC(=C(C)C)CC1)C</chem>
35	β -elemene	33880-83-0	251-713-0	<chem>CC(=C)C1CCC(C(C1)C(=C)C)(C)C=C</chem>
36	γ -elemene	-	-	<chem>CC(=C1CCC(C(C1)C(=C)C)(C)C=C)C</chem>
37	Methylethyl ketone	78-93-3	201-159-0	<chem>CCC(=O)C</chem>
38	Methylisobutyl ketone	108-10-1	203-550-1	<chem>CC(C)CC(=O)C</chem>
39	Trans-2-hexanal	6728-26-3	229-778-1	<chem>CCCC=CC=O</chem>
40	Trans-2-hexanol	928-95-0	218-972-1	<chem>CCCC=CCO</chem>
41	Germacrene D	23986-74-5	639-624-9	<chem>CC1=CCCC(=C)C=CC(CC1)C(C)C</chem>
42	β -caryophyllene	87-44-5	201-746-1	<chem>CC1=CCCC(=C)C2CC(C2CC1)(C)C</chem>
43	Sabinene	3387-41-5	222-212-4	<chem>CC(C)C12CCC(=C)C1C2</chem>
44	α -pinene	80-56-8	201-291-9	<chem>CC1=CCC2CC1C2(C)C</chem>
45	β -pinene	18172-67-3	242-060-2	<chem>CC1(C2CCC(=C)C1C2)C</chem>
46	Limonene	138-86-3	205-341-0	<chem>CC1=CCC(CC1)C(=C)C</chem>
47	Furfurol	98-00-0	202-626-1	<chem>C1=COC(=C1)CO</chem>
48	Asparagine	70-47-3	200-735-9	<chem>C(C(C(=O)O)N)C(=O)N</chem>
49	Glycine	56-40-6	200-272-2	<chem>C(C(=O)O)N</chem>
50	Proline	4305-67-3	205-702-2	<chem>C1CC(NC1)C(=O)O</chem>
51	Tyrosine	60-18-4	200-460-4	<chem>C1=CC(=CC=C1CC(C(=O)O)N)O</chem>

Compound Number	Compound Name	CAS Number	EC Number	SMILES
52	Valine	72-18-4	200-773-6	<chem>CC(C)C(C(=O)O)N</chem>
53	Leucine	61-90-5	200-522-0	<chem>CC(C)CC(C(=O)O)N</chem>
54	Phenilalanine	63-91-2	200-568-1	<chem>C1=CC=C(C=C1)CC(C(=O)O)N</chem>
55	Vitamin E	59-02-9	233-466-0	<chem>CC1=C(C2=C(CCC(O2)(C)CCCC(C)CCCC(C)CCCC(C)C)C(=C1O)C)C</chem>
56	Vitamin C	50-81-7	200-066-2	<chem>C(C(C1C(=C(C(=O)O1)O)O)O)O</chem>
57	Pro-vitamin A	7235-40-7	230-636-6	<chem>CC1=C(C(CCC1)(C)C)C=CC(=CC=CC(=CC=CC=C(C)C=CC=C(C)C=CC2=C(CCCC2(C)C)C)C)C</chem>
58	Hamamelitol	-	-	<chem>C(C(C(C(CO)(CO)O)O)O)O</chem>

4. RESULTS

4.1. Protective Effects against Inflammation Assay Results

Protective effects against inflammation of different concentrations of the extract were tested by measuring nitrite levels and cell viability in LPS-induced RAW 264.7 macrophage cells. As seen in Figure 4-1, all concentrations of *H. helix* extract significantly reduced the nitrite levels in the macrophages compared to M+LPS. All concentrations of the extract showed similar effects in terms of nitrite level reduction when compared with indomethacin and NOS inhibitor L-NAME. Nitrite inhibition rates of different concentrations of the extract were between $25.13 \pm 1.54\%$ to $43.61 \pm 1.63\%$, while the inhibition percentages with indomethacin and L-NAME were $44.90 \pm 2.29\%$ and $42.41 \pm 0.80\%$, respectively and shown in Table 4-1.

Table 4-1 also includes the cell viability rates seen with all concentrations of the extract. Medium was suggested as the control with a cell viability rate of 100%. There was a concentration-dependent decrease with all concentrations of the extract compared to the control. The highest cell viability was demonstrated with 0.03 mg/ml extract with a rate of $99.49 \pm 1.55\%$ which was very similar to the control, while 1 mg/ml of the extract caused the lowest cell viability of $74.63 \pm 2.52\%$.

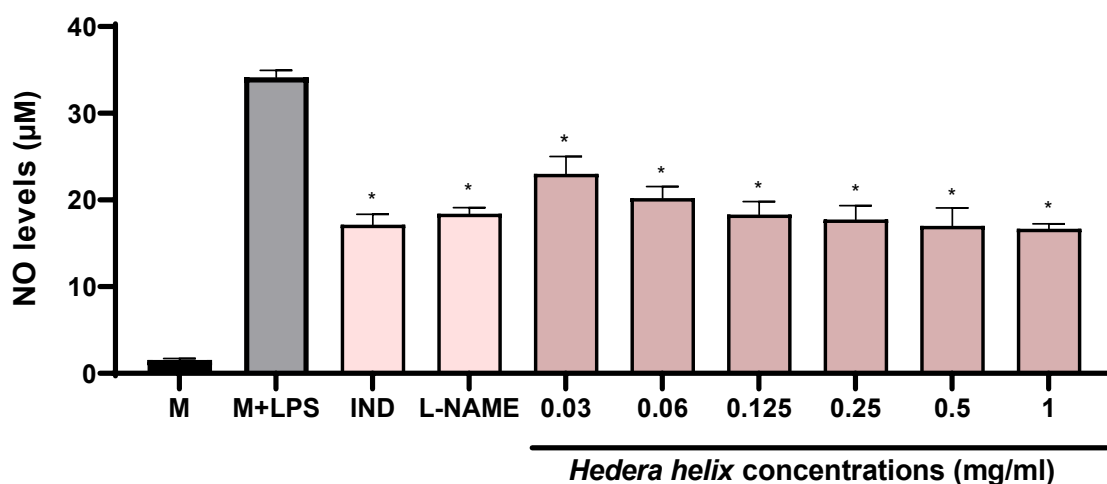


Figure 4-1 The nitrite levels of different concentrations of *Hedera helix* extract, positive controls as indomethacin and L-NAME and negative control as medium

The significant difference was defined with * $p < 0.0001$ vs. M+LPS.

Table 4-1 The cell viability and nitrite inhibition percentages of the study groups

Groups	Cell viability %	Nitrite inhibition %
Control (Medium)	100 ± 1	No inhibition
<i>Hedera helix</i> 0.03 mg/ml	99.49 ± 1.55	25.13 ± 1.54
<i>Hedera helix</i> 0.06 mg/ml	95.43 ± 1.79	35.20 ± 1.55
<i>Hedera helix</i> 0.125 mg/ml	93.75 ± 1.72	40.28 ± 1.57
<i>Hedera helix</i> 0.25 mg/ml	88.73 ± 1.53	42.50 ± 1.26
<i>Hedera helix</i> 0.5 mg/ml	84.19 ± 1.53	42.87 ± 0.62
<i>Hedera helix</i> 1 mg/ml	74.63 ± 2.52	43.61 ± 1.63
Indomethacin 100 µM	-	44.90 ± 2.29
L-Name 100 µM	-	42.41 ± 0.80

4.2. Mutagenicity and Antimutagenicity Assay Results

In this study, mutagenic and antimutagenic activities of the extract at concentrations of 1, 10, 100, 1000, and 5000 µg/plate were analyzed in *Salmonella* strains TA98 and TA100 with and without metabolic activation (S9).

The results of the mutagenic activity assay including the number of revertants and mutagenic index were shown in Table 4-2. There was no significant increase in the number of revertants with any of the tested concentrations of the extract in the TA98 and TA100 strains with/without S9. In both TA98 and TA100 strains, the mutagenic ratios calculated for all tested concentrations of the extract were all below 2 representing the non-mutagenic activity of the tested compound.

Table 4-3 and Figure 4-2 represent the results of the antimutagenic activity assay with the inhibition rates. The revertant colony numbers significantly decreased with the concentration of 5000 µg/plate of *H. helix* extract compared to positive control in the strains TA98 and TA100 with S9, respectively. No significant change was demonstrated with other concentrations of the extract compared with the positive control. *H. helix* extract showed strong antimutagenic activity against indirect mutagen only at the concentration of 5000 µg/plate with an inhibition rate of 52% in the experiment conducted in the TA100 strain with S9. While 1000 µg/plate of the extract exhibited moderate antimutagenic activity with an inhibition rate of 25% in the presence of S9 in TA100 strain, 1000 and 5000 µg/plate of the extract had moderate activities (inhibition rates of 28% and 27% respectively) in the TA98 strain without S9. On the other hand, there was no antimutagenic activity with the concentrations of 1000 and 5000 µg/plate in the TA98 strain with S9 and TA100 strain without S9. The extract concentrations of

1, 10, and 100 µg/plate showed weak antimutagenicity in both strains with and without S9.

Table 4-2 Mutagenic index of *Hedera helix* extract in *Salmonella* strains TA98 and TA100 with and without S9

Without S9	TA98		TA100	
	Number of revertant/plate	Mutagenic Index (MI)	Number of revertant/plate	Mutagenic Index (MI)
Negative Control	25.00 ± 4.24		131.00 ± 1.41	
<i>Hedera helix</i> (µg/plate)				
1	24.00 ± 8.48	0.96	131.50 ± 4.95	1.00
10	20.00 ± 4.24	0.80	142.50 ± 10.61	1.09
100	25.50 ± 10.61	1.02	129.00 ± 8.48	0.98
1000	19.50 ± 9.19	0.78	131.00 ± 9.90	1.00
5000	23.50 ± 2.12	0.94	112.50 ± 17.68	0.86
With S9				
Negative Control	36.00 ± 2.83		112.00 ± 7.07	
<i>Hedera helix</i> (µg/plate)				
1	38.00 ± 1.41	1.06	132.50 ± 0.71	1.18
10	33.50 ± 3.54	0.93	118.50 ± 7.78	1.06
100	40.00 ± 7.07	1.11	126.50 ± 4.95	1.13
1000	47.00 ± 4.24	1.31	124.00 ± 19.80	1.11
5000	39.50 ± 9.19	1.10	129.00 ± 5.66	1.15

Table 4-3 Antimutagenic activity of *Hedera helix* extract in *Salmonella* strains TA98 and TA100 with and without S9 against a positive mutagen

Without S9	TA98		TA100	
	Number of revertant/plate	Inhibition (%)	Number of revertant/plate	Inhibition (%)
Negative Control	20.00 ± 7.07		119.00 ± 2.83	
Positive Control	1018.00 ± 144.20		405.00 ± 5.66	
<i>Hedera helix</i> (µg/plate)				
1	822.50 ± 159.10	19	391.00 ± 16.97	5
10	883.00 ± 39.60	13	392.00 ± 2.83	4
100	818.50 ± 44.55	20	381.50 ± 4.95	8
1000	740.00 ± 38.18	28	392.00 ± 2.83	4
5000	746.50 ± 9.19	27	388.00 ± 8.48	6
With S9				
Negative Control	29.50 ± 3.54		161.00 ± 19.80	
Positive Control	1029.00 ± 24.75		604.5 ± 61.52	
<i>Hedera helix</i> (µg/plate)				
1	905.00 ± 14.14	12	597.50 ± 4.95	1
10	943.50 ± 72.83	8	577.50 ± 9.19	6
100	1017.00 ± 4.95	1	572.50 ± 38.89	7
1000	968.00 ± 4.24	6	492.50 ± 60.10	25
5000	891.00 ± 72.12*	14	373.50 ± 3.54**	52

*p<0.03, **p<0.002 vs. positive control.

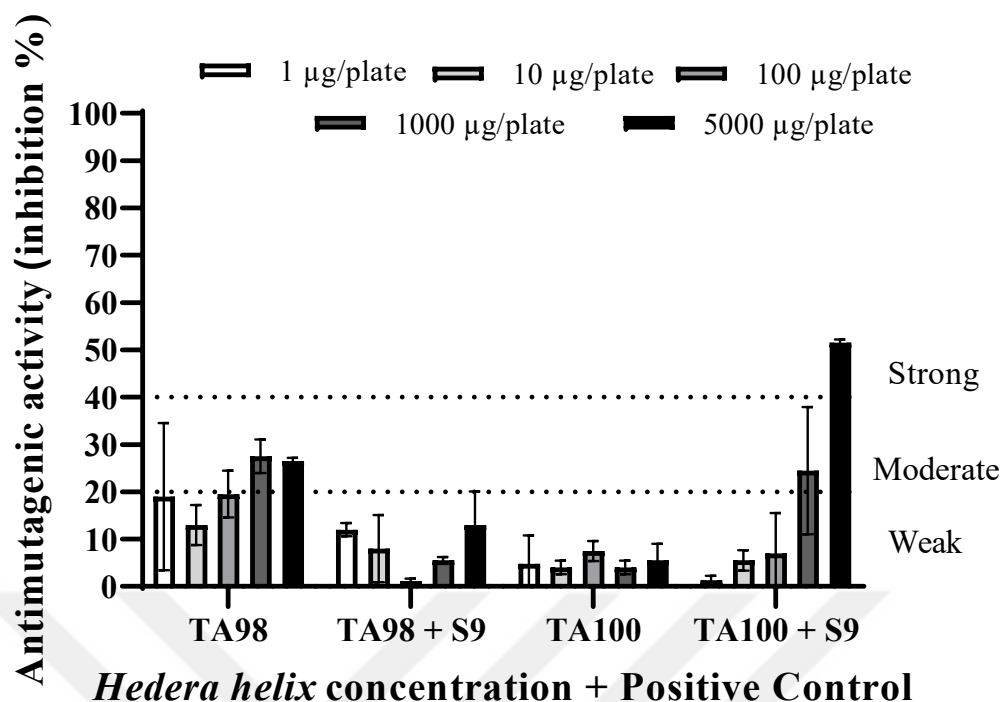


Figure 4-2 Antimutagenic activity (inhibition %) of *Hedera helix* extract against a positive mutagen

4.3. *In Silico* Analysis Results

As listed in Table 4-4, 47 of 58 compounds were found to have negative predictions for the Ames test excluding the results presented as N/A. Two of the five computational toxicology freeware provided predictions with additional positive experimental data indicating quercetin, kaempferol, and pro-vitamin A are potentially mutagenic. Additionally, predictions yielded mostly positive Ames results for trans-2-hexanal inconsistent with the experimental data obtained from Danish (Q)SAR Database and T.E.S.T.

We ran additional predictions for rutin, isoquercitrin, astragalin, nicotiflorin, emetin, furfural, and trans-2-hexanol for which there were inconsistent findings in the five freewares listed below. In the VEGA (Q)SAR platform, rutin, nicotiflorin, and trans-2-hexanol were predicted to be non-mutagenic without any structural alert with reliable results, emetin, and furfural were non-mutagenic without any structural alert with good reliable results and isoquercitrin and astragalin were mutagenic without any structural alert with moderately reliable results.

Table 4-4 The Ames test predictions and experimental data (if available) from different platforms

Compound Number	Danish (Q)SAR	pkCSM	OCHEM	ProTox-II	T.E.S.T.	Experimental
1	Negative	Negative	Negative	Negative	Negative	-
2	Negative	Negative	Negative	Couldn't be screened	Negative	-
3	Negative	Negative	Negative	Negative	Negative	-
4	Negative	Negative	Negative	N/A	Negative	-
5	Negative	Negative	Negative	Negative	Negative	-
6	Negative	Negative	Negative	Negative	Negative	-
7	Negative	Negative	Negative	Negative	Negative	-
8	Negative	Negative	Negative	Negative	Negative	-
9	Negative	Negative	Positive	N/A	Positive	Positive ^{a, b}
10	Negative	Negative	Positive	N/A	Positive	Positive ^{a, b}
11	Negative	Negative	Positive	Negative	Negative	Negative ^a
12	Negative	Negative	Positive	Negative	Positive	-
13	Negative	Negative	Positive	Negative	Negative	-
14	Negative	Negative	Positive	Negative	Negative	-
15	N/A	Negative	Negative	N/A	Negative	-

Compound Number	Danish (Q)SAR	pkCSM	OCHEM	ProTox-II	T.E.S.T.	Experimental
16	Negative	Negative	N/A	Negative	Negative	-
17	Negative	Negative	Negative	Negative	Negative	-
18	Negative	Negative	Negative	Negative	Negative	Negative ^b
19	Negative	Negative	Negative	Negative	Negative	Negative ^{a, b}
20	Negative	Negative	Negative	Negative	Negative	Negative ^{a, b}
21	Negative	Negative	Negative	Negative	Negative	-
22	Negative	Negative	Negative	Negative	Negative	-
23	Negative	Negative	Negative	Negative	Negative	-
24	Negative	Negative	Negative	Negative	Negative	-
25	Negative	Negative	Negative	Negative	Negative	-
26	Negative	Negative	Negative	Negative	Negative	Negative ^b
27	Negative	Negative	Negative	Negative	Negative	-
28	Negative	Negative	Negative	Negative	Negative	Negative ^b
29	Negative	Negative	Negative	Negative	Negative	-
30	Negative	Negative	Negative	Negative	Negative	Negative ^b
31	Negative	Negative	Negative	Negative	Negative	-
32	Negative	Negative	Negative	Negative	Negative	Negative ^b

Compound Number	Danish (Q)SAR	pkCSM	OCHEM	ProTox-II	T.E.S.T.	Experimental
33	Negative	Negative	Negative	Negative	Positive	-
34	N/A	Negative	Negative	Negative	N/A	-
35	Negative	Negative	Negative	Negative	N/A	-
36	Negative	Negative	Negative	Negative	N/A	-
37	Negative	Negative	Negative	Negative	Negative	Negative ^{a, b}
38	Negative	Negative	Negative	Negative	Negative	Negative ^{a, b}
39	N/A	Positive	Negative	Positive	Positive	Positive ^{a, b}
40	Negative	Negative	Negative	Negative	Positive	-
41	Negative	Negative	Negative	Negative	N/A	-
42	Negative	Negative	Negative	Negative	Negative	Negative ^{a, b}
43	Negative	Negative	Negative	Negative	N/A	-
44	Negative	Negative	Negative	Negative	Negative	Negative ^{a, b}
45	Negative	Negative	Negative	Negative	Negative	Negative ^{a, b}
46	Negative	Negative	Negative	Negative	N/A	Negative ^{a, b}
47	N/A	Negative	Negative	Negative	Positive	Negative ^{a, b}
48	Negative	Negative	Negative	Negative	Negative	-
49	N/A	Negative	Negative	Negative	Negative	Negative ^{a, b}

Compound Number	Danish (Q)SAR	pkCSM	OCHEM	ProTox-II	T.E.S.T.	Experimental
50	Negative	Negative	Negative	Negative	Negative	-
51	Negative	Negative	Negative	Negative	Negative	Negative ^b
52	Negative	Negative	Negative	Negative	Negative	Negative ^b
53	Negative	Negative	Negative	Negative	Negative	-
54	Negative	Negative	Negative	Negative	Negative	Negative ^{a, b}
55	Negative	Negative	Negative	Negative	Negative	Negative ^{a, b}
56	Negative	Negative	Negative	Negative	Negative	Negative ^a
57	N/A	Positive	Negative	Positive	Negative	Positive ^b
58	Negative	Negative	Negative	Negative	Negative	-

^aDanish (Q)SAR, ^bT.E.S.T.

As listed in Table 4-5, 28 of 58 compounds were found to have negative predictions for other *in vivo/in vitro* genotoxicity/cancer endpoints in the Danish (Q)SAR Database excluding the results presented as N/A. For the rest of the compounds, additional predictions were run in the VEGA (Q)SAR platform in the selected endpoints of chromosomal aberration and micronucleus assay as there were inconsistencies between the results.

For chromosomal aberration endpoint, this additional analysis showed that palmitic acid, emetin, β -elemene, γ -elemene, and pro-vitamin A were inactive with reliable results, proline and vitamin C were inactive with moderate reliable results and hederasaponin B, hederacoside C, hederasaponin D, and p-coumaric acid were inactive with poor reliable results. Vitamin E was inactive both in experimental data and model prediction. Caffeic acid were active in experimental data, rutin, isoquercitrin, astragalin, nicotiflorin, chlorogenic acid, neochlorogenic acid, 4,5-O-dicaffeoyl-quinic acid, rosmarinic [(R)-(+)] enantiomer] acid, and furfural were active with moderate reliable results and hederasaponin F, scopolin, 3,5-O-dicaffeoyl-quinic acid, protocatechuic acid, tyrosine, and valine were active with poor reliable results.

For *in vitro* micronucleus activity, furfural was inactive in both experimental and model prediction, palmitic acid and β -elemene were inactive with reliable results, γ -elemene and vitamin E were inactive with moderate reliable results and rosmarinic [(R)-(+)] enantiomer] acid and emetin were inactive with poor reliable results. On the other hand, vitamin C was active in both experimental data and model prediction. Hederasaponin B, hederacoside C, and hederasaponin D were found to be active with reliable results, hederasaponin F, scopolin, chlorogenic acid, neochlorogenic acid, 3,5-O-dicaffeoyl-quinic acid, 4,5-O-dicaffeoyl-quinic acid, tyrosine and pro-vitamin A were active with moderate results, rutin, isoquercitrin, astragalin, nicotiflorin, caffeic acid, protocatechuic acid, proline, and valine were active with poor reliable results. p-coumaric acid could not be predicted.

Table 4-5 The predictions for other *in vitro* and *in vivo* genotoxicity/cancer endpoints in the Danish (Q)SAR database

Compound Number	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 <i>Drosophila</i> m.	Micronucleus Test in Mouse Erythrocytes	Dominant Lethal Mutations in Rodents	Sister Chromatid Exchange in Mouse Bone Marrow Cells	Comet Assay in Mouse
1	Negative	Negative	N/A	N/A	Negative	Negative	Negative	Positive	N/A	N/A
2	Negative	Negative	N/A	N/A	Negative	Negative	Negative	Positive	N/A	N/A
3	Negative	Negative	N/A	N/A	Negative	Negative	Negative	Positive	N/A	N/A
4	Negative	Negative	Positive	N/A	Negative	Negative	Negative	Positive	N/A	N/A
5	Negative	Negative	N/A	Negative	Negative	Negative	Negative	N/A	Negative	Negative
6	Negative	Negative	N/A	Negative	Negative	Negative	Negative	Negative	Negative	Negative
7	Negative	N/A	Negative	Negative	N/A	Negative	N/A	N/A	Negative	Negative
8	Negative	N/A	Negative	Negative	N/A	Negative	N/A	N/A	Negative	Negative
9	Positive Positive*	Positive Positive*	Positive Positive*	Negative* Negative*	N/A Positive*	Negative	Positive Positive*	N/A	Positive	N/A
10	Positive	Positive	Positive	Negative Negative*	N/A Negative*	Negative	Positive Positive*	N/A	Positive	N/A
11	N/A Negative*	Positive	Positive	N/A	Negative	Negative	Positive	N/A	Positive	N/A
12	Positive	Positive	Positive	N/A	Negative	Negative	Positive	Negative	Positive	N/A
13	Negative	N/A	Positive	Positive	Negative	Negative	Positive	Negative	Positive	N/A
14	Negative	N/A	Positive	Positive	Negative	Negative	Positive	N/A	Positive	N/A

Compound Number	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
15	Positive	Positive	Positive	N/A	Negative	N/A	Positive	Negative	Positive	N/A
16	N/A	N/A	Negative	Negative	N/A	Negative	Negative	N/A	N/A	N/A
17	Negative	Negative	Negative	N/A	Negative	Negative	Negative	N/A	Negative	N/A
18	Negative	Positive	Negative	N/A	N/A	Negative	N/A	N/A	N/A	N/A
19	Positive	Positive Positive*	N/A	Negative	Negative	Negative	Negative	Positive	Negative	Negative
20	Positive	Positive Positive*	N/A	Negative	Negative	Negative	Negative	Positive	Negative	Negative
21	Positive	Positive	N/A	Negative	Negative	Negative	Negative	N/A	Negative	N/A
22	Positive	Positive	N/A	Negative	Negative	Negative	Negative	Positive	Negative	Negative
23	Positive	Positive	N/A	N/A	N/A	Negative	N/A	N/A	N/A	N/A
24	Positive	N/A	Negative	N/A	N/A	Negative	Negative	Negative	Positive	Negative
25	Negative	Positive	Negative	N/A	N/A	Negative	N/A	Negative	N/A	N/A
26	Negative	Negative Negative*	Negative	Negative	Negative Negative*	N/A	Negative	Positive	Negative Negative*	Negative Negative*
27	Negative	Negative Negative*	Negative	Negative	N/A Negative*	Negative	Negative Negative*	N/A	Negative Negative*	Negative Negative*
28	Negative	Negative	Negative	Negative	N/A	Negative	Negative	N/A	Negative	Negative
29	Negative	Negative	N/A	Negative	N/A	Negative	Negative	N/A	Negative	Negative

Compound Number	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
30	Negative	Negative	Negative	Negative	N/A	Negative	Negative	N/A	Negative	Negative
31	Negative	Negative	N/A	Negative	N/A	Negative	Negative	N/A	Negative	Negative
32	Negative	Negative	Negative	Negative	N/A	Negative	Negative	N/A	Negative	Negative
33	Positive	N/A	N/A	N/A	N/A	N/A	Positive	N/A	N/A	N/A
34	Negative	N/A	Negative	N/A	N/A	N/A	N/A	N/A	N/A	Negative
35	N/A	N/A	N/A	N/A	N/A	Negative	N/A	N/A	Positive	N/A
36	N/A	N/A	N/A	N/A	N/A	Negative	N/A	N/A	Positive	N/A
37	N/A	Negative	Negative	N/A	Negative	N/A	Negative Negative*	N/A	Negative	Negative
38	N/A	Negative	Negative	N/A	Negative	N/A	Negative	N/A	Negative	Negative
39	Negative	N/A	Negative	Negative	N/A	N/A	Negative	N/A	N/A	N/A
40	Negative	N/A	Negative	N/A	N/A	Negative	Negative	N/A	Negative	N/A
41	Negative	Negative	N/A	Negative	N/A	N/A	N/A	N/A	Negative	Negative
42	Negative	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
43	Negative	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
44	Negative	N/A	Negative	Negative	N/A	N/A	N/A	N/A	N/A	Negative

Compound Number	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
45	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
46	Negative	N/A	Negative	N/A	N/A Negative*	N/A	N/A	N/A	Negative	Negative
47	N/A	Positive	N/A	N/A	N/A	N/A	Negative Negative*	N/A	N/A	N/A
48	N/A	Negative Negative*	Negative	N/A	Negative	N/A	Negative	Negative	Negative Negative*	Negative Negative*
49	Negative Negative*	Negative Negative*	Negative	N/A	Negative	Negative	Negative Negative*	Negative	Negative Negative*	Negative
50	N/A	N/A Negative*	N/A	N/A	Negative Negative*	N/A	N/A Negative*	Positive	Negative Negative*	Negative Negative*
51	Positive	N/A Negative*	N/A	Negative	Negative	Negative	Negative Negative*	N/A	Negative Negative*	Negative
52	Negative Negative*	Negative Negative*	Negative	N/A	Negative Negative*	Negative	Negative Negative*	Positive	Negative Negative*	Negative Negative*
53	Negative	Negative Negative*	Negative	Negative	Negative Negative*	Negative	Negative Negative*	N/A	Negative Negative*	Negative Negative*
54	Negative	Negative Negative*	Negative	Negative	Negative	Negative	Negative Negative*	N/A	Negative Negative*	Negative
55	Negative Negative*	Negative Negative*	N/A	N/A	Negative Negative*	N/A	Negative Negative*	Negative	Positive Negative*	Negative Negative*
56	Negative Negative*	N/A Negative*	Positive	N/A	Negative Negative*	Negative Negative*	Positive	Negative	Negative Negative*	N/A Negative*
57	Negative Negative*	Negative Negative*	Negative	Negative	Positive	N/A	N/A	N/A	Negative Negative*	Negative Negative*
58	Negative	Negative	Negative	Negative	Negative	N/A	Negative	Negative	Negative	Negative

In this study, we also ran predictions for several activities using PASS online freeware to determine whether *H. helix* extract had any of these activities. In Table 4-6, the prediction values (probability to be active) of the selected activities for each compound are listed. According to our results, 37 of 58 compounds previously reported from *H. helix* were predicted as having anti-inflammatory activities. α -hederin and hederacoside C were the most tested compounds in various studies. They also had high prediction values of 0.84 for both of them in our study which were consistent with the previous experimental studies. 16 of total compounds including α -hederin and hederacoside C demonstrated positive predictions for antimutagenic activity. Positive predictions for antioxidant properties, activity like a nitric oxide antagonist and activity like a nitrite reductase (NO-forming) inhibitor appeared in a total of 23, 18, and 15 compounds, respectively. These parameters were not well-established in experimental studies. There was no reliable result for the activity like nitric-oxide synthase inhibitor with no predictions for most of the compounds.

Table 4-6 The predictions for selected activities in the PASS online freeware

Compound Number	Antiinflammatory	Antimutagenic	Antioxidant	Nitric oxide antagonist	Nitric-oxide synthase inhibitor	Nitrite reductase (NO-forming) inhibitor
1	0.82	-	0.72	0.82	-	-
2	0.84	-	0.67	0.83	-	-
3	0.84	-	0.64	0.82	-	-
4	0.84	-	0.64	0.82	-	-
5	0.84	-	0.57	0.83	-	-
6	0.84	-	0.52	0.82	-	-
7	0.89	-	N/A	0.83	-	-
8	0.84	-	N/A	0.79	-	-
9	0.69	0.94	0.87	0.62	-	N/A
10	0.68	0.95	0.86	0.59	-	N/A
11	0.73	N/A	0.92	0.70	-	-
12	0.74	0.91	0.76	0.69	-	-
13	0.75	0.70	0.91	0.68	-	-
14	0.74	N/A	0.92	0.69	-	-
15	0.71	N/A	0.68	0.53	-	-
16	N/A	0.66	N/A	N/A	N/A	N/A
17	0.66	N/A	N/A	0.60	N/A	N/A
18	0.65	0.84	0.60	N/A	-	0.65
19	0.60	N/A	0.78	N/A	-	N/A
20	0.60	N/A	0.78	N/A	-	N/A
21	0.66	0.96	0.78	N/A	-	N/A
22	0.66	0.95	0.80	N/A	-	N/A
23	N/A	0.72	0.54	N/A	-	N/A
24	0.54	0.83	N/A	N/A	-	0.67
25	0.64	0.89	0.55	N/A	-	0.69

Compound Number	Antiinflammatory	Antimutagenic	Antioxidant	Nitric oxide antagonist	Nitric-oxide synthase inhibitor	Nitrite reductase (NO-forming) inhibitor
26	0.51	0.78	N/A	N/A	N/A	0.83
27	0.57	-	N/A	N/A	-	-
28	N/A	-	N/A	N/A	-	-
29	0.54	-	N/A	N/A	-	-
30	N/A	-	N/A	N/A	-	-
31	N/A	-	N/A	N/A	-	-
32	N/A	-	N/A	N/A	-	-
33	-	-	-	N/A	N/A	-
34	0.53	N/A	-	N/A	N/A	N/A
35	0.82	-	N/A	0.69	-	N/A
36	0.75	-	N/A	0.53	-	N/A
37	0.51	0.56	N/A	-	-	0.73
38	N/A	N/A	N/A	-	-	0.64
39	N/A	0.54	N/A	N/A	N/A	-
40	N/A	0.68	N/A	N/A	N/A	0.76
41	N/A	-	-	N/A	-	N/A
42	0.74	N/A	N/A	N/A	N/A	N/A
43	0.85	-	-	N/A	-	N/A
44	N/A	N/A	-	N/A	N/A	N/A
45	0.61	-	-	N/A	-	N/A
46	0.61	N/A	N/A	N/A	N/A	N/A
47	N/A	N/A	N/A	N/A	N/A	0.58
48	-	N/A	N/A	-	N/A	0.58
49	Could not be screened*					
50	-	N/A	-	-	N/A	N/A
51	-	0.53	N/A	N/A	N/A	0.52

Compound Number	Antiinflammatory	Antimutagenic	Antioxidant	Nitric oxide antagonist	Nitric-oxide synthase inhibitor	Nitrite reductase (NO-forming) inhibitor
52	N/A	N/A	-	-	N/A	0.64
53	-	N/A	N/A	-	N/A	0.61
54	-	N/A	-	-	N/A	0.57
55	0.81	-	0.97	N/A	N/A	-
56	0.78	N/A	0.93	-	-	0.55
57	0.69	N/A	0.77	N/A	-	N/A
58	-	N/A	N/A	-	-	0.65

*could not be estimated because of the carbon number less than 3.

5. DISCUSSION and CONCLUSION

Hedera helix is a well-known ornamental and medicinal plant that is a member of the Araliaceae family (1, 2). It is a climber evergreen woody plant covering the walls or other objects with its leaves (13). The plant's fresh leaves and fruits have toxic effects leading to bloody diarrhea, gastrointestinal irritation, and death at high amounts. The polyacetylenes found in the leaves of *H. helix*, falcarinol, and didehydrofalcarinol are allergens with powerful irritating and sensitizing properties (4). Thus, contact dermatitis has been reported in several cases (8).

Even though inflammation is a defense mechanism for the immune system, destructive effects on the physiology and structure of tissues may be observed due to excessive inflammation (14). Steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) are currently used for the treatment of inflammatory diseases. NSAIDs inhibit arachidonic acid metabolism by both lipoxygenase and COX enzyme pathways. Although they are commonly used worldwide, they often have severe adverse effects such as gastrointestinal bleeding. Hence, safer compounds are warranted (8). There are many studies investigating the anti-inflammatory effects of dry extract of *H. helix* leaves. For many years, dry extract of *H. helix* leaves has been one of the options in the treatment of respiratory diseases with productive cough. Several preparations containing *H. helix* leaves dry extract were investigated in terms of safety and tolerance. The extract exerts secretolytic and bronchospasmolytic effects which are led by the main active compounds like saponins with α -hederin being the most important compound for the extract's therapeutic activity (39). EA 575[®] is one of the preparations containing *H. helix* leaves dry extract which is used to improve symptoms in chronic inflammatory bronchial diseases and acute respiratory tract inflammation with cough with proven efficacy by clinical studies. The extract leads to an increase in the respiratory tract β_2 -adrenergic responsiveness via inhibition of phosphorylation of activated β_2 -adrenergic receptors. This results in the inhibition of internalization of β_2 -adrenergic receptors (11). This increased β_2 -adrenergic activity can also result in a more sensitive response to spasmolytics leading to a bronchodilating effect. Moreover, more surfactant is generated by type II alveolar epithelial cells and mucus viscosity is reduced. In one study, the dry extract of *H. helix* leaves showed non-inferior outcomes in clinical and lung function endpoints compared to Ambroxol (39).

A few studies of acute and/or chronic inflammation induced by carrageenan/cotton pellet/formalin exploring the effects of *H. helix* extract or saponins extracted from *H. helix* were conducted in rat or mice models. These studies confirmed the inflammatory activity of the plant extract on serotonin, histamine, and bradykinin release (3, 8, 12, 13). In one of the studies, crude saponin and saponin's purified extracts of the plant showed better efficacy in the first phase compared to the second phase in the acute inflammation model. An inhibitory effect on macrophages and fibrosis was demonstrated which were likely the cause of the observed anti-inflammatory activity in this model. Block of some inflammatory mediators may be the focus of *H. helix*'s mode of action (8).

There are also studies examining the effects of *H. helix* extract on inflammatory mediators. A dose-dependent reduction in IL-6 release from LPS-induced murine macrophages was observed (9). This finding was confirmed by another study investigating hederacoside C activity in acute lung inflammation. Pro-inflammatory cytokine levels such as TNF- α , IL-6, IL-1 β , and IL-10 are elevated along with increased severity of lung inflammation. Hederacoside C inhibited the secretion of pro-inflammatory cytokines and suppressed inflammation (14). Additionally, a reduction in the synthesis of immunoglobulin E and cytokines like IL-4 and IL-5 by chlorogenic acid was shown in a mouse model of allergic asthma as well as protective effects in mice against LPS-induced lung injury in another study (11). Moreover, the anti-inflammatory activity of Hederacoside C on *Staphylococcus aureus* induced inflammation was shown in *in vivo* and *in vitro* studies. Hederacoside C reduced pulmonary edema and mammary gland tissue pathology and inhibited pro-inflammatory cytokine production and TLR 2 and 4 (10, 14). Besides, it was shown that the EA 575[®] had a regulatory effect on the NF- κ B pathway and the possible reason was the specificity switch of I κ B kinase β (11).

Even though the anti-inflammatory activity of *H. helix* extract was tested in different aspects, especially against cytokine secretion, there were no studies evaluating the effect of the extract on nitrite levels in LPS-induced RAW 264.7 macrophage cells. NO modulates several physiological and pathophysiological processes in the human body including host defense. Increased synthesis of NO has been observed in inflammatory conditions. Several studies revealed the role of NO in the regulation of inflammatory processes like vascular responses, signaling pathways, transcription factors, leukocyte migration and rolling, cytokine synthesis, apoptosis, and infection control. Anti-inflammatory activities via NO synthesis inhibition (predominantly

selective iNOS inhibitors) have been demonstrated in a number of experimental models of inflammation like colitis and arthritis (42). Evidence supports its modulatory effects on airway function and pro-inflammatory and immunomodulatory effects in pulmonary disease (43). In our study, all concentrations of *H. helix* extract significantly reduced the nitrite levels in the macrophages compared to negative control without causing severe toxicity. The observed effects on nitrite levels were similar with indomethacin and L-NAME.

Diet contains multiple chemicals including both carcinogens and mutagens and compounds preventing carcinogenesis and mutagenesis (45). Chronic inflammation is considered an important element in the progression of neoplastic processes, as it has been shown to hasten cancer progression (13). It is important to discover mutagenicity and cancer inhibitors and understand their mode of action in order to make available preventive measures against cancer. Many substances found in nature including plant extracts have antimutagenic activity. A decrease in the mutagenicity of well-known mutagens by some plant compounds has been confirmed. Short-term mutagenicity assays are effective to analyze potential mutagens and carcinogens. Additionally, potential antimutagens and anticarcinogens can be identified with the same assays (45). Mutagenic and antimutagenic activities of several saponins isolated from leaves of *Hedera helix* were tested in different studies. In one study, it was shown that three saponins isolated from *H. helix* had no mutagenic activity (44). Our study also revealed the non-mutagenicity of all tested concentrations of *H. helix* leaves extract with negative Ames test results on *Salmonella* tester strains TA98 and TA100 with and without S9 activation. Additionally, antimutagenic activity of a few saponins from *H. helix* including α -hederin which is one of the main compounds representing biological activity was tested in two different studies (44, 45). The tested saponins had a significant dose-dependent antimutagenic activity against benzo[a]pyrene and smoker's urine in a *Salmonella*/microsomal assay (44). In our study, we observed strong antimutagenic activity against indirect mutagen only at the concentration of 5000 $\mu\text{g}/\text{plate}$ with an inhibition rate of 52% in the experiment conducted in the TA100 strain with S9. The antimutagenic activity ratios observed with the lower concentrations were moderate or weak. Another study also indicated that α -hederin exerted antimutagenic activity against clastogenicity caused by doxorubicin in human lymphocytes at concentrations tested (1.3×10^{-2} , 0.13, 1.3 and 13 nmol/ml). The findings of this study

suggested the possibility of its effect through inducing metabolic enzymes to act as metabolic inhibitors of mutagens (45).

Plant extracts are mixtures of a variety of phytochemicals found in nature. Many types of extracts or individual compounds obtained via isolation from a plant are available to be intended to be used in different products for consumer use (80). We found 58 different compounds isolated from the *H. helix* leaves so far and carried out an *in silico* analysis of the genotoxicity, mutagenicity, and several biological activity-related endpoints to confirm the consistency of the findings with experimental data and obtain overall data for all the compounds in the extract. The findings of compound 23, compound 26, compound 27, and compound 33 were not included in our final assessment as they were not in the constituents list of the extract according to the assessment report on *Hedera helix* L., folium by European Medicines Agency (81). Ames test was one of our endpoints to make a decision on the safety in terms of the mutagenicity of the extract. Forty-eight of 54 compounds yielded negative predictions for the Ames test including 43 absolute negative findings and five negative findings that needed additional confirmation via the VEGA (Q)SAR platform later on. Quercetin, kaempferol, pro-vitamin A, and trans-2-hexanal were predicted to be potentially mutagenic while isoquercitrin and astragalin were mutagenic without any structural alert with moderately reliable results based on the additional screening in the VEGA (Q)SAR platform. Consequently, the whole extract can be suggested to be Ames negative based on the predictions performed. The prediction was consistent with a previous experimental study (44) and our current study.

Regarding the antimutagenic activity of the plant extract, 14 of the total of 54 compounds demonstrated positive predictions for antimutagenic activity in the PASS online freeware. Previous studies demonstrated antimutagenic activity of 3 saponins and α -hederin isolated from the *H. helix* leaves (44, 45). Our results also indicate consistent results for the presence of antimutagenic activity of α -hederin and hederacoside C as the most tested compounds. Moreover, our experimental study showed moderate and/or strong antimutagenic activities of the plant extract at the concentrations of 1000 and 5000 $\mu\text{g}/\text{plate}$. Based on the additional data obtained via *in silico* analysis, this topic can be further addressed in other studies to be conducted.

In the analysis of other *in vivo/in vitro* genotoxicity/cancer endpoints, our study combined the results of the two databases Danish (Q)SAR Database and the VEGA (Q)SAR platform. 34 of 54 compounds showed negative predictions including β -

elemene, γ -elemene, p-coumaric acid, and vitamin E which were predicted as negative in most of the endpoints in both of the databases used. Proline, tyrosine, and valine were also included in this number. Chromosomal aberration prediction for proline was supported by the VEGA (Q)SAR platform as negative. Despite positive results in the VEGA (Q)SAR platform, valine and tyrosine were also considered to be negative in chromosomal aberration and/or *in vitro* micronucleus activity endpoints due to the experimental negative results in the Danish (Q)SAR Database. These compounds were suggested as potentially non-genotoxic.

The saponins hederasaponin B, C and D and pro-vitamin A, vitamin C, and protocatechuic acid were found to be predicted as positive in only two different endpoints. The predictions for vitamin C in chromosomal aberration and *in vitro* micronucleus activity endpoints were positive in both of the databases. The VEGA (Q)SAR platform confirmed the positive prediction for protocatechuic acid in the chromosomal aberration endpoint. However, there was inconsistency between the predictions for *in vitro* micronucleus activity endpoint. The prediction was considered to be negative as the positive result in the VEGA (Q)SAR platform was poorly reliable. These compounds were also considered to reveal potentially non-genotoxic activities.

Three positive predictions were indicated for 3,5-O-dicaffeoyl-quinic acid and caffeic acid because of the positivity in the VEGA (Q)SAR platform in chromosomal aberration and/or *in vitro* micronucleus activity endpoints. Positive results with moderate reliability in the *in vitro* micronucleus activity endpoint in the VEGA (Q)SAR platform turned 4,5-O-dicaffeoyl-quinic acid, chlorogenic acid, and neochlorogenic acid to have a total of four positive predictions in the related endpoints. Despite having a positive result in chromosomal aberration endpoint in the VEGA (Q)SAR platform, an experimental negative result was taken into consideration for rutin and it had also a total of four positive predictions in the related endpoints.

The predictions for scopolin and isoquercitrin were concordant between both of the databases and these compounds were potentially genotoxic according to the overall prediction with five positive and two or three negative results. Nicotiflorin and astragalol were also considered to be potentially genotoxic as they had also a total of 5 positive predictions due to moderately reliable positive prediction in chromosomal aberration endpoint in the VEGA (Q)SAR platform. The VEGA (Q)SAR platform identified a positive prediction for furfural in the chromosomal aberration endpoint with

a total of two positive and one negative result which made this compound potentially genotoxic.

The overall evaluation for hederasaponin F was not clear. This compound was positive in both chromosomal aberration and *in vitro* micronucleus activity endpoints in the VEGA (Q)SAR platform while it was predicted to be mostly negative in the Danish (Q)SAR Database.

In our experimental study, *H. helix* extract exerted non-mutagenic activity on all of the strains tested. *In silico* predictions run in five different computational software and VEGA (Q)SAR platform for contradictory results supported the non-mutagenicity of the extract leaves with a total of 48 Ames negative compounds. Regarding predictions for other *in vivo/in vitro* genotoxicity/cancer endpoints, 27 of 54 compounds were found to have negative predictions in the Danish (Q)SAR Database. A total of 39 compounds were evaluated as potentially non-genotoxic considering the additional results obtained with VEGA (Q)SAR platform. On the other hand, strong antimutagenic activity was only demonstrated at the concentration of 5000 µg/plate. Positive predictions for antimutagenic activity were only for 14 of the total compounds including α-hederin and hederacoside C. In terms of anti-inflammatory activity of the *H. helix* leaves extract, the highest nitrite inhibition rate was 43.61 ± 1.63 at the concentration of 1 mg/ml even though the extract showed similar effects when compared with indomethacin and L-NAME. Moreover, the predictions run for several activities using PASS online freeware to determine whether *H. helix* extract had any of these activities demonstrated anti-inflammatory activities of 34 compounds isolated from *H. helix* including α-hederin and hederacoside C as the currently known active compounds with high prediction values for both of them. However, the predictions for an activity like a nitric oxide antagonist and nitrite reductase (NO-forming) inhibitor were only positive for a total of 18 and 14 compounds, respectively, while there were no predictions for the activity of nitric-oxide synthase inhibitor for most of the compounds. All of these findings suggested that the anti-inflammatory activity of the *H. helix* leaves extract was not likely seemed to be only dependent on NO inhibition. These results indicated a correlation with other experimental studies in which different aspects of the anti-inflammatory activity of *H. helix* extract were discovered. Further research may be warranted to address the other contributory anti-inflammatory mode of action of the *H. helix* leaves extract as well as its major secondary metabolites. Additionally, a comparative study would be beneficial to conduct with the extracts prepared with

different extraction methods to be able to assess efficacy of possible different constituent profiles.



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7. APPENDICES

7.1. Curriculum Vitae

Personal Informations

Name	Makbule Kübra	Surname	Doğan
Place of Birth			
Nationality			
E-mail			

Education

Degree	Department	The name of the Institution Graduated From	Graduation year
Doctorate			
Master			
University	Biology	İstanbul Üniversitesi	2011
High school	-	Şehremini Lisesi	2007

Languages	Grades (#)
İngilizce (YDS)	85 (B)

All the grades must be listed if there is more than one (KPDS, ÜDS, TOEFL; EELTS vs),

Work Experience (Sort from present to past)

Position	Institute	Duration (Year - Year)
Senior Regulatory Affairs Specialist	Colgate-Palmolive Turkey	2022 -
Quality Assurance – Medical Devices Supervisor	Kansuk Laboratuvarı San. ve Tic. A.Ş.	2016 - 2022
Medical Specialist	Celtis İlaç Tanıtım ve Pazarlama Tic. A.Ş.	2011 - 2013

Computer Skills

Program	Level
MS Office	Excellent

*Excellent , good, average or 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)

Pedagogical Education Certificate
Several Training Certificates for Medical Devices