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

The Ameliorative Effect of Rosmarinic Acid and Epigallocatechin Gallate Against Doxorubicin-Induced Genotoxicity

PhD THESIS

Sinem HELVACIOĞLU AKYÜZ, Pharm.

İSTANBUL-2022

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

The Ameliorative Effect of Rosmarinic Acid and Epigallocatechin Gallate Against Doxorubicin-Induced Genotoxicity

PhD THESIS

Sinem HELVACIOĞLU AKYÜZ, Pharm.

Supervisor

Assoc.Prof. Dr. Muhammed HAMİTOĞLU

İSTANBUL-2022

THESIS APPROVAL FORM

Institute : Yeditepe University Institute of Health Sciences

Programme : Pharmaceutical Toxicology

Title of Thesis: The ameliorative effect of rosmarinic acid and epigallocatechin gallate against Doxorubicin-induced genotoxicity

Owner of the Thesis: Sinem HELVACIOĞLU AKYÜZ

Examination Date : 17.06.2022

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

	Title, Name-Surname (Institution)
Chair of the Jury:	Prof.Dr. Ahmet Aydın Yeditepe University
Supervisor:	Assoc.Prof. Dr. Muhammed HAMİTOĞLU Yeditepe University
Member:	Assoc.Prof. Dr. Aylin YABA UÇAR Yeditepe University
Member:	Prof.Dr. Semra Şardaş İstinye University
Member:	Prof.Dr. Yalçın Duydu Ankara University

APPROVAL

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

Prof. Dr. Bayram YILMAZ

Director of Institute of Health Sciences

DECLARATION

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

17.06.2022

Sinem HELVACIOĞLU AKYÜZ

Dedication

This thesis is dedicated to my beloved family with deepest love and thanks

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude and praise to my advisor and mentor, Assoc.Prof. Dr. Muhammed Hamitoğlu, for his patience, precious and constructive suggestions, unlimited guidance, and support during my academic life. I will remain forever indebted to him for his encouragement and guidance.

I am also deeply grateful to head of Toxicology department Prof. Dr. Ahmet Aydın for his guidance, positive encouragement, and endless support. I would like to give my sincerest thanks to him for giving me chance as a part of his team. It was an excellent chance for me to be his student. I will always be grateful to him.

I also would like to present my sincerest thanks to Prof. Dr. Hande Sipahi for helping and guiding me any time without hesitation.

I thank Prof. Dr. Helga Stopper for giving me a platform to improve my research interest in Genetic Toxicology. I also would like to thank Dr.Ezgi Eylül Bankoğlu for guiding me. It was a wonderful learning experience.

I also would like to my gratitude to Assoc.Prof. Dr. Aylin Yaba Uçar for her contributions and positive encouragement.

I would like to thank you Dr. Gülşah Selin Akyüz for our morning coffees, our conversations about life and your incredible support and positive energy. I would also like to thank my friends Pharm. Rima Konya, Pharm. Murat Erdoğan, Dr. Mümin Yiğit İnan and Dr.Bengisu Turgutalp who supporting me to finish my thesis.

I thank my colleague, Pharm. Yağmur Özhan for her support. I'm also glad for the brilliant ideas we've come up with together. I would like to thank Assoc.Prof. Dr. Etil Güzelmeriç for her advices and support.

Last but not least, I thank my dear big family, my father Ömer Helvacıoğlu, my mother Suzan Helvacıoğlu, my sisters Elif Yıldırım, Özlem Cam and Didem Helvacıoğlu, mother-in-law Esra Zeynep Akyüz, father in law Murat Akyüz, my nephews Selin Yıldırım, Öykü Cam, my brothers-in-law Ali Yıldırım and Necmi Cam for their endless support, emotional and financial supports and always being there for me. Finally, I would like to thank my dear husband, who has always helped and supported me in my doctoral thesis process, as in every moment of my life. Aras, I have always felt your moral support and love with me. I dedicate this work to my family. Without their support, this study would not be possible.

TABLE OF CONTENTS

THESIS APPROVAL FORM ii
DECLARATION iii
DEDICATION iv
ACKNOWLEDGEMENTS v
TABLE OF CONTENTS vi
LIST OF TABLES x
LIST OF FIGURES xi
ABBREVIATIONS xiii
ABSTRACT xv
ÖZET xvi
1. INTRODUCTION AND AIM 1
2. GENERAL INFORMATION 4
2.1. Definition and History of Cancer 4
2.2. Cancer Epidemiology 5
2.3. Cancer Etiology 5
2.4. Cancer Treatment 9
2.4.1. Radiotherapy 10
2.4.2. Surgical Methods 10
2.4.3. Immunotherapy 11
2.4.4. Hormone Therapy 11
2.4.5. Cancer Vaccines 12
2.5. Classification of Antineoplastic Drugs 13
2.5.1. Alkylating Agents 13
2.5.2. Antimetabolites 14
2.5.3. Topoisomerase Inhibitors 14
2.5.4. Antimicrotubule Agents 15
2.5.5. Antibiotics 15
2.5.5.1. Anthracyclines 16
2.6. Doxorubicin 16
2.6.1. Chemical structure of Doxorubicin 17

2.6.2. Pharmacokinetics of Doxorubicin 18
2.6.3. Treatment with Doxorubicin 18
2.6.4. Mechanism of action 19
2.6.5. Development of Doxorubicin Resistance 21
2.6.6. Toxicity of Doxorubicin 22
2.6.7. Toxicity in the Heart 25
2.6.8. Toxicity in the Brain 25
2.6.9. Toxicity in the Liver 25
2.6.10. Toxicity in the Kidneys 26
2.6.11. Dox-Induced Secondary Cancers 26
2.6.12. Dox-Induced Genotoxicity 27
2.7. Doxorubicin and Photochemicals 29
2.7.1. Rosmarinic Acid 31
2.7.1.1. Protective effects of Rosmarinic acid 33
2.7.2. Epigallocatechin gallate 35
2.7.2.1. Antimutagenic and Anticarcinogenic Potential of EGCG 38
2.8. Genotoxicity studies 39
2.8.1. Usage Areas of Genetic Toxicity Tests 40
2.8.2. *Salmonella*/Microsome Mutagenicity Test 41
2.8.3. Micronucleus (MN) Test 44
2.8.4. Comet Analysis (Single Cell Gel Electrophoresis) 45
2.9. Cytotoxicity and Cytotoxicity Determination Method 41
2.9.1. Dye Exclusion Assays 41
2.9.2. Colorimetric Assays 41
2.9.3 WST-1 Assay 47
2.9.4. Fluorometric assays 47
2.9.5. Luminometric assays 47
2.9.6. Flow cytometric assays 48
2.9.7. Mitochondria assays 48
3. MATERIALS and METHODS 49
3.1. Materials 49
3.1.1. Chemicals 49
3.1.2. Equipment and Instruments 51

3.2. Methods 53
3.2.1. Preparation of Samples 53
3.2.2. Cell lines and culture conditions 54
3.2.3. Cell viability assay 54
3.2.4. Comet assay 55
3.2.5. Evaluation of the cellular superoxide anion concentration 56
3.2.6. Ames test 57
3.2.6.1. Mutagenic index (MI) 61
3.2.6.2. Antimutagenicity assay 62
3.2.7. WST-1-based cytotoxicity assay 62
3.2.7.1. Cytotoxicity assay for suspension cells (TK6 cells) 63
3.2.7.2. WST-1-based cytotoxicity assay for adherent cells 62
3.2.7.3. Calculation of IC50 Value 63
3.2.7.4. Selectivity index (SI) calculation 63
3.2.7.5. *In vitro* drug interaction analysis 63
3.2.8. Mitochondrial membrane potential assay 65
3.2.9. Micronucleus (MN) assay 66
3.2.10. Statistical analysis 67
4. RESULTS 68
4.1. *In vitro* Genetic Toxicology Tests Results 68
4.1.1. Comet Assay 68
4.1.1.1. Results of vitality test 76
4.1.2. Micronucleus Assay Results 82
4.2 Microscopic analysis of ROS production 95
4.3 Mitochondrial Membrane Potential Assay 98
4.4 Mutagenicity and Antimutagenicity Assays 104
4.4.1 Bacterial Reverse Mutation Test Results 104
4.4.2 Antimutagenicity Assay Results 109
4.5 Anticancer Activity 113
4.5.1 Determination of IC50 value by WST-1 Assay 113
4.5.2 Selectivity Indexes (SI) of Samples 113
4.5.3 Evaluation of Combination Effect by Coefficient of drug interaction (CDI) 114
5. DISCUSSION 118

6. CONCLUSION 132
7. REFERENCES 133

LIST OF TABLES

Table 1. Genotypes of widely used bacterial strains in the Ames test 43
Table 2. The determination of Synergy 64
Table 3. Percentage of the living cells that was determined on CHO-K1 cells after 4 hours of Dox incubation. 77
Table 4. Percentage of the living cells that was determined on TK6 cells after 4 hours of Dox incubation. 78
Table 5. Percentage of the living cells that was determined on CHO-K1 cells after 4 hours of Dox, RA and combination of both incubation. 78
Table 6. Percentage of the living cells that was determined on TK6 cells after 4 hours of Dox, RA, combination of Dox and RA incubation. 79
Table 7. Percentage of the living cells that was determined on CHO-K1 cells after 4 hours of Dox, EGCG and combination of Dox and EGCG incubation. 79
Table 8. Percentage of the living cells that was determined on TK6 cells after 4 hours of Dox, EGCG and combination of Dox and EGCG incubation. 80
Table 9. Percentage of the living cells that was determined on CHO-K1 cells after 4 hours of Dox, EGCG,RA and combination of Dox, RA and EGCG incubation. 80
Table 10. Percentage of the living cells that was determined on TK6 cells after 4 hours of Dox, EGCG,RA and combination of Dox, RA and EGCG incubation. 81
Table 11. Mutagenic activity expressed as the mean and standard deviation of the number of revertants/plate in *Salmonella typhimurium* TA98, TA97a, TA100, TA102, and TA1535 strains exposed to Dox at various concentrations, with and without S9 metabolic activation. 105
Table 12. Mutagenicity results of RA in *Salmonella typhimurium* TA98 and TA102 strains exposed to RA at various concentrations (1-1000 µg/plate), with and without S9 metabolic activation 107
Table 13. Mutagenicity results of EGCG in *Salmonella typhimurium* TA98 and TA102 strains exposed to EGCG at various concentrations (1-1000 µg/plate), with and without S9 metabolic activation 108
Table 14. The concentrations of the studied compounds that inhibit 50% of cell survival (IC50). 113
Table 15. Calculated SI of compounds using MCF12A cells as reference 114
Table 16. Determination of CDI in MDA-MB-231cells by co-administration of Dox and EGCG 115
Table 17. Determination of CDI in MDA-MB-231cells by co-administration of Dox and RA 116
Table 18. Determination of CDI in MCF-7 cells by co-administration of Dox and EGCG 116
Table 19. Determination of CDI in MCF-7 cells by co-administration of Dox and RA 117

LIST OF FIGURES

Figure 1. Chemical structure of Dox 17
Figure 2. Organs affected by Dox 24
Figure 3. Chemical structure of Rosmarinic acid 32
Figure 4. The potential functions of Rosmarinic acid 33
Figure 5. Chemical structure of Epigallocatechin gallate 36
Figure 6. The characteristics and applications of EGCG 37
Figure 7. Application of the Ames test.....42
Figure 8. Examples images of comet assay on CHO-K1 cells with and without Dox…68
Figure **9.** Concentration dependent increase in DNA damage after 4 hours Dox treatment in CHO-K1 cells. 69
Figure 10. DNA damage induction by Dox treatment with or without RA inhibition in CHO-K1 cells. 70
Figure 11. DNA damage induction by Dox treatment with or without EGCG in CHO-K1 cells. 71
Figure 12. DNA damage induction by Dox treatment with or without RA and EGCG combination in CHO-K1 cells. 72
Figure 13. Concentration dependent increase in DNA damage after 4 hours Dox treatment in TK6 cells. 73
Figure 14. DNA damage induction by Dox treatment with or without RA in TK6 cells. 74
Figure 15. DNA damage induced by Dox treatment with or without EGCG in TK6 cells. 75
Figure 16. Dox induced DNA damage and the treated TK6 cells were incubated with RA and EGCG for 4 h. 76
Figure 17. Representative pictures of viable and dead cells under the microscope. 77
Figure 18. Representative images showing TK6 cells stained by Giemsa under a light microscope. 83
Figure 19. Micronucleus and cell viability tests of Dox in TK6 cells. 85
Figure 20. Number of MNi and cytotoxicity biomarkers in TK6 cells treated with Dox, RA and their combinations. 87
Figure 21. Number of MNi and cytotoxicity biomarkers in TK6 cells treated with Dox, EGCG and their combinations. 89
Figure 22. Number of MNi and cytotoxicity biomarkers in TK6 cells treated with Dox, RA,EGCG and their combinations. 91
Figure 23. TK6 cells were treated with different concentrations of Dox for 24 h, and viability was assessed by WST-1. 92
Figure 24. The effects of EGCG on cell viability of TK6. 93
Figure 25. The effects of RA on cell viability of TK6. 93
Figure 26. The protective effect of the combination of EGCG and RA against Dox induced cell viability. 94
Figure 27. Representative pictures of DHE stained CHO-K1cells 95
Figure 28. Superoxide production was detected using the dye DHE in CHO-K1 cells. 96

Figure 29. Superoxide production and its inhibition by RA in CHO-K1 cells. 97
Figure 30. Intracellular ROS after treating the cells with RA 1mM and EGCG (50, 100 μM) 98
Figure 31. Dose-dependent effects of Dox on mitochondrial membrane potential.....99
Figure 32. Dose-dependent effects of EGCG on mitochondrial membrane potential..100
Figure 33. Dose-dependent effects of RA on mitochondrial membrane potential.....101
Figure 34. Mitochondrial membrane potential of TK6 cells after 24 h of treatment with 100 nM Dox and/or RA.....102
Figure 35. Mitochondrial membrane potential of TK6 cells after 24 h of treatment with 100 nM Dox and/or EGCG (6.25 and 12.5 μM). 103
Figure 36. Mitochondrial membrane potential of TK6 cells after 24 h of treatment with 100 nM Dox and combination of EGCG and RA. 104
Figure 37. Inhibitory effect of RA, EGCG also combination of RA and EGCG against the mutagenicity of Dox (5μg/plate) to *Salmonella typhimurium* TA98 strain. 110
Figure 38. Inhibitory effect of RA, EGCG also combination of RA and EGCG against the mutagenicity of Dox (5 μg/plate) to *Salmonella typhimurium* TA102 strain. 112

ABBREVIATIONS

2-AA: 2-Amino Anthracene

2-AF: 2-Aminofluorene

A549: Lung cancer

AC: After Christ

AMA: Antimycin A

AML: Acute myeloid leukemia

ATCC: American Type Culture Collection

ATP: Adenosine triphosphate

BC: Before Christ

BCRP: Breast cancer–resistance protein

BN: Binucleated

BSA: Bovine Serum Albumin

CBPI: Cytokinesis block proliferation index

CDI: Coefficient of Drug Interaction

CHO-K1: Chinese Hamster Ovary cell line

DHE: Dihydroethidium

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic Acid

DOX: Doxorubicin

EDTA: Ethylenediaminetetraacetic acid disodium salt dihydrate

EGCG: Epigallocatechin Gallate

EGCG: Epigallocatechin Gallate

EMS: Ethyl Methanesulfonate

FBS: Fetal Bovine Serum

FDA: Fluorescein diacetate

FDA: Food and Drug Administration

GSH: Glutathione

HCl: Hydrochloric Acid

HT-29: Colorectal cancer

iNOS: Incucible nitric oxide synthases

IARC: The International Agency for Research on Cancer

IC_{50}: The half aximal inhibitory concentration

IUPAC: The International Union of Pure and Applied Chemistry

IV: Intravenous

LRP: Lung resistance protein

MCF-12A: Normal Human Breast Epithelial Cells

MCF-7: Human Breast Cancer cell line

MDA-MB-231: Human Breast Cancer cell line

MDR-1: Multi-Drug Resistance 1

MGA: Minimal Glucose Agar

MI: Mutagenic Index

MN: Micronucleus

MNi: Micronuclei

MRP: Multidrug resistance protein

MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

NDEA: N-nitrosodiethylamine

NDI: Nuclear Division Index

NPD: 4-nitro-o-phenylenediamine

P-gp: P-glycoprotein

PBS: Phosphate-Buffered Saline

RA: Rosmarinic acid

RI: Replication Index

RNS: Reactive nitrogen species

ROS: Reactive Oxygen Species

SA: Sodium Azide

SI: Selectivity Index

TK6: Human Lymphoblastoid cell line

TMRE: Tetramethylrhodamine, ethyl ester

WHO: The World Health Organization

WST-1:(4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate)

ABSTRACT

Akyüz Helvacıoğlu, S. (2022). The ameliorative effect of rosmarinic acid and epigallocatechin gallate against Doxorubicin-induced genotoxicity. Yeditepe University, Institute of Health Sciences, Deparment of Pharmaceutical Toxicology, Ph.D. Thesis, İstanbul.

Doxorubicin (Dox) is an anticancer agent that is effective against a variety of human cancers. However, one of the most adverse effects of Dox treatment is its genotoxic effect on normal cells. Antitumor, antioxidant, and antigenotoxic properties of phenolic compounds have been extensively investigated, and their use has expanded significantly in recent years. The phenolic compounds rosmarinic acid (RA) and epigallocatechin gallate (EGCG) are widely seen in plants. In this study, the protective effect of RA and EGCG alone and in combination on Dox induced genotoxicity was investigated with various *in vitro* test systems, including comet assay, cytokinesis-block micronucleus, and Ames test. It has been shown that free radicals are involved in Dox-induced genotoxicity. For these reasons, the formation of reactive oxygen species was also investigated in this study. Furthermore, the synergistic/antagonistic interaction of these combinations on chemotherapeutic effect of Dox was investigated in breast cancer cell lines. Our results show that EGCG and RA did not exerted genotoxic effect but Dox increased genotoxicity in CHO-K1 and TK6 cells. EGCG and RA significantly reduced the genotoxic effects of Dox in the comet assay, micronucleus, and ames assays. The antioxidant properties of EGCG and RA, as well as their ability to interfere with DNA repair pathways, may be involved in their genoprotective effect. Furthermore, RA decreased the intracellular reactive oxygen species generation in CHO-K1 cells. However, EGCG did not protect against oxidative stress and damaged cells due to its pro-oxidant properties at high concentrations. Dox was more selective against MCF-7 cells, EGCG and RA were found to be more selective against MDA-MB-231 cells. The synergistic effect was observed when EGCG or RA was administered together with Dox to breast cancer cells. In this study, EGCG and RA were found to be effective in decreasing Dox-induced genetic damage as well as inducing Dox's cell viability-reducing effect in breast cancer cells.

Key words: Doxorubicin, Rosmarinic acid, Epigallocatechin gallate, Genotoxicity, Chemoprotection.

ÖZET

Akyüz Helvacıoğlu, S. (2022). Doksorubisin'in neden olduğu genotoksisiteye karşı rosmarinik Asit ve epigallokateşin gallatın koruyucu etkileri. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmasötik Toksikoloji ABD, Doktora Tezi, İstanbul.

Doksorubisin (Doks), birçok kanser türüne karşı etkili bir antineoplastik ilaçtır. Fakat, Doks'un normal hücreler üzerindeki genotoksisitesi istenmeyen sonuçlara yol açabilmektedir. Fenolik bileşiklerin antitümör, antioksidan ve antigenotoksik etkileri yoğun olarak araştırılmış ve son yıllarda kullanımları oldukça artmıştır. Rosmarinik asit (RA) ve epigallocatechin gallate (EGCG), bitkiler aleminde yaygın olarak bulunan fenolik bileşiklerdir. Bu çalışmada, RA ve EGCG'nin tek başına ve kombinasyon halinde Doks kaynaklı genotoksisite üzerindeki koruyucu etkisi, komet testi, sitokinez-blok mikronükleus testi ve Ames testi de dahil olmak üzere çeşitli *in vitro* test sistemleri ile araştırıldı. Doks kaynaklı genotoksisitede serbest radikallerin de rol oynadığı gösterilmiştir. Bu nedenlerle reaktif oksijen türlerinin oluşumu da bu çalışmada incelenmiştir. Ayrıca, bu kombinasyonların Doks'un kemoterapötik etkisi üzerindeki sinerjistik/antagonistik etkileşimi, tümör hücre hatlarında araştırılmıştır. Bulduğumuz sonuçlar, EGCG ve RA'nın genotoksik etki göstermediğini, ancak Doks'un CHO-K1 ve TK6 hücrelerinde genotoksisiteyi arttırdığını göstermektedir. EGCG ve RA, komet, mikronükleus ve Ames testlerinde Doks'un neden olduğu genotoksik etkileri önemli ölçüde azaltmıştır. EGCG ve RA'nın antioksidan özellikleri ve ayrıca DNA tamir mekanizmalarına etki etme yetenekleri, genoprotektif özelliği kazandırmış olabilir. Ayrıca, RA, CHO-K1 hücrelerinde hücre içi reaktif oksijen türlerinin oluşumunu azaltmıştır. Ancak EGCG'nin yüksek konsantrasyonlarının sahip olduğu pro-oksidan etkiden dolayı oksidatif strese ve hasarlı hücrelere karşı koruma sağlayamamıştır. Doks, MCF-7 hücrelerine karşı daha seçici iken, EGCG ve RA, MDA-MB-231 hücrelerine karşı daha selektif olarak bulunmuştur. EGCG veya RA, Doks ile birlikte meme kanseri hücrelerine uygulandığında her iki meme kanseri hücre hattında sinerjik etki gözlenmiştir. Bu çalışmada EGCG ve RA'nın meme kanseri hücrelerinde Doks'un hücre canlılığını azaltıcı etkisini indüklemede ve Doks'un neden olduğu genetik hasarı azaltmada etkili olduğu bulunmuştur.

Anahtar kelimeler: Doksorubisin, Rosmarinik asit, Epigallokateşin gallat, Genotoksisite, Kemoprevensiyon.

1. INTRODUCTION AND AIM

Cancer is a fast expanding health concern also it is the second biggest reason of death, after cardiovascular diseases globally (1). There are different ways for cancer therapy. Chemotherapy uses a wide range of agents with various properties in the treatment of cancer. Such agents are used to shrink and destroy cancerous cells. In this way, they try to prevent the proliferation and metastasis of cancer cells by various mechanisms. However, these drugs do not only affect the targeted cancerous cells, but also they can create similar effects in normal and healthy cells, triggering the occurrence of secondary cancers. Anti-cancer drugs have genotoxic effects, causing genetic damage and epigenetic changes since they are designed to damage the DNA structure in tumor cells. However, one of the most unpleasant adverse effect of chemotherapy is the genotoxicity of anti-cancer drugs on normal cells, which have potential to cause secondary cancers.

Doxorubicin (Dox) is a commonly used antineoplastic agent. Dox has been demonstrated to be metabolically activated to reactive oxygen species (ROS) which can cause DNA and protein damage, and also initiate lipid peroxidation (2). DNA intercalation and disruption of DNA repair is another genotoxic mechanism of Dox (3). The genotoxicity and mutagenicity of Dox have been investigated in numerous *in vivo* and *in vitro* studies. Alexander et al. (2017) reported that Dox has increased the mutant frequency in strains of *Salmonella typhimurium* especially TA98 and TA102 with and without metabolic activation. Additionally, micronucleus formation was induced by Dox treatment in CHO-K1 cells in that study (4). It was also reported that Dox increased the frequency of micronuclei in mouse bone marrow erythrocytes in a dose-dependent manner (5).

Chemopreventive phytochemicals have an important place in the prevention or reduction of adverse effects that may occur due to exposure to antineoplastic drugs. In addition, due to their chemopreventive effects, almost half of anticancer drugs are derived from herbal products or derivatives (6). Polyphenols are a class of phytochemicals that includes various compounds found naturally in mostly edible plants. These compounds contribute to the prevention of diseases and the preservation of genomic stability by showing antioxidant, antimutagenic and anticarcinogenic effects. These natural agents

have the potential to reduce the genotoxic effects of chemotherapeutics on healthy cells. Polyphenols increase cell viability with their antioxidant effects, and prevent tumor growth by stimulating apoptosis with their pro-oxidant effects (7).

Rosmarinic acid (RA) is a common phenolic compound found in plants, which has properties such as anti-oxidant, anti-genotoxic and anti-carcinogenic activities (8). Limited studies investigated the capability of RA to prevent chemotherapeutic agent-induced DNA damage. Furtado et al. (2009a) demonstrated that RA reduces chromosome breakage and DNA damage induced by Dox in V79 cells. The same research group revealed that RA is also effective in reducing the chromosome damage induced by Dox in the mouse micronucleus assay. Although the antigenotoxic mechanisms of RA are not completely understood, the antioxidant activity of RA can contribute to the decrease of DNA damage. Also, Furtado et al. (2009b) indicated the importance of an optimum dose for the maximum protective effect of RA. Hence the assessment of dose response for antigenotoxic effects of RA against Dox and better characterization of the action mechanism of RA is being necessary for a rational administration of chemoprotective strategies (2,9).

Another phenolic compound, epigallocatechin gallate (EGCG) is the most plentiful catechin and powerful antioxidant in tea leaves. EGCG has been found to inhibit various malignancies in animal models (10). Although both EGCG and RA are phenolic compounds, they belong to different chemical classes. EGCG is tannin with flavan-3-ol structure esterified with gallic acid, while RA is a phenolic acid. As EGCG contains more phenolic –OH groups than RA, it was shown to have higher scavenging activity when compared to RA in a study conducted by Soobrattee et al. (2005), (11). Thereby, it is expected that EGCG exert stronger antioxidant and consequently antigenotoxic activity against Dox. To our knowledge, protective effect of EGCG against genotoxicity of Dox in normal cells was not investigated previously. The main goal of this thesis is to evaluate the possible protective effect of RA, EGCG alone and in combination against Dox-induced genotoxicity in normal cells.

The chemopreventive effect of phenolic compounds against cancer has also been demonstrated in several studies. Sadzuka et al. (2000) proposed that EGCG could improve Dox induced anticancer activity and raise the Dox concentrations in tumors through the inhibition of Dox outflow (12). In another study, the influence of EGCG was

investigated on Dox-resistant murine sarcoma and human colon carcinoma cell lines. EGCG showed a sensitizing effect on the cell lines if they had been treated with Dox (13). In a recent study, RA prevents the formation and multiplicity of aberrant crypt foci also tumor formation in rat colon cancer model, spares the antioxidant enzymes' activity and induces apoptosis in tumor cells by the induction of p53 and caspase pathways, thereby preventing tumor cell proliferation and evasion of apoptosis (14). Since activation of p53 in tumor cells is one of the chemotherapeutic mechanisms of Dox and EGCG sensitize cancer cell to Dox, combination of Dox, RA and EGCG can be a novel strategy that has potential in the treatment of cancer patients. Furthermore, a Dox dose adjustment to lower levels with same efficacy and fewer side effects may be possible thanks to a synergic effect of this combination along with anti-genotoxic activity. Therefore, the second aim of the thesis is to prove whether RA, EGCG alone or in combination modulate the antineoplastic activity of Dox.

In this study, it is expected that the intake of these compounds as a nutraceutical decrease the side effects of antitumor agent and raises the efficacy of the chemotherapeutic index, and this efficacy is important for enhancing the quality of life on chemotherapy. For this aim, the dose response ability of RA and EGCG alone and in combination to prevent Dox induced DNA damage investigated in CHO-K1 and TK6 cell lines using a variety of *in vitro* genotoxicity test methods including comet assay and cytokinesis-block micronucleus test. *Salmonella typhimurium* strains were used for bacterial reverse mutation assay. Also, formation of ROS as a mechanism of oxidative-DNA damage investigated in Dox, Dox-RA, Dox-EGCG and Dox-RA-EGCG treated cells.

After dose optimization of RA, EGCG and RA-EGCG combination against Dox induced genetic damage, the synergistic/antagonistic interaction of these combinations on chemotherapeutic effect of Dox investigated in breast carcinoma cell lines. Moreover, investigation of possible synergistic effect of these natural compounds on Dox anticancer effect is beneficial for improving the quality of life on chemotherapy.

2. GENERAL INFORMATION

2.1. Definition and History of Cancer

Cancer occurs as a consequence of the change or mutation of normal and healthy cells. It is a disease caused by the uncontrolled proliferation of these altered cells. It is a multifactorial disease with complex development. The primary cause of its development is Deoxyribonucleic acid (DNA) damage that prevents the DNA from being matched correctly (15). Although the words cancer and tumor are used synonymously, they are not synonyms. The tumor can be benign or malignant, but the term cancer is used for malignant tumors (16). The origin of the word 'cancer' is from Latin language. It is derived from the word 'cancrum', its literal meaning is crab, which means it does not leave its grip. Hippocrates (460-375 BC), the father of medicine, used the word cancer to describe malignant tumors, non-healing tumors and ulcers. In ancient Rome, Galen (200 AC) classified tumors and differentiated malignant tumors and explained the occurrence of cancer in a similar way to Hippocrates. Galen used 'oncos' term for cancer which means swelling. Galen's views were valid for 1300 years, and diet therapy for cancer was administered during this period (17).

Cancer has been a wide concern in the history of humans and animals from the beginning of the time. Even in dinosaur fossils dating back 50 million years BC it has been found evidence of bone tumors (18). Considering the history of cancer, it is seen that it is as old as human history. Descriptions of tumors are first found in Egyptian papyri, Babylonian cuneiform and tablets, also ancient Indian scripts. In the Edwin Smith papyrus, information about breast cancer was described in eight cases (3000 BC), soft tissue tumors and cancer in the skin, uterus, stomach and rectum were mentioned for the first time in Ebers papyrus (1500 BC), and it was stated that tumor treatment could be lethal. Tumors and tumor removal are described in Babylon tablets (19). There are over 100 different types of cancer. Cancer types are commonly named for the organs or tissues where the cancers are occured. Leukemia, brain and other central nervous system cancers are the most commonly diagnosed cancer types in children aged 0-14 years (20). Breast and thyroid cancer are the most common cancer types in 25-to 39-year-olds. In adolescents also young adults, the most common cancers are bone, brain and other central nervous system tumors, breast, cervical, colorectal, leukemia, lymphoma, melanoma, sarcomas, bone, soft tissue sarcoma, testicular and thyroid (21).

2.2. Cancer Epidemiology

The concept of cancer epidemiology emerged in the 18th century. It is a public health field that examines the prevalence of cancer cases and the causes of their occurrence. In 1713, the Italian doctor, Bernard Ramazzini, explained whether the causes of cervical cancer depend on the lifestyle or not, and in 1775 Percival Pott in chimney sweeps in London that scrotum cancer is a cancer type consisting of occupational carcinogenic risks. Geographical spread, ethnicity, age, socioeconomic class, and occupational characteristics are all factors that determine the epidemiological characteristics of cancer. It is known that approximately 70 % of cancer deaths occur in low and middle income countries (22).

Cancer is a leading cause of death and major obstacle to increasing life expectancy. According to the data announced by the World Health Organization (WHO), around 10 million people died from cancer in 2020. In the same year, the most common types of cancer causing death were lung (1.80 million deaths), colon and rectum (935000 deaths), liver (830000 deaths), stomach (769000 deaths) and breast (685000 deaths) (23). According to the Global Cancer Obervatory data for 2020, 19.3 million new cancer cases and 10 million cancer deaths were estimated. Breast cancer in women has overtaken lung cancer as the most often diagnosed cancer in women. The most common types of cancer in 2020 were breast cancer (11.7%) followed by lung cancer (11.4%), colorectal (10.0%), prostate (7.3%) and stomach (5- 6%) cancers. Lung cancer accounts for 18%, colorectal cancer for 9.4%, liver cancer for 8.3%, stomach cancer for 7.7% and breast cancer for 6.9% of cancer-related deaths of 1.8 million people. Men had a 19% higher global prevalence rate for all cancers (222 per 100.000) than women (186 per 100.000) in 2020, while rates varied widely between regions (24).

2.3. Cancer Etiology

Although cancer is among the most serious health issues in the world, its incidence varies by race, society and gender. Health scientists argue that if adequate precautions are not taken, cancer will be the leading cause of death in 2030 (25). The first step to preventing cancer is to determine the causes of cancer in humans. Cancer-causing substances are called carcinogens. Cancer formation is called ‘carcinogenesis’. Genetic damage underlies carcinogenesis. There are different factors that cause such damage.

Today it is accepted that cancer occurs not by a single factor, but by more than one factor which means cancer occurance has different reasons. The cause of cancer is multifactorial.

Diet, physical activity and obesity are important factors related to carcinogenesis. Food can show carcinogenic effects due to cooking techniques, storage conditions and food additives. It is known that adequate and balanced nutrition has an important effect in preventing cancer. It is thought that 30-40% of all cancer types can be prevented simply by changing one's lifestyle and eating habits. Obesity, impaired glucose metabolism, excessive consumption of red meat, poor fiber intake, insufficient intake of omega 6 and omega 3 fatty acids, and nutritionally malnourished diets such as concentrated sugars and refined flour products all contribute to an increased risk of cancer (26). Obesity, which occurs due to excessive nutrition and lack of physical activity, is a problem encountered in one-third of women and one-fifth of men in the world. As a result of obesity, an increase in inactive fat storage occurs, which increases biologically active polypeptides and regulator proteins called adipokines. This increase causes metastasis by increasing cell proliferation as a result of angiogenesis through a carcinogenic process. In addition, obesity affect the carcinogenic process by changing the synthesis and metabolism of sex steroid hormones. Overweight and obesity is predicted to account for 14 percent in men and 20 percent in women of all cancer deaths in a new report from a prospective cancer prevention cohort. Obesity has been linked to an increased risk of death from cancers of the esophagus, cervix, uterus and ovary, colon and rectum, gallbladder, kidney, liver, pancreas, stomach, prostate, breast (27,28). Selenium, vitamin B-12, folic acid, vitamin D, chlorophyll, and antioxidants are all protective components in a cancer prevention diet. Ascorbic acid has its advantage when used orally, but intravenously it can be extremely beneficial (29,32).

Another factor affecting the formation of cancer is age. Since the prevalence of most cancers rises with age, particularly in midlife, cancer may be considered an age-related illness. Cancer is seen at all ages. It has been determined that cancer deaths increase with age and are mostly increased in the 45-64 age range (33). Overall, cancer incidence rates increase significantly with age, from less than 25 cases per 100.000 in the under-20 age group to over 350 cases per 100.000 in the 45–49 age group and more than 1.000 cases per 100.000 in the 60 age group. According to the most recent statistics from the National Cancer Institute's Program, the median age of people diagnosed with cancer is 66.This suggests that half of cases of cancer occur with those under the age of 60, with

the other half occurring in people over 60. Numerous different types of cancer follow a similar pattern. Breast cancer, as example, is diagnosed at a median age of 62 years, whereas colorectal cancer is diagnosed at a median age of 67 years, lung cancer is diagnosed at an average age of 71 years, and prostate cancer is diagnosed at a median age of 66 years. On the other hand, cancer can occur at any age. Children and young people (under the age of 20), for example, account for around one-fourth of all cases of bone cancer. Furthermore, 12 percent of brain and other nervous system tumors affect children and teenagers, compared to just 1% of all malignancies diagnosed in this age group (34).

Sex has a significant role in the prevalence, prognosis, and mortality of a variety of cancers (35). Between 2009 and 2013, the prevalence of cancer was roughly 20% greater in males than in women in the United States, while the mortality rate was 40% higher in men (36). At the genetic/molecular level, gender variations affect cancer susceptibility. Sex hormones may potentially have a positive or negative impact on cancer growth. In the United States, men are more likely than women to get prostate, ovarian, and colorectal cancer, while women are more likely to develop lung, colorectal and breast cancer (35). Cancer occurrence in sex-specific organs such as the prostate and ovary has been documented, as well as sex variations in cancers of the colon, liver, and lung. Females are more likely than males to have thyroid cancer. Estrogen has been attributed to an enhanced risk of thyroid cancer in women (37). Sex-related genetic and biochemical variations frequently impact the degree of pharmacological response (38).

People who have a family history of cancer are more prone to developing it. Inherited genetic mutations have a great impact in around 5% to 10% of all cancers. The genetic structure of the person is one of the factors affecting the formation of cancer because of its response to environmental factors and its predisposition to cancer development. Changes in the sequence of cancer susceptibility genes (oncogenes, tumor suppressor and risk modifier genes) which increase the risk of cancer are inherited. It is known that the sensitivity genes of breast and colon cancers are among the types of cancer that may occur due to sequence changes (39).

Infectious agents such as viruses, bacteria, and parasites may also cause cancer or raise the risk of cancer developing. Some viruses may disrupt the signaling pathways that control cell growth and proliferation. Moreover, some viruses damage the immune system, causing the body susceptible to other cancer-causing infections. Chronic inflammation, which can lead to cancer is often caused by certain viruses, bacteria, and parasites. Epstein–Barr virus, a type of herpes virus, causes mononucleosis, as well as

some forms of lymphoma and cancers of the nose and mouth. Chronic hepatitis B and hepatitis C viruses infections may lead to liver cancer. Human immunodeficiency virus (HIV) impairs the immune system, leaving it more vulnerable to other cancer-causing pathogens. Many malignancies, including Kaposi sarcoma, lymphomas (including non-Hodgkin lymphoma and Hodgkin disease), and cancers of the cervix, lung, anus, throat, and stomach, are made more likely by HIV infection. Cervical cancer is usually caused by high-risk HIV. They also cause the majority of anal cancers, as well as a variety of oropharyngeal, penile ,vulvar and vaginal cancers. *Helicobacter pylori*, a bacteria, can cause noncardia stomach cancer (40-42).

Psychosocial factors have long been recognized to play a role in the progression and persistence of cancer.In particular, epidemiological and clinical research over the last 30 years has found significant indication of links between depression, social isolation and stress as well as cancer growth.Relevant signaling mechanisms that influence cancer growth and metastasis have been established in recent cellular and molecular studies (43).

The International Agency for Research on Cancer (IARC) has been assessing epidemiological and scientific data for the carcinogenicity of substances, mixtures of chemicals, manufacturing practices, lifestyle and cultural patterns. Hundreds of chemicals have been shown to cause cancer or tumors in laboratory animal. If a carcinogen binds covalently to cellular DNA, it is called genotoxic. If the affected DNA is not restored, it can induce mutations by causing base misincorporation during DNA replication. Genotoxic carcinogens may work directly on DNA or need metabolic activation to become reactive. Alkyl and aryl epoxides, nitrosamides, nitrosoureas, also some sulfonate and sulfate esters are examples of direct-acting carcinogens. Polycyclic aromatic hydrocarbons, alkyl nitrosamines, aflatoxin B1 and aromatic amines are examples of indirect-acting carcinogens. To induce a tumorigenic reaction, numerous chemical carcinogens require bioactivation (44,45).

It is known that smoking is a factor that triggers many cancers such as lung cancer, larynx, pharynx, esophagus, liver, myeloid leukemia, kidney, bladder, stomach, pancreas and oral cavity. About 60 substances in cigarette smoke have carcinogenic properties. Some of these carcinogens (such as radon, bismuth, lead and polonium) are radioactive. These substances with carcinogenic properties cause mutation and errors in DNA replication. Factors such as the starting age of smoking, the amount of cigarettes smoked daily, and the duration of smoking directly affect the development of cancer. It is considered that smoking was caused for 21% of cancer-related deaths in the world in

2000. Thirty two percent of adult deaths due to cancer are related to smoking in males and 8% in females. It is estimated that 1.3 billion people smoke worldwide and more than half of this number are men (46,47).

It is known that the type of radiation that causes cancer is mostly ionizing radiation. Ionizing radiation locally releases enough energy to break down biologically important chemical bonds. Cancer development due to radiation exposure is directly linked to the exposure dose also age of exposure to radiation. The carcinogenic effects of radiation are as follows; oncogene activation resulting from gene amplifications, chromosome translocations and point mutations, chromosome deletions, tumor suppressor gene losses and free radical formation mechanisms (48). Ionizing radiation raises the risk of cancer as a result of exposure to other medical and industrial sources of ionizing radiation. It induces the risk of many neoplasms such as leukemia, thyroid and breast cancer. In addition to ionizing radiation, many studies have been conducted on the risk of cancer development due to electromagnetic radiation resulting from the use of mobile phones. Although studies have suggested that prolonged and intensive use of mobile phones is associated with the formation of glioma, acoustic neuroma, and parotid gland tumors, these results have not been clarified yet (49,50).

Hormones also play significant role in the development of some of the world's most frequent cancers, such as endometrial, ovarian and breast cancers in women, and prostate cancer in men. Hormones are thought to influence cancer risk by regulating the rate of cell proliferation, cell division and the number of susceptible cells. Hormones have a heavy impact on cell differentiation in the endometrium; oestrogens promote mitosis, whereas progestins inhibit it. Endometrial cancer risk rises with late menopause, oestrogen replacement therapy, and obesity, and falls with parity and oral contraceptive use; hence, risk rises in relation to the length of unopposed oestrogen exposure, perhaps because unopposed oestrogens promote endometrial cell division. Early menarche raises the risk of breast cancer (51).

2.4. Cancer Treatment

Cancer is a fatal disease that is widespread in societies and is tried to be treated with different methods. Cancer treatment is determined according to the histological structure of the tumor, the presence of metastases and the stage of the disease. Cancer treatment is performed to treat patients, to keep the disease under control or to extend the

life of the patient palliative and to increase the quality of life. Although chemotherapy, radiation, and surgery are the most prevalent cancer treatment procedures, additional treatments like as hormone therapy and the use of biological methods can be employed in combination with or as a stand-alone treatment. Because each therapy has its own set of benefits and drawbacks, cancer is a unique disease with therapies that vary from person to person (52).

2.4.1. Radiotherapy

Radiotherapy is a cancer treatment that uses ionizing radiation to eliminate cancerous cells. Treatments might be targeted to a specific part of the body, or they can be treated to the entire body. The main goal in the development of these method is to create systems that have the most effect on cancer cells that need to be eliminated while causing the least amount of damage to normal cells. Intensity modulated radiotherapy systems are generally used in head and neck, brain, prostate and breast cancers, and it is a method that provides irradiation of different strength to the area to be treated, thus protecting the healthy area. In the Stereotactic Ablative Radiotherapy system, very high doses of radiation can be delivered to very small areas with great precision and increase the success rate of the treatment. Radiation therapy is usually applied before the surgical procedure to reduce the tumor. After the dosage calculations by the radiation oncologist, it is aimed to shrink the tumor area and kill the cancerous cells after the radiation given to the tissue or organ. However, one of the biggest disadvantages of radiotherapy is that while it kills harmful cells, it also kills healthy ones. Furthermore, common adverse effects such as hair loss, vomiting, weakness, diarrhea, skin changes and loss of appetite can affect the patient's quality of life. Other therapeutic methods could be considered as a result (53,54).

2.4.2. Surgical Methods

It can be used alone as a common therapy method or in combination with radiotherapy and /or chemotherapy. It is frequently used as a prophylactic in the prevention of cancer development, in diagnosing biopsy and in removing the cancerous tissue from the body in cases where there is no metastasis or growth. In cases where it is not possible to remove the mass without damaging other tissues, it may prefer to reduce the mass with chemotherapy or radiotherapy. Surgery can finally be used in the restoration and reconstruction of the damaged tissue or area (55).

2.4.3. Immunotherapy

Immunotherapy is a biological treatment. The immune system's reaction to internal and external stimuli is utilized in this sort of treatment.In this therapy, three primary molecular groups are widely utilized.These are antibodies, cytokines, and cells. The treatment's goal is to stimulate the immune system to target cancerous cells. This can be accomplished either through the body's natural immune system or through the use of synthetic stimulants like monoclonal antibodies. Cancer is treated by a variety of immunotherapies. Immune checkpoint inhibitors work by inhibiting checkpoint proteins from binding to their target proteins (56). Therefore, T cells do not get the 'off' signal, leading them to kill cancer cells. The CTLA-4 checkpoint enzyme is the target of one of these medications. Other immune checkpoint inhibitors work by targeting the checkpoint proteins PD-1 and PD-L1. T-cell transfer treatment enhances T cells' natural capacity to fight cancer. This treatment involves removing immune cells from the tumor. Those that are most effective against cancer are chosen or changed in the lab to better target cancer cells, then injected back into the body through a vein needle. Vaccines for cancer treatment are a type of immunotherapy that enhances the body's inherent anti-cancer responses.Treatment vaccines may enable the immune system to recognize and respond to antigens, helping cancer cells to be killed. Cytokines (interferons, interleukins), which are proteins generated by white blood cells, are examples of immune-modulating agents. Pain, swelling, soreness, redness, itchiness, and rash are typical side effects in all forms of immunotherapy. Further possible adverse effects include edema and weight gain due to fluid retention, heart palpitations, nasal congestion, diarrhea, infection risk, and organ inflammation (57,58).

2.4.4. Hormone Therapy

Hormones can be used as drugs in cancer treatment. These are generally sex hormones used to treat prostate, endometrial and breast cancers. However, these drugs resemble chemotherapy drugs, the biggest difference is that the hormones produced by the body naturally cannot bind to the cell and prevent the growth of cancer cells. Drugs used in hormone therapy can generally be divided into 3 subgroups. These are hormone synthesis inhibitors, hormone receptor antagonists and hormone supplementation. Hormone synthesis inhibitors, work by suppressing synthesis where the hormone is produced. They can be divided into two within themselves. Aromatase inhibitors and

gonadotropin-releasing hormone analogs. Aromatases are enzymes responsible for converting androgens to estrogen in menopausal women. Aromatase inhibitors inhibit this conversion, reducing estrogen levels and inhibiting cell growth. Drugs such as anastrazole and letrozole are used to treat breast cancer patients with positive estrogen receptors and reduce death due to breast cancer. Gonadotropin-releasing hormone analogues are used to treat prostate and ovarian cancers by chemically sterilizing organs. The production of testosterone in men and estrogen in women is suppressed (59,60). Hormone receptor antagonists are molecules that bind to the receptor of a particular hormone on the cell, preventing that hormone from binding, and thus prevent the growth of the cell from getting a growth signal into the cell and stopping the growth of the cell. They are divided into anti-androgens and anti-estrogens. Testosterone needs 17α-hydroxylase to be produced. Anti androgens block 17α-hydroxylase and inhibit testosterone production. Drugs such as bicalutamid and flutamide can be used in this therapy. Treatment can be performed using drugs known as selective estrogen receptor modulator and drugs such as hormone receptor positive breast cancer (Tamoxifen), metastatic breast cancer (Fulvestrant), and progressed breast cancer (Toremifen) in postmenopausal women (61,62).

As a supplement of hormone, synthetic progestogens such as progestin are used in breast, prostate and endometrial cancers. Also, they can be used to treat prostate cancer by suppressing testosterone production with drugs such as synthetic estrogens (diethylstilbestrol) (63).

2.4.5. Cancer Vaccines

Cancer vaccines are similar to conventional vaccines that use attenuated molecules to stimulate the immune system (B or T cells) and prevent diseases. The only difference is that cancer cells are targeted. Vaccines produced against viruses such as hepatitis B and human papilloma virus trigger B lymphocytes to produce antibodies. To date, no cancer vaccine has been produced with proven effectiveness. The FDA has approved only 2 vaccines (Gardasil and Cervarix) developed against HPV that causes uterine cancer. The vast majority of uterine cancers develop due to human papillomavirus infection, and type 16 and type 18 constitute the high risk group (64).

2.5. Classification of Antineoplastic Drugs

Cancer drugs affect cancerous cells by different mechanisms. Anticancer drugs may affect cancer cells, extracellular matrix, endothelium, the immune system or host cells at various levels. This effect is often to induce cell death (cytotoxicity), less frequently to inhibit its growth without killing the cell (cytostatic effect). The least common effect is the induction of differentiation in the cancerous cell. The combination of surgery, radiotherapy and chemotherapy applications, which is now called a multidisciplinary approach, increases the success in the treatment of many cancers. Systemic approaches have an important place in the treatment of cancer, which is accepted as a systemic disease. In the systemic treatment of cancer, there are two main uses. These are cancer chemotherapy, one of which includes cytotoxic drugs and hormones; the other is immunotherapy methods and biological therapy with anti-angiogenic agents (65).

2.5.1. Alkylating Agents

Alkylating agents are the most commonly used anticancer medications and they are a big part of combination chemotherapy. Alkylating agents bind to cellular proteins and DNA, are highly reactive molecules.Cross-linking of DNA strands is the key mechanism of action for the most alkylating agents. Cisplatin, carboplatin, oxaliplatin, chlorambucil, cyclophosphamide, mechlorethamine, and melphalan are alkylating agents which are used widely in chemotherapy . The nitrosoureas, tetrazines, aziridines (thiotepa and mitomycin C), and nonclassic alkylating agents such as procarbazine and hexamethylmelamine are all known as alkylating agents. Alkylating agents are used to treat cancers such as breast cancer, lymphoma, multiple myeloma, Hodgkin's disease and others. Some alkylating agents have been related to clinical toxicities, including bone marrow depression (including leukopenia, anemia and thrombocytopenia), nausea and vomiting. Gonadal dysfunction and late leukemias can be caused by chlorambucil, mechlorethamine, melphalan, and procarbazine. Pulmonary fibrosis may be caused by busulfan, chlorambucil, carmustine and lomustine. Cyclophosphamide and ifosfamide can trigger hemorrhagic cystitis, as well as bladder cancer in a small percentage of patients. Renal damage may be caused by cisplatin, carmustine, lomustine, and streptozocin. Ototoxicity and peripheral neuropathy are potential side effects of carboplatin and cisplatin. Procarbazine is a monoamine oxidase inhibitor with a low

potency. When combined with sympathomimetic agents and tricyclic antidepressants, it may induce hypertensive reactions (66,67).

2.5.2. Antimetabolites

The antimetabolites are a class of anticancer agents that are structurally similar to natural substrates. Antimetabolites act by mimicry or interfering with pyrimidines and purines, that are necessary to synthesis of DNA and subsequently to inhibit DNA synthesis. Antimetabolites affect cells in the S phase of the cell cycle during DNA replication. Methotrexate, 5-fluorouracil, capecitabine, floxuridine, gemcitabine, cytarabine, decitabine, and vidaza are some of the most important medicines in this group. Colorectal, bladder, and pancreatic cancers, as well as leukemia, are often treated with antimetabolites. Antimetabolites have the potential to cause significant side effects. Antimetabolites influence all proliferating cells, resulting in immunosuppression, vomiting, nausea, ulcerative stomatitis, diarrhea, hemorrhagic enteritis, and intestinal perforation, although there are few long-term end-organ consequences that affect anesthetic management (68,69).

2.5.3. Topoisomerase Inhibitors

Topoisomerase inhibitors are chemotherapeutic drugs that block the topoisomerase enzymes (topoisomerase I and II), which regulate DNA structure changes. Topoisomerase I and II are natural enzymes which are required for cellular division and DNA replication and they are found in the nucleus of mammalian cells. Topoisomerase activity is very strong in cancer cells and rapidly dividing cells. Topoisomerase inhibitors inhibit ligation stage of the cell cycle, which causes apoptotic cell death and DNA single strand breaks and double strand breaks. Topoisomerase inhibitors are derived from natural sources and act by interfering with DNA to cause cytotoxicity. Topoisomerase I inhibitors include topotecan, irinotecan and camptothecin, whereas topoisomerase II inhibitors include doxorubicin, etoposide and epirubicin. Colorectal, small-cell lung, ovarian, and hematological tumors are also treated with topoisomerase inhibitors. Camptothecin is a topoisomerase I inhibitor which is commonly recognized as the most effective anticancer drug in the world. Topotecan and irinotecan are two varieties of camptothecins that have been approved to treat lung, colon and ovarian cancer by the

FDA. The most common side effects of topoisomerase inhibitors are hematologic (neutropenia, anemia, thrombocytopenia) and gastrointestinal (diarrhea, nausea) (70-72).

2.5.4. Antimicrotubule Agents

Antimicrotubule drugs act by stabilizing microtubules and subsequently reducing cell division. Microtubules are involved in numerous cellular functions, including the development of the mitotic spindle during the mitotic phase. Cancer treatment has had success with these highly dynamic mitotic-spindle microtubules. Antimicrotubule agents are antimitotic chemicals obtained from plants that inhibit cell proliferation. These agents, act by the polymerization of the tubulin protein in the structure of the strands that provide mitotic division within the cell, make them unstable and ultimately stop mitosis. It is a phase specific agent effective in the M phase of cell division. Antimicrotubule agents such as vinca alkaloids and taxanes cause microtubule instability. Vinca alkaloids including vincristine and vinblastine bind to tubulin dimers and inhibit them from polymerizing, blocking tubulin polymerization. Taxanes like paclitaxel and docetaxel, on the other hand, have opposing modes of action. Microtubules are stabilized by these agents, which adhere to them and keep them from depolymerizing. In this way, mitosis is delayed or stopped during the metaphase-anaphase transition, resulting in apoptotic cell death. Asymptomatic sinus bradycardia and first-degree atrioventricular block are the most frequent side effects of paclitaxel (73).

2.5.5. Antibiotics

Antibiotics with pro-apoptotic, anti-proliferative and anti-epithelial-mesenchymal-transition properties have been used in the treatment of cancer. Antibiotics are secondary metabolites formed in the course of life by microorganisms (such as actinomycetes, bacteria and fungi) as well as higher animals and plants that have anti-pathogen or other functions and can interact with growth of other living cells. Antibiotics can induce apoptosis of cancer cells, prevent cancer proliferation also inhibit metastasis of cancer cells, according to research findings. They are widely used to succeed in the cancer treatment for these purposes. Anticancer antibiotics are substances formed by microorganisms that have anticancer properties. They are mostly peptides and anthraquinones in structure, and they have a powerful inhibitory effect on malignant cancers' proliferation, rapid development, and metastasis (74).

2.5.5.1. Anthracyclines

It is well known that anthracyclines constitute a group of the most widely used and effective antineoplastic agents in the world. In the 1960s, anthracycline antibiotics were first isolated from the *Streptomyces peucetius* and *Streptomyces caesius* fungi and used in clinical oncology (75). The composition of anthracycline antibiotics is dependent on the anthracyclinone. The anthracyclinone (anthraquinone) or (naphthaquinone) center is surrounded by quinones, phenolic groups, and other substituents on the reasonating ring structure A, B, and C, as well as the saturated D ring. In antibiotics with antitumoral properties, the effect occurs in two ways. First, the intercalation to DNA and the inhibition of DNA synthesis in this way; the second is through inhibition of topoisomerase I and II enzymes. They interact with helical unwinding of DNA by inhibiting topoisomerase I and II, that are necessary to replicate DNA. Breast cancers, leukemia, aggressive lymphomas, non-Hodgkin's lymphoma, lung cancers and many solid and hematologic malignancies are mostly treated with anthracyclines. Anthracyclines, mitomycin, bleomycin, actinomycin, guanorycin, and endiyne are among the most popular anticancer antibiotics. Furthermore, their anticancer properties are both complex and efficient. Anticancer antibiotics including anthracyclines include epirubicin, mitoxantrone, daunorubicin and doxorubicin (74).

2.6. Doxorubicin

In the 1950s, cancer treatment was began to search from soil-based microorganisms. *Streptomyces peucetius*, a recent strain that produces a bright red pigment, has been isolated and shown to have anti-tumor effect in mice. A new antibiotic has been produced from this bacterium. The new antibiotic, called daunorubicin, has been used to treat lymphoma and acute leukemia successfully (76). Doxorubicin (Dox) was isolated from a mutant strain obtained from *Streptomyces peucetius* in Italy in 1967 (Farmitalia Research Laboratories). *Streptomyces peucetius var. caesius*, derived from *S. peucetius*, differs from parent culture by the mutagenesis, vegetative and due to its ability to produce antibiotics (77).

Dox is a drug used in cancer chemotherapy. It is an anthracycline derivative antibiotic. Dox was used in treatment in 1967-1969 and is the most widely used member of this group (78). Dox, is a cytotoxic chemotherapy agent, also known as Adriamycin

that is commonly used as the first line of defense in the treatment of cancer, either alone or together with other anticancer agents or radiation and surgery (79).

2.6.1. Chemical structure of Doxorubicin

Dox is an anthracycline-structured antibiotic, which consists of a chromophoric tricyclic ring and two identical pentapeptide rings attached to it. Anthracycline molecule consists of tetracyclic core and amino sugar (daunosine). The tetracyclic core gives the drug its red color. All members of the group have quinone and hydroquinone groups adjacent to the tetracyclic ring. Dox is formed by the attachment of the hydroxyl group to the carbon number 14 in the daunorubicin molecule (80). The substituent moieties and the quinone-hydroquinone system define a rigid Dox structure. The amino group of the amino-sugar group is the most reactive Dox group, which allows Dox to bind with various substances that may differ in affinity to water. Thanks to this feature, Dox can show different amounts of accumulation in different cell structures (81).

The chemical structure of Dox are shown in Figure 1. Systematic name is (7S,9S)-7-[(2R,4S,5S,6S)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8,10-dihydro-7*H*-tetracene-5,12-dione. $C_{27}H_{29}NO_{11}$ is molecular formula of Dox (82).

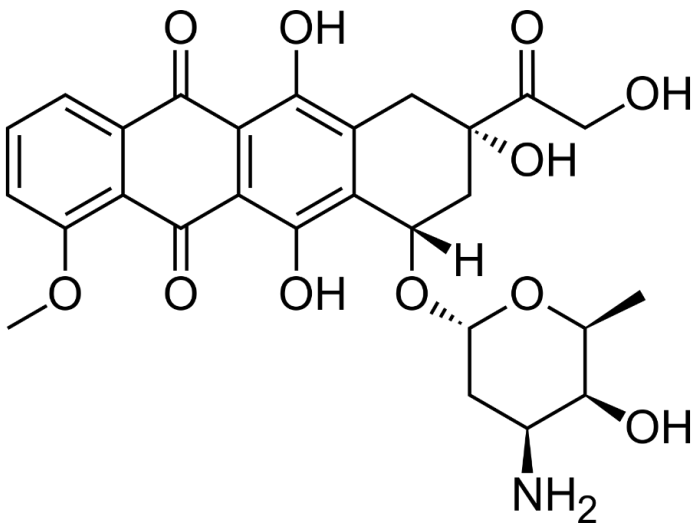

Figure 1. Chemical structure of Dox

2.6.2. Pharmacokinetics of Doxorubicin

Dox is not well absorbed from the gastrointestinal tract, therefore it is administered intravenously (IV). Its main metabolic properties are the reduction of a ketone group to a hydroxyl group. A less active derivative -ol is usually formed from this parent component. It is mainly metabolized in the liver. It breaks down rapidly after IV application. As a result of this break down Doxorubicinol and daunorubicinol are formed. The main metabolite of Dox is 13-OH-doxorubicinol produced by aldoketo reductases, which have a certain antitumor activity. Dox is identified by a fast dispersion stage and a slow elimination stage. Dox is quickly taken up by tissues outside of the brain. Although it is rapidly metabolized in the liver, it remains in the body for a long time and its effect is long since it binds to tissues and is released from there slowly; most of it is excreted in bile. If liver function is impaired due to liver metastasis or primary tumor or other reason, its elimination slows and accumulates in the body if the dose is not reduced. The aglycon part that is partially released in the body as a result of hydrolysis is not effective. Dox is eliminated by converting to various less active or inactive products such as alcohol derivative, aglycone ring and other derivatives. It is excreted from the liver and kidneys. It is excreted by biotransformation from the liver and excretion from the bile. The excreted substance is doxorubicinol and daunorubicinol. In the liver, reduction occurs first. The half-life of Dox is 12-18 hours and it is dispersed within this period. Plasma protein binding of Dox is approximately 75%. However, it cannot cross the blood-brain barrier. The total plasma clearance is aproximately 30 l/h/m^2. At steady state, the total volume of distribution is around 15 l/kg (83,84).

2.6.3. Treatment with Doxorubicin

One of the most commonly used chemotherapeutic drugs is Dox. It is preferred in first-line treatment in children and adult patients. It is also indicated for superficial bladder tumors after transurethral resection (preventive treatment) and when administered intravesically for therapeutic purposes. It is used in the treatment of cancer types listed below (85).

- Acute Lymphoblastic Leukemias
- Acute Myeloblastic Leukemia
- Endometrium Cancer
- Soft Tissue Sarcomas

- Urothelial Carcinoma
- Bronchogenic Carcinoma
- Stomach Carcinoma
- Kaposi's Sarcoma
- Hodgkin Lymphoma
- Metastatic Breast Cancer
- Non-Hodgkin Lymphoma
- Multiple Myeloma
- Mycosis Fungoides
- Neuroblastoma
- Metastatic Ovarian Cancer
- Ovarian Cancer
- Bone Sarcoma
- Sezary Syndrome
- Soft Tissue Sarcoma
- Thyroid Carcinoma
- Waldenstrom's Macroglobulinemia
- Wilms Tumor
- Uterine Sarcomas
- Thymoma

It is commonly used in hematological malignancies and solid tumors due to Dox's broad spectrum antineoplastic effect (86).

2.6.4. Mechanism of action

Cytotoxic and cytostatic effects of anthracyclines on tumors and organs have been explained by four main mechanisms (87).

a. Cytotoxic and cytostatic effects of anthracyclines on tumors and organs have been explained by four main mechanisms. Binding to DNA by forming a complex: It enters the DNA and forms the DNA topoisomerase II-DNA-anthracycline complex. Inhibits DNA replication and RNA transcription and synthesis. It also causes DNA strand breaks.
b. Inhibition of RNA and DNA polymerases: It disrupts RNA transcription and DNA replication and by inhibiting the functions of DNA and RNA polymerases.

c. Effects on the cell membrane: By binding to the cell membrane, it disrupts the membrane function.
d. Dox also causes DNA oxidative damage caused by free radicals, reducing DNA synthesis even further (78).

Dox's mechanism of action is not completely understood because of its complexity, it is thought that it interacts with DNA through intercalation and inhibits DNA synthesis. Dox, the planar chromophore part intercalates non-covalently by entering between two base pairs in DNA. It binds itself to DNA with high efficiency by a mechanism called intercalation, where DNA base pairs are placed between and linked in parallel to the base pairs. Dox inhibits DNA replication and RNA transcription by intercalation (88,89).

Topoisomerase II is a common target for anticancer drug families that intercalate into DNA. Topoisomerase II works dependent on adenosine triphosphate (ATP). Its basic biological function is the opening of superhelix DNA strands and the replication fork's formation. Therefore, it has a significant function in DNA replication. Following chromosomal replication, they allow the separation of DNA molecules that are interlocked and intermingled. It is required for DNA replication of eukaryotic and prokaryotic cells. Topoisomerase I cuts a strand of the DNA double helix, allow it to relax, and afterwards reconnect the truncated helix. Topoisomerase I enzymes do not need ATP for hydrolysis. Topoisomerase II enzymes break double strands of DNA and add two super-annular turns to DNA by changing the number of links. At clinically relevant and cytotoxic concentrations, Dox stabilizes DNA and complexity of enzyme. During transcription, the progress of the enzyme Topoisomerase II, which opens the helix structure of DNA, is inhibited. Higher concentrations of Dox inhibit topoisomerase II activity, but once the drug exits the DNA, topoisomerase II inhibiton is reversed. As cells begin dividing, levels of topoisomerase II raises across the genome. There are three factors inhibit this enzyme; low temperature, high salt concentration and high drug concentrations. It is a fact that all these factors regulate the cytotoxicity of Dox (90). Drugs that interact with topoisomerase I and II are used in cancer treatment and are promising for future use. However, these agents themselves can cause mutations and cancer by showing genotoxic effects in normal cells (91).

Dox can also inhibit the activity of the polymerase enzyme, affect gene expression regulation also DNA damage caused by free radicals. Dox itself has an anticancer effect against most of cancers. Dox is not a cell cycle specific drug. Depending on the molecular structure of Dox, the anticancer mechanism is related to its quinone structure. When

oxido-reductive enzymes such as the quinone component of anthracyclines are present, cytochrome P450 reductase, xanthine oxidase and NADH dehydrogenase, they can undergo redox reactions where they produce excessively ROS. Free radicals are formed during the conversion of quinone to semi-quinone. Hydroxyl radicals, superoxides, and peroxides are formed when free radicals combine with oxygen. Furthermore, cellular iron catalyzes redox processes and produces ROS. Excessive ROS that cannot be detoxified causes lipid peroxidation ,oxidative stress and DNA damage which leads to cell death (92).

2.6.5. Development of Doxorubicin Resistance

The ineffectiveness of a treatment, such as an antineoplastic, is called drug resistance. Cancer cells can develop resistance to treatments. Although many types of cancer are initially sensitive to chemotherapy, they can develop resistance over time through a number of mechanisms (93). There are different resistance mechanisms that develop in cancer cells against chemotherapeutic agents such as Dox. Some of these mechanisms are multi-drug resistance (MDR-1) or P-glycoprotein (P-gp). There are mechanisms mediated by drug transport proteins such as breast cancer resistance protein (BCRP), multidrug resistance-associated protein family (MRP) and lung resistance protein (LRP) (94,95). Another resistance mechanism is related to the resistance to apoptosis induced by radiation and cytotoxic agents in tumor. Resistance mechanisms include inhibition of the chemotherapeutic action producing apoptosis, mutations in cytostatic drug target enzymes, and alterations in the p53 gene. P-gp functions in an ATP-dependent way, and it has been discovered that it decreases the quantity of drug in the cell by forcing the drug to be pumped out. Resistance to numerous anticancer medicines, including the hydrophobic Dox, can develop when P-gp levels increase. P-gp is present in a variety of tissues in humans. Although many tumor cells express P-gp, additional research has revealed that P-gp is connected to the ATP-dependent carrier protein (ABC proteins), which causes multi-drug resistance and is involved in transport (96). The ABC transporter family is encoded by approximately 49 genes. According to their sequence similarities, they were divided into seven subgroups as ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, ABCG (94,97). Among these groups, 14 members are responsible for the transportation of drugs. ABC transporter proteins are located in the cell membrane and are the primary active transporters operating in the presence of ATP, enabling the

transport of various endogenous compounds and xenobiotics across membranes. ABC transporters are expressed in both normal tissues and many cancerous tissues. MRP-1 is a member of the ABCC subfamily of the carrier ABC family. This protein is 190 kDa in size and is encoded by chromosome 16. Significant resistance to Dox occurs as a result of overexpression of MRP-1(3). MRPs generally carry anionic organic drugs. It is from the ATP-dependent carrier protein family, as in MRP and P-gp in BCRP. Overexpression of BCRP reduces the amount of intracellular drug, which causes drug resistance, albeit indirectly. Mechanisms of resistance to anticancer drugs are (98):

- Multi-Drug Resistance
- Decreased Intake of the Drug into Cells
- Decreased Biotransformation of the Drug to its Active Metabolite
- Accelerating the Repair of DNA Damage
- Epithelial-Mesenchymal Transition and Metastasis
- Increasing the Synthesis of the Target Enzyme
- Decreased Tendency of Cell to Apoptosis
- Increase of Glutathione or Glutathione-S-Transferase Enzyme in Cancerous Cell
- Epigenetic Mechanisms
- Cancer Cell Heterogeneity

Resistance to Dox is another problem that limits the use of the drug. The development of Dox resistance is multifactorial. One of these mechanisms is the increase in the activity of the efflux pumps which throw Dox out of the cancer cell and the decrease in the accumulation of the Dox. One of the most common changes in Dox resistance is raised genes expression for proteins which is involved in the efflux (99).

2.6.6. Toxicity of Doxorubicin

Dox is the most effective antineoplastic drug among antibiotics with antitumor activity. Dox causes acute and chronic toxic side effects that are dosage dependant. Significant side effects of anthracycline drugs are cytotoxicity and cardiotoxicity. It has the potential to induce multi-organ toxicity in various patients (100). Dox has been commonly used as an anticancer medication for more than four decades in the treatment of a variety of solid tumor and hematological malignancies, with a proposed maximum cumulative dose of 500 - 450 mg/m^2 of body surface area (101). Dox has a cardiotoxic cumulative dosage of 550 mg/m^2. The main disadvantage of Dox is the toxicity on healthy

cells. Even though it is approved as an chemotherapeutic agent, possible cardiotoxicity. Cardiotoxicity is a cardiomyopathy or heart failure and it can develop years after ending of cancer treatment and remission of the tumor (102). Children treated for lymphoblastic leukemia with Dox therapy develop cardiovascular problems in adulthood about 15–20 years following treatment (103).

The most likely mechanism of Dox toxicity is due to the increase in oxidative stress. Acute vomiting, nausea, gastrointestinal disorders, hair loss, and neurological disturbances (commonly hallucinations and lethargy) were prominent adverse effects in early clinical evaluations (phase II and III studies) of Dox in the 1970s (104). Dox is a significant exogenous ROS producer that, when taken in cancer treatment, may cause delayed cardiomyopathy. Dox-induced ROS over-production is mediated by mitochondrial NADPH oxidase activity and occurs in mitochondria (105). Oxidative stress was found to be important for Dox-induced systemic damage in a previous study (106). ROS are formed during the oxidative stress process as a result of Dox treatment, which is associated with organ injury and cell death. Dox-induced ROS generation is expected to trigger an apoptotic cascade (107). In this way, the generation of H_2O_2 by Dox (a process that is regulated by the GSHPx enzyme, which is found in both the cytosol and the mitochondria) has a significant effect in the onset of cardiac apoptosis, as it does in mammalian endothelial cells (108). The quinone structure associated with the cytotoxic activity of Dox not only has anticancer properties, but also causes the formation of free radicals. The enzyme cytochrome P-450 reductase in the microsomes reduces Dox to the free radical intermediate semi-quinone. While this unstable metabolite reverts to its Dox form, it reduces the oxygen molecule, leading to the formation of superoxide anion radical and hydrogen peroxide, which cause breaking in DNA strands. It has been reported that these free radicals cause DNA damage and oxidative stress in cells (3,109).

Dox is not specific to tumor cells and it can effect healthy cells as well. As a result, the number of immune cells decreases, the immune system weakens, leaving the patient more vulnerable to microbial infections, fatigue, and a longer recovery period.The severity and frequency of these side effects are determined by the Dox dose and the patient's bone marrow's regenerative capacity. When Dox is continuously injected into a small artery, phlebosclerosis can develop, and if Dox erupts to surrounding organs or tissues, necrosis could develop. Cellulitis, thrombophlebitis, and painful saturation are other extravasation side effects that restrict joint movement (104,110). Figure 2 shows the organs where Dox causes toxicity.

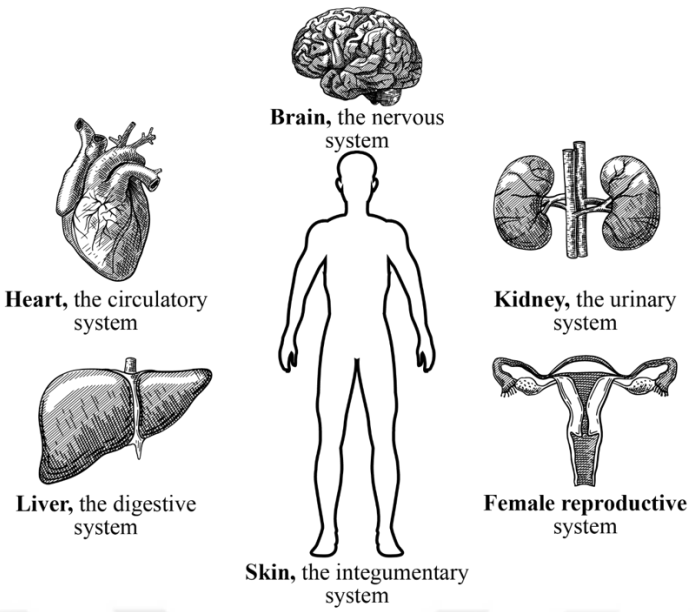

Figure 2. Organs affected by Dox

Adverse effects which may occur while using Dox are;

- Secondary Malignancies
- Cardiomyopathy and Arrhythmia
- Liver changes
- Skin and nail hyperpigmentation
- Severe Myelosuppression
- Hypersensitivity – Anaphylaxis
- Gastrointestinal Problems
- Tumor Lysis Syndrome
- Peripheral Sensory and Motor Neuropathy
- Phlebosclerosis, phlebothrombosis, and thrombophlebitis
- Extravasation and Tissue Necrosis
- Conjunctivitis, Keratitis, Lacrimation
- Increased risk of getting an infection
- Tiredness and fatigue
- Alopecia
- Weight gain

2.6.7. Toxicity in the Heart

Cardiotoxicity is the most serious form of Dox-induced toxicity, it can be caused by a variety of pathways. As a result, Dox must be administered in a dose-restricted manner. There is evidence to show that Dox-induced cardiotoxicity is associated to inflammation, apoptosis and oxidative stress (111). Blood biochemical parameters such as cardiac superoxide dismutase, brain natriuretic peptide and fatty acid-binding protein were all affected by Dox. Dox-induced cardiotoxicity is caused by a decrease in the antioxidant system as well as an increase in oxidative stress. Patients are impacted in different manners, with acute and chronic illnesses. Dox causes structural changes in the cardiomyocytes of the heart, causing them to enlarge (112).

2.6.8. Toxicity in the Brain

While investigating on Dox sourced brain toxicity, it's crucial to note that Dox can't pass the blood–brain barrier, and therefore any toxicity to the brain is indirect (113). TNF- α is stimulated by Dox, which causes microglial cells in the brain to release inflammatory cytokines. TNF-α stimulates the production of inducible nitric oxide synthase (iNOS), which causes an increase in the the levels of reactive nitrogen species (RNS) when it is generated in excess. Proteins undergo nitration when RNS levels rise, and after protein nitration drives ROS production, mitochondria permeability changes. Apoptosis causes the release of cytochrome c from mitochondrial pores, resulting in cell death. Finally, these activities are responsible for cognitive impairment in numerous parts of the brain (114).

2.6.9. Toxicity in the Liver

The liver is another prominent location for Dox-induced tissue damage and cell death, with 40 % of patients experiencing some type of liver impairment after therapy. Liver is known for detoxification and metabolism activity and it metabolizes high concentrations of Dox, producing high amounts of ROS. As a result, ROS produces an excessive amount of damage, including DNA damage, lipid peroxidation, a decrease in vitamin E levels and reduced glutathione (GSH), and an imbalance in oxidative processes (115).

2.6.10. Toxicity in the Kidneys

Dox-induced nephropathy develops when the Dox interferes with mitochondrial function and reduces the activity of mitochondrial complexes I and IV. As a result of lipid peroxidation, the levels of superoxides, triglycerides and citrate synthase rise, while the levels of antioxidant compounds and vitamin E decrease. Hypertension, steroid resistance, and proteinuria are all symptoms of glomeruli-affecting illness, which can progress to renal failure (104).

2.6.11. Dox-Induced Secondary Cancers

It is accepted that malignant secondary tumors different from primary tumors may develop due to treatment during or after chemotherapy. Today, secondary tumors constitute 16% of cancers. Secondary cancers are among the most common causes of treatment-related deaths. The most common secondary tumor in treatment with DOX is acute myeloid leukemia (AML). Therapy-associated acute myeloid leukemia (t-MDS/t-AML) and myelodysplastic syndrome are thought to be the direct result of mutations induced by anticancer drugs. Translocations in bands involving t-AML, chromosome 11q23 or 21q22 have been characterized in patients administered topoisomerase II inhibitors (116).

Dox is classified as a category 2A carcinogen by the IARC in a 1987 report (highly probable carcinogen in humans) (117). The National Toxicology Program indicated in its 14th report issued in 2016 that Dox has the potential to be a human carcinogen based on carcinogenicity data in experimental animals (118). However, there are no epidemiological studies evaluating the relationship between cancer development and exposure to Dox in humans. Although, leukemia and bone tumor development have been reported in some patients with the use of Dox in combination with alkylating agents and radiotherapy (119). IARC (1987) concluded that the compound was carcinogenic to rats after intravenous and subcutaneous injection. Tumor formation in the bladder has been observed after intravesical instillation of Dox (120). Bertazolli et al. (1971) evaluated the carcinogenicity of Dox was administered to female Sprague-Dawley rats in a single dosage (8 mg/kg) IV.In a short time, the incidence of breast cancers increased. However, no tumors were observed in control animals (121). Marquardt et al. (1976) confirmed the rise in the incidence of breast tumors found by Bertazolli et al (1971). In this study, a single dose IV injection containing 5 mg/kg Dox was applied to Sprague Dawley rats, it

was reported that 16 fibroadenoma and 3 adenocarcinoma occurred in 17 Dox-administered rats after 1 year of observation (122). In the study of Bucciarelli (1981), 5 and 10 mg/kg Dox was applied to female and male Sprague-Dawley rats intravenously. Numerous breast tumors, mainly adenocarcinoma, were observed (123). Jang et al. (1987) investigated the oncogenic potential of Dox in Sprague-Dawley female and male rats. Dox 2 and 10 mg/kg doses were applied as a single IV injection or repeated dose of 2 mg/kg/day. Several mammary tumors, mostly fibroadenomas, were seen in 30-41% of female rats with a single dose (2 mg/kg) or repeated doses (5x2 mg/kg). Tumor incidence increased by 8% compared to the control group. In the low dose group, two male rats developed breast cancers. In addition, renal cell tumors were also observed in the low and single dose administered rats (124). In another study, the incidence of nodular hyperplasia and papilloma showed a significant increase in female Fischer F344 rats administered 1 mg/ml intravenous Dox compared to the control group (125).

2.6.12. Dox-Induced Genotoxicity

Genotoxicity and myelosuppression , that can cause to secondary malignancy, as well as dose-dependent cardiotoxicity, are serious side effects of Dox treatment. Increased oxidative damage, apoptosis and inflammation are the mechanisms behind Dox toxicity. The production of free radicals is thought to be the fundamental mechanism behind Dox's genetic damage. It induces mutations and the development of secondary malignant neoplasms by causing DNA damage and disrupting DNA replication. Dox's cytotoxic effect is based on DNA intercalation, which is caused by a decrease in topoisomerase II enzyme activity. DNA intercalation occurs when two adjacent base pairs in a DNA strand come into contact, interrupting DNA synthesis and transcription and leading apoptosis (126).

Drug safety evaluations need a variety of preclinical tests. Among these assays, genotoxicity and carcinogenicity tests are critical for defining and predicting chemical safety (127). The Ames test was performed to determine Dox's mutagenicity in the presence and absence of metabolic activation in the TA98, TA100, and TA1537 strains at four different concentrations (6.5, 32.5, 65 and 130 mM). Furthermore, using a Chinese hamster ovary cell line, the cytogenetic effect of Dox was assessed using chromosomal aberration and sister chromatid exchange assays. All bacterial strains used in the absence of metabolic activation were shown to be mutagenic as a consequence of this research.

Chromosome breaks and sister chromatid exchange have also been recorded (128). The Ames test was performed on *Salmonella* TA 98 and 100 strains in another study investigating the genotoxicity of Dox. In the presence and absence of S9, Dox showed a significant mutagenic effect in the TA98 strain. In the same study, Dox caused chromosome breaks and sister chromatid exchange in V79 cell line. In addition, in the *in vivo* micronucleus test, Dox (1.25 mg/kg) caused an increase in the micronucleus frequency (129). In another study in the V79 cell line, Dox caused an increase in mutation frequency dose-dependently compared to the control group (122). Dox's mutagenic and cytotoxic effects was tested in the V79 cell line and *Salmonella typhimurium* TA1535, TA1538, TA100, and TA98 bacterial strains in another research paper. While there was no notable rise in the number of revertant colonies in strain TA1535, the number of colonies in strain TA1538 increased significantly (130). In another Ames test evaluating the mutagenicity of Dox, it was strongly mutagenic against frame-shift-sensitive strain TA98 without S9. The effects observed in TA98 were not seen with TA102, a strain sensitive to oxidation mechanisms (131). Clements et al. (1984) researched the mutagenicity of Dox in *Drosophila* germ cells. Dox induced sex-related recessive lethal mutations at concentrations between 250 µg/ml and 1 mg/ml. The highest concentration (1 mg/ml) was found to be toxic to flies. However, a lethality frequency of 4.40% was observed in this experiment, suggesting that Dox has a significant mutagenic effect on mature sperm (132). Another study conducted on patients who used Dox for therapeutic purposes and did not receive any other chemotherapy or radiotherapy. Blood samples were taken from patients before and after treatment. Structural chromosomal changes and sister-chromatid exchange were investigated in short-term phytohemagglutinin-stimulated lymphocytes. In patients who were given a single 800 mg IV Dox dosage, a proportional increase was found in the frequency of both parameters examined. The increase in the number of sister-chromatid exchanges was seen to persist several months after the end of treatment (133). In recent years, the link between the cardiotoxic effect of Dox and its genotoxicity has been investigated. Dox was applied to Wistar rats at a dose of 15 mg/kg intraperitoneally and DNA strand breaks in heart, liver and kidney tissue samples were evaluated in Comet assay. When compared to the control group a significant increase in tail and tail density was observed in heart, liver and kidney tissues. According to the results, Dox-induced cardiotoxicity may cause genotoxicity by forming DNA strand breaks in cardiac tissue (134). As a result, Dox was genotoxic in a variety of *in vivo* and *in vitro* assays.

2.7. Doxorubicine And Photochemicals

Since 1940, diet and nutritional factors have been a focus of research in the etiology of cancer. Nutrition is an important part of cancer treatment. During cancer treatment, a decrease in the risk of infection, rapid recovery, better tolerance of treatment-related side effects, and better feeling are provided with nutrition (135). Antineoplastic chemotherapy's main goal is to prevent or eliminate the tumor's development and proliferation without damaging the patient's healthy cells. However, because most anticancer medicines are not selective, they harm both healthy and malignant cells. It has been argued that medicines having systemic toxicity, such as Dox, cannot be given at levels that will impact cancer cells without harming healthy cells. As a result, it has been determined that using combination treatments that permit the medication to be effective without damaging normal tissues is appropriate. Agents with antigenotoxic or antimutagenic effects can be used for chemoprevention purposes. US National Cancer Institutes data show that, there are 400 agents for chemoprevention. These agents, synthetic or natural, delay, prevent or slow down the progress of carcinogenesis. Chemoprevention is divided into 3 groups as primary, secondary and tertiary. Agents in the tertiary chemoprevention group protect cancer patients from secondary tumors (136).

Phytochemicals naturally found in herbal products and processed foods obtained from them can be used for chemoprevention purposes. Phenolic compounds, flavonoids, alkaloids, organosulfur compounds, nitrogen-containing compounds, and carotenoids are all types of phytochemicals.Phytochemicals play a function in oxidative damage protection, cell cycle control gene modulation and DNA repair, apoptosis induction, angiogenesis inhibition, tumor cell invasion and spread regulation also epigenetic alterations. Furthermore, by directly engaging with a mutagenic agent, it can prevent the mutagenic substance from connecting with DNA (137).

The studies in the literature on herbal products showing a protective effect against genotoxicity caused by Dox are as follows.

Copaifera lansdorffii Desf. is a type of tree known as 'copaiba' or 'copaiva' and grows in parts of Brazil. The genotoxic potential and the antigenotoxic potential of the hydroalcoholic extract of the leaves of this tree were assessed using the mouse peripheral blood MN test induced by Dox. The major heteroside flavonoids were found to be quercetin and afzelin in the analysis by high pressure liquid chromatography. In antigenotoxic assessment, animals were given different concentrations of the

hydroalcoholic extract at the same time 15 mg/kg of Dox were administered intraperitoneally. Blood samples were taken 24 and 48 hours after Dox application. The results showed that the number of MN in animals treated with extract and Dox was much lower than in animals treated with only Dox. One or more active chemicals' antioxidant activity was considered to have a role in this plant's protection against Dox-induced genotoxicity (138).

Bixin, found in the seed of the annatto tree (*Bixa Orellana* L.) is a natural red dye which is commonly used in food industry. The impacts of bixin on the genotoxicity and cytotoxicity induced by Dox in the promyelocytic leukemia cell line HL60 were investigated. Bixin showed cytotoxic effects at doses greater than 0.3 mg/ml, but no mutagenic or genotoxic effects were found in HL60 cells. Also, bixin decreased DNA damage caused by Dox. In HL60 cells, Dox and bixin had no apoptotic impact, but their simultaneous combination treatments increased the number of apoptotic cells (139).

Cell viability and apoptosis were investigated on the combination of silymarin (*Silybum marianum* extract) and Dox. MCF-7 cell line was used and this cell line is estrogen-dependent breast cancer. Trypan blue staining was used to determine cell viability and apoptosis after 16, 24, and 48 hours. It has been stated that silymarin has a synergistic impact on Dox's therapeutic potential, and that it may be more effective at increasing Dox's therapeutic potential while reducing its dose-limiting adverse effects (140).

The possible protective effects of thymoquinone, which has antioxidant and antimutagenic effects, on chromosomal damage induced by 0.15 µg/ml Dox in isolated human lymphocyte culture were investigated. L-carnosine and curcumin, whose antigenotoxic and antioxidant effects are well known, were compared with the possible antigenotoxic effects of thymoquinone. At the end of 72 hours of lymphocyte culture, oxidative stress parameters and 8-hydroxydeoxyguanosine, a biomarker of oxidative genotoxicity, were measured. Dox caused a significant increase in oxidative stress. Thymoquinone, L-carnosine and curcumin provided the highest protection against Dox-induced toxicity, respectively (141).

Apigenin is one of the best known flavonoids. It is the main flavonoid in medicinal plants and spices. Apigenin has been found as an antioxidant and antimutagenic chemopreventive agent. Due to these properties, the protective effects of apigenin against genotoxicity caused by Dox were investigated. In the bacterial reverse mutation test, it has been stated that apigenin has a protective effect against mutation induced by 0.2

μg/plaque Dox in the concentration range of 10-400 μg/plaque in TA98 and TA100 *Salmonella typhimurium* strains (142).

Grapes and grape products have been the subject of research because they are a source of flavonoids and have antioxidant properties. Grape seed extract is a polyphenol mixture that includes catechin, epicatechin also their gallate derivatives. Its protective role against Dox-induced genotoxicity and cardiotoxicity was investigated in mice by micronucleus and chromosomal aberration tests. Malondialdehyde and glutathione levels, which are indicators of lipid peroxidation, were measured in the heart tissue. Dox caused heart damage due to a significant alteration in cardiac histology. In the Dox treated group with Dox, a significant increase in the level of malondialdehyde and glutathione were found in the heart tissue. Dox induced genotoxicity by increasing micronucleus and chromosomal aberrations. Cardiac tissue was significantly preserved in the group that received the extract before treatment with Dox, and this protection was thought to protect the heart tissue thanks to the antioxidant properties of the extract. It also provided protection effect against Dox caused genotoxicity (143).

In a study, the effect of RA on preventing Dox-induced chromosome breaks and DNA damage was investigated. For this, micronucleus and Comet analysis were performed on the V79 cell line. Cell culture was applied at concentrations of 0.28-1.12 mM with RA alone or in combination with 0.5 μg/ml concentration of Dox. The results showed that RA had no genotoxic effect. It was observed that the three concentrations tested significantly reduced micronucleus frequency and DNA damage caused by Dox. RA's antioxidant activity was thought to play a role in reducing DNA damage which induced by Dox (9).

2.7.1. Rosmarinic Acid

Rosmarinic acid (RA) is an ester of caffeic acid and 3, 4-dihydroxyphenyl lactic acid. RA is most widely found in members of the Boraginaceae family, the Lamiaceae subfamily and Nepetoideae as well as in lower plants like hornworts and ferns (144). Scarpatti and Oriente, Italian scientists, discovered and described RA from rosemary (*Rosmarinus officinalis* L.) in 1958, and named it after them (145). Systematic name is (2*R*)-3-(3,4-dihydroxyphenyl)-2-[(*E*)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxypropanoic acid. $C_{18}H_{16}O_8$ is the chemical formula of RA. The chemical structure of RA was illustrated in Figure 3.

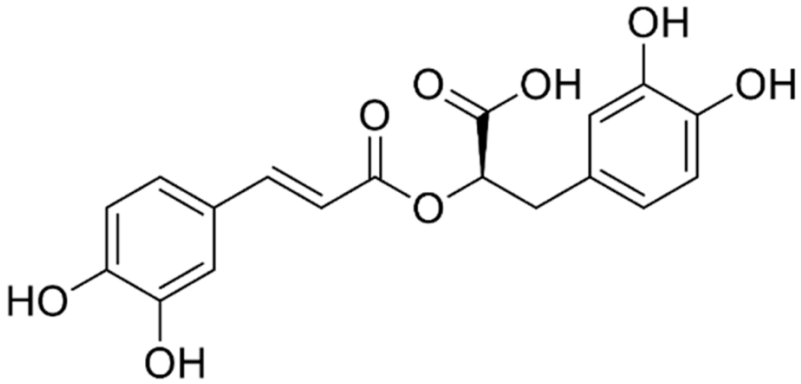

Figure 3. Chemical structure of Rosmarinic acid

RA is a health-promoting compound found in spices, medicinal plants and herbs. RA is one of the polyphenolic substances found in plants such as rosemary (*Rosmarinus officinalis* L.), perilla (*Perilla frutescens* L.), peppermint (*Mentha arvense* L.), sage (*Salvia officinalis* L.) and basil (*Ocimum basilicum* L.). Polyphenols are a group of chemicals known to affect carcinogenesis, tumor cell proliferation, apoptosis, metastasis and inflammation. RA is thought to act as a preformed constitutively accumulating defense component in plants (144). RA is well absorbed from the gastrointestinal tract and skin. Consumed RA may be in a non-systemically metabolized state or may also exist as numerous metabolites such as *m*-phenylhydroxypropionic acid, *m*-coumaric acid, sulfated caffeic, ferulic and coumaric acid forms (146).

Anticancer, antioxidant, anti-inflammatory and antimutagenic effects are only a few of the biological effects of RA (147). RA, as well as several isolated chemicals from rosemary, including carnosic, carnosol and ursolic acids have been found to reduce the possibility of tumor formation in a variety of organs, including the stomach, colon, breast and liver. Various therapeutic potentials of RA are shown in Figure 4.

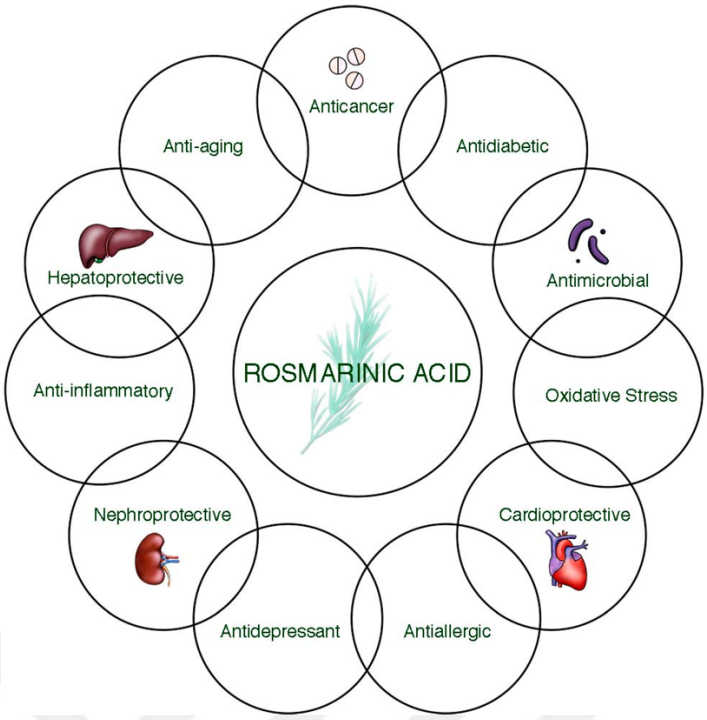

Figure 4. The potential functions of Rosmarinic acid

2.7.1.1. Protective effects of Rosmarinic acid

Many studies have shown that RA has anticancer properties via multiple molecular pathways. RA can be absorbed via bone, muscle, and skin it also suppresses epidermal inflammatory reactions and decreases prostaglandin production. As a result, RA is used to prevent skin cancer (148).

RA decreases cyclooxygenase-2 promoter activity and protein levels in colorectal (HT-29) cells and inhibits their growth. Furthermore, because of its anti-inflammatory characteristics, RA has anti-colon cancer capabilities (149). RA treatment suppressed lung cancer (A549) cells by up to 50%, mediated by COX-2 activity suppression via hydrogen bonding at Ser353 and Arg120 in COX-2 active sites (150). RA was found to reduce activity of DNA methyltransferase in the MCF7 cell line (151). Human ovarian cancer cells showed sensitivity to RA, which was increased when RA and cisplatin were combined. Cell growth was inhibited after several stages of the cell cycle were stopped. There was also the induction of apoptosis (152).

Whether RA causes genotoxicity or not, has been investigated in various studies with different methods. The genotoxic effect of RA on peripheral blood and brain tissue was investigated with comet assay. Animals were treated with i.p. injection of RA at dose

level of 2 or 8 mg kg^{-1}. The comet tests showed that RA had no effect on DNA damage. At 2 and 8 mg kg^{-1} concentrations of RA, it did not show any genotoxic effect in both tissues (153). In the study conducted on ethanolic extract of Tuscan *Rosmarinus officinalis*, rosmarinic acid was found as its main constituents. The Ames test demonstrated that ethanolic extract was not genotoxic towards both TA98 and 100 strains (154).

Cytotoxicity and genotoxicity of *Rosmarinus officinalis* total extract were evaluated in Balb-C mice's erythrocytes with the micronucleus assay. The extract was given orally in three dosages (30, 100, and 300 mg/kg). Micronucleated erythrocytes and micronucleated polychromatic erythrocytes did not increase. In the same study, *Rosmarinus officinalis* was also investigated for its ability to protect against cyclophosphamide-induced DNA damage. A dose of 100 mg/kg of extract was applied orally to evaluate the DNA protecting effect. Following that, cyclophosphamide (50 mg/kg) was administered. *Rosmarinus officinalis* extract was able to decrease micronucleated erythrocytes and micronucleated polychromatic erythrocytes frequency by reducing cyclophosphamide caused DNA damage. The extract's lack of genotoxic and cytotoxic effects, as well as the fact that it decreases the genotoxicity induced by cyclophosphamide, indicates its potential therapeutic use (155).

Another study investigated the protective effects of RA against DNA damage by ethanol in mice. The comet assay was used to analyze peripheral blood and brain cells, while the micronucleus assay was used to evaluate bone marrow cells. In peripheral blood and brain cells, the mean damage frequency and damage index values of ethanol following pre-treatment with RA were considerably lower than the ethanol group.The findings suggest that RA can reduce DNA damage induuced by ethanol (156).

Studies have also been conducted with aqueous and ethanol extracts of *Rosmarinus officinalis* on human lymphocyte against DNA damage induced by H_2O_2. The comet test was used to determine the damage of DNA lesions. The DNA damage induced by 100 µM H_2O_2 was reduced by the ethanol extract of rosemary at the dosages tested. Antioxidant components in rosemary ethanol extract can prevent DNA damage in human lymphocytes (157).

In a study using V79 cells, the capacity of RA to inhibit chemically induced chromosomal loss or breakage as well as DNA damage was investigated using comet and micronucleus tests.The DNA-damaging agent was Dox (0.5 µg/ ml), a chemotherapeutic drug. The cells were given different concentrations of RA alone or in combination with

Dox. RA did not exhibited genotoxicity, although it did decrease the number of micronuclei and the level of Dox-induced DNA damage (9).

In another study, the micronucleus assay was used to assess the antimutagenicity and/or mutagenic activity of RA on Swiss mice's peripheral blood cells. The different doses of RA were used with the chemotherapeutic drug Dox (15 mg/kg) in antimutagenicity test. After treatment, micronucleated polychromatic erythrocytes were examined in peripheral blood. In mice given different dosages of RA, the frequency of micronuclei was not increased. In comparison to mice treated with Dox alone, the frequency of micronuclei was dramatically reduced when varied doses of RA were combined with Dox (2).

The antimutagenic and DNA protective effects of RA was investigated in another study. In this study, *Salmonella typhimurium* tester system and the supercoiled DNA strand scission assay were performed. In the Ames test, the protective effect of RA against potent mutagens N-methyl-N-nitro-N-nitrosoguanidine and sodium azide also how RA affects the DNA repair system were investigated. RA was found to have antimutagenic activity against N-methyl-N-nitro-N-nitrosoguanidine and sodium azide in the TA100 strain. It was found that RA (500 μg/plate) reduced the mutagenicity induced returns of *S. typhimurium* colonies by 20%. Using a supercoiled DNA test, the effectiveness of RA treatments to protect supercoiled DNA against 2,2-azobis (2-amidinopropane) dihydrochloride-induced oxidative DNA damage was investigated. In the group administered with RA, the DNA supercoiled was preserved by 95 percent (158).

RA has an antigenotoxic mechanisms which are not fully understood. However, the antioxidant activity of RA may contribute decrease DNA damage (159). All cell lines included in the studies with RA showed a proliferative effect instead of cytotoxic activity. A limited number of researches have investigated the ability of RA to prevent DNA damage caused by chemotherapeutic agents.

2.7.2. Epigallocatechin gallate

Green tea is known for its health benefits,which is an infusion of the tea plant. In East Asia, green tea has been consumed as a beverage for centuries. Green tea contains numerous polyphenols with similar chemical structures, with the exception of the heterocyclic C ring. Green tea is mostly consists of flavanols. Flavanol catechins's weight

is nearly one-third of the dry leaf. This drink contains high levels of antioxidants, namely polyphenols.

Epigallocatechin gallate (EGCG) is a phenolic antioxidant that may be found in many plants, including black and green tea. EGCG belongs to the catechin family of phytochemicals and is the ester of gallic acid and epigallocatechin (160). Systematic name is [(2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2H-chromen-3-yl] 3,4,5-trihydroxybenzoate. The molecular formula of EGCG is $C_{22}H_{18}O_{11}$. Green tea's most abundant catechin is EGCG. Catechins have been observed to have antioxidant, anticancer, cardiovascular, and neurodegenerative properties, as well as the ability to scavenge ROS and RNS (161). While the two gallocatechol rings in its structure give EGCG a strong antioxidant and chelating properties, each of these rings can capture free radicals directly from the environment with high efficiency. EGCG has a higher antioxidant activity than other green tea catechins and is effective in radical scavenging, according to studies[160]. As seen in Figure 5, it is a complex molecule structure consisting of a gallate ester, a gallocatechol group and a flavanol core (flavan-3-ols) (162).

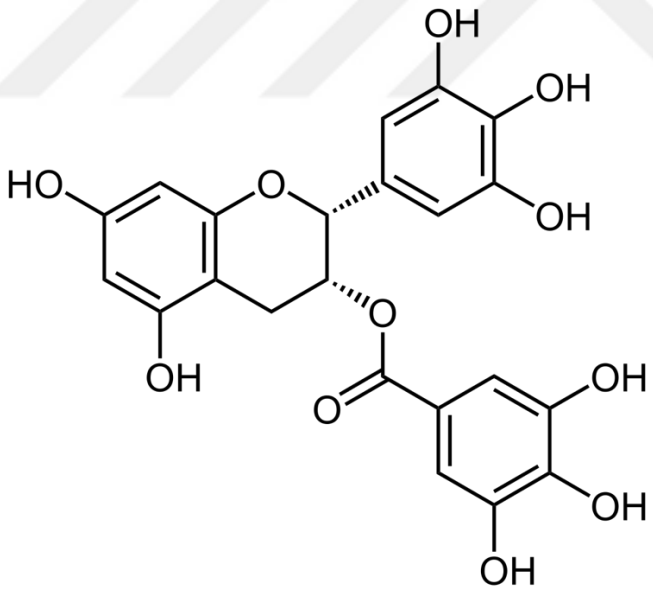

Figure 5. Chemical structure of Epigallocatechin gallate

Studies have shown that EGCG has inhibitory effects on numerous of aberrant alterations which include antioxidant, anticancer, anti-inflammatory, antifibrosis effects and anticollagenase, among others. The characteristics and applications of EGCG is shown in Figure 6.

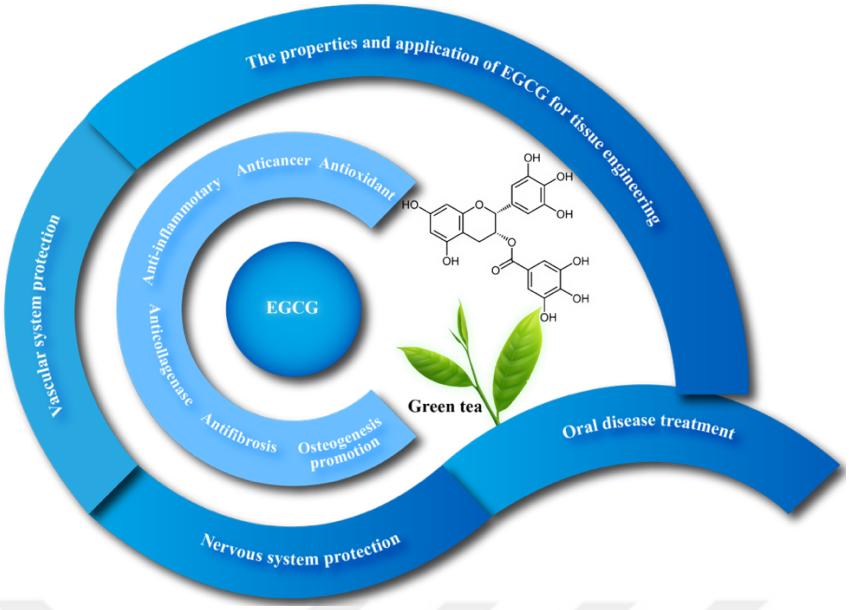

Figure 6. The characteristics and applications of EGCG

Green tea is the most common source of EGCG, although it may also be found in small amounts in other foods including nuts (pecans, hazelnuts, and pistachios), fruits (cranberries, strawberries, blackberries, cherries, apples, pears, peaches, kiwis and avocados), white, oolong, and black teas (163).

EGCG has 25 times more antioxidant activity than vitamin E and 100 times more than vitamin C. The bioavailability of catechins is limited because their absorption is low and their elimination is high. The catechin with the longest half-life among catechins is EGCG (173 min). EGCG is poorly absorbed when taken orally. EGCG, which is the equivalent of 8-16 cups of green tea per day, might cause nausea and heartburn in some people. In 1.7 hours after ingestion, the blood level of EGCG reaches its maximum level. Although absorbed EGCG has a plasma half-life of around 5 hours, most unaltered EGCG is eliminated in the urine within 0 to 8 hours (164). It's crucial to remember that EGCG isn't completely risk-free. Supplements containing EGCG have been linked to serious adverse effects such as liver and renal failure, anemia, low blood sugar and dizziness (165). EGCG may interfere with the absorption of several prescription medicines, such as cholesterol-lowering and psychiatric medications (166).

In experimental animal studies and *in vitro* studies, EGCG has been shown to have antimutagenic, antiproliferative, pro-apoptotic and antiangiogenic effects. As a conventional adjuvant in cancer therapy, EGCG might minimize dangerous side effects

and provide addictive or synergistic benefits. However, EGCG can also lower therapeutic effect to some extent, limiting its practical use (167).

2.7.2.1. Antimutagenic and Anticarcinogenic Potential of EGCG

According to the National Cancer Institute , EGCG is a potential anti-cancer agent. EGCG suppresses enzymes associated with the expression of cancer-associated genes, for example, telomerase and DNA methyltransferase. *In vitro* and *in vivo* studies have demonstrated that EGCG can induce apoptosis and suppress cancer development in a wide range of malignancies, such as kidney, colon, breast, and brain tumors, as well as leukemia (168). The ability of EGCG to decrease cancer cell growth, cell cycle arrest and induce apoptosis without damaging healthy cells is linked with its chemopreventive effects (169).

Green tea consumption has been linked to a lower risk of breast cancer in epidemiological, laboratory, and clinical studies. In a double-blind study on 103 postmenopausal women, 400 mg of EGCG and 800 mg of EGCG were given to one group once a day for 2 months. These clinical data and in in vivo and in vitro study findings support the consume of tea polyphenols as chemopreventive agents in adjuvant therapy in breast cancer. EGCG has been shown to have beneficial effects on LDL cholesterol and blood sugar levels.The effect of green tea against weight gain after breast cancer therapy has also been studied because of its potential for cardiovascular disease prevention and weight control. Improvement in carbohydrate metabolism, slight decrease in weight and increase in HDL-cholesterol concentration values were observed in overweight breast cancer patients (170,171).

The pro-apoptotic effects of EGCG have also been shown in *in vivo* and *in vitro* studies of green tea extract and tea catechins in tumor cells. EGCG showed moderate pro-apoptotoic activity in different breast cancer cell lines. Although, in 4T1 cells, a higher rate of cell apoptosis was found in the treatment with EGCG and taxol compared to the effect of taxol alone. EGCG is chemically a metal chelating agent and has pro-oxidant and antioxidant activity depending on its doses. Other studies have demonstrated that green tea inhibits angiogenesis, and green tea extracts and EGCG dose-dependently reduce the transcription of vascular endothelial growth factor. Furthermore, EGCG has been found to inhibit breast cancer cells and the proliferation of human umbilical vessel endothelial cells (172,173). EGCG induces apoptosis in many human cell lines like

human prostate cancer cell lines, human epidermoid carcinoma cells, breast carcinoma cells, melanoma cells, and pancreatic cancer cells (174–177).

In another study, it was investigated the efficacy of EGCG on genetic and epigenetic factors regulating autophagy in chronic myeloid leukemia cells (K-562). EGCG administration caused a significant reduce in the expression level of genes that are positive regulators of autophagy in K-562 cells. In line with the data obtained, it was determined that EGCG causes autophagy inhibition in chronic myeloid leukemia cells by genetic and epigenetic regulation (178).

EGCG suppresses the induction of apoptosis, inhibition of cell replication, development and growth of the neoplasm. EGCG also inhibits urokinase, a proteolytic enzyme. Urokinase is the enzyme that cancer cells use to metastasize. EGCG inhibits mellatoproteinase-2 and metalloproteinase-9. These two enzymes are involved in tumor metastasis and invasion. EGCG has both anti-matrix metalloproteinase and anti-angiogenesis effects (179).

Different studies have been done to evaluate the antimutagenic effect of EGCG. A study was conducted investigating the antimutagenicity of EGCG. The goal of this study was to see how EGCG affected the development of DNA damage and micronucleus in Chinese hamster cells caused by N-methyl-N′-nitro-N nitrosoguanidine and H_2O_2 . The role of EGCG in apoptosis was also investigated in the human leukemic cell line. The findings demonstrate that EGCG both protects cells from genotoxicity also promotes tumor formation by apoptosis (180).

Morley et al. analyzed the effect of EGCG, which is known to have a chemopreventive effect, on UV-caused DNA damage in cultured human cells using comet test. They stated that EGCG dose-dependently reduced DNA damage in lung fibroblasts, skin fibroblasts and epidermal keratocytes (181).

2.8. Genotoxicity studies

Genotoxicity is a common term that refers to damages to the nucleus, chromosomes and DNA structure, including DNA breaks, DNA attachments,gene mutations, chromosomal abnormalities, aneuploidy and clastogenicity. Genotoxic effects are defined as genotoxic compounds which interact with enzymes or DNA. Agents that generate DNA mutations have an effect on DNA either directly or indirectly by adhering to proteins produced based on genetic information.

In eukaryotic organisms, damage to the somatic cells's genetic material can cause to cancer. The genetic damage of germ cells, on the other hand, may result in heritable mutations that cause birth defects. Any type of mutation can occur, including deletion, insertion and duplication of nucleotides. These mutations have the potential to cause a wide range of health problems, including cancer (182).

One of the best solutions to control DNA damage sourced from carcinogens or mutagens is to identify antimutagens and anticlastogens that directly inhibit or suppress the mutagenicity as well as demutagens that remove mutagens (183).

2.8.1. Usage Areas of Genetic Toxicity Tests

Since the 1970s, genetic toxicity tests have been used to evaluate the carcinogenic potential of genotoxic and mutagenic chemicals (184). Genotoxicity and mutagenicity studies are crucial for determining the risks of pharmaceuticals, cosmetics, medical devices, food additives, agrochemicals, industrial compounds, natural toxins and nanomaterials for regulatory purposes. *In vitro* and *in vivo* assays have been developed to identify direct or indirect damage to genetic material caused by a variety of mechanisms. These tests end up different genotoxicological results such as changes in the structure and number of chromosomes also point and frameshift mutations to evaluate genotoxicity and mutagenicity.

The basic goals of genetic toxicology are to identify mutagens by genotoxicity studies, determine risk in humans, and avoid unnecessary exposure to these compounds. It's critical to assess a compound's potential genotoxicity and mutagenecity early on in the drug development process (185). It is widely accepted that in drug development, genotoxicity data should be examined before beginning phase I trials, and that clinical research should only begin if genotoxicity is not present. Medications that have the potential to induce direct DNA damage should only be used in life-threatening situations (186). In addition, these tests are used as biomonitoring tests in cancer prevention, determination and follow-up of cancer susceptibility (187). *In vitro* genotoxicity is applicable in primary cells, bacteria, primitive eukaryote and cell lines. *In vivo* genotoxicity testing are used to confirm the results of chromosomal tests performed *in vitro.* Genotoxicity tests *(in vivo* and *in vitro*) may be used to provide information about the compound's potential to induce mutations directly or indirectly, as well as a warning about the possibility of carcinogenicity. among the usage areas; to monitor populations

in terms of disease and environmental exposures, to determine the effects of occupational exposures, and to reveal the toxicity mechanisms of various factors. Even though assays are cheap, they have good statistical power, can be replicated and can detect a wide range of genotoxic end-points. In order to be used in a tiered standard approach, genotoxicity tests have been accepted in globally verified recommendations. Recently, it has also been used to evaluate the possible mutagenic or genotoxic effects of plants origins as well as their antimutagenic and genoprotective effects.

The most widely used standard *in vitro* and *in vivo* mutagenicity tests, which provide a correlation between the mutagenic and carcinogenic potentials of the substances to be investigated using genotoxicity tests that are Ames test, comet assay and micronucleus test.

2.8.2. *Salmonella*/Microsome Mutagenicity Test

The *Salmonella*/microsome mutagenicity test, also known as Ames test, is one of the bacterial test systems used in the investigation of chemical substances' mutagenic effects. This widely used method was developed by Bruce N. Ames in 1972. Although many tests are used for the determination of genotoxicity, the Ames test is often preferred because it provides standard and reliable results. Since 1970s, the procedure of the test has become precise and its results are widely valid. Although the reliability of this test varies depending on the carcinogenicity and toxicological infrastructure of the mutagenic chemical, it is mostly used alone in the evaluation of the genotoxicity of the chemicals.

It also has a wide range of applications since it is rapid, inexpensive, and simple to implement. This test is also commonly used to detect point mutations in tumor suppressor genes in somatic cells and experimental animals, as well as to identify antimutagenic and anticarcinogenic compounds that prevent chemicals from interacting with DNA and thus eliminating their mutagenic and carcinogenic effects (188).

Salmonella typhimurium mutant strains with different mutations in various regions of the histidine operon are used in Ames test. It is performed in the presence or absence of cytochrome P-450 enzymes from rat liver post mitochondrial supernatant (S9). Counting self-mutating colonies that can develop in histidine-free media is used to measure mutagenicity (Figure 7). The frequency of bacterial colonies growing via reverse mutation rises statistically considerably when a positive mutagen chemical is present in the environment (189,190).

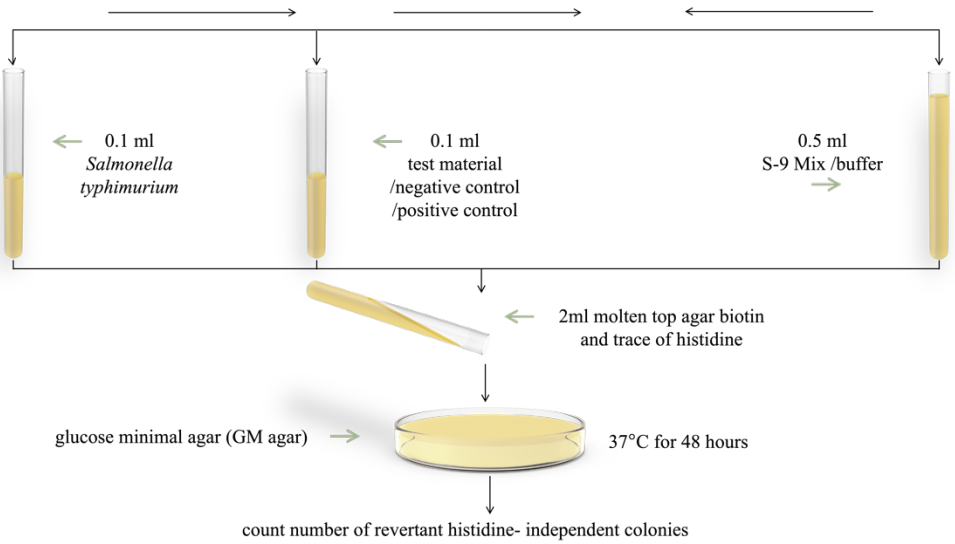

Figure 7. Application of the Ames test

Each test strain carries either a base pair replacement mutation or a frameshift mutation in the histidine operon. In order to increase the susceptibility of initially developed his$^-$ mutants to various chemicals, some mutations have been added to these strains.

The Rfa mutation occurs in genes encoding the lipopolysaccharide layer of the bacterial cell wall. As a result of the Rfa mutation, which partially destroys the lipopolysaccharide barrier of the cell wall, the entry of large molecules that normally cannot pass through the cell wall is facilitated (191).

The UvrB mutation is caused by the deletion of the UvrB gene, which encodes the enzyme that undertakes the task of excision repair in the DNA repair system. UvrB mutation results in increased susceptibility to reveal many mutagens. However, during the truncation of the UvrB gene for technical reasons, this deletion extends to the biotin gene. Therefore, besides histidine, biotin is also needed for bacteria to grow (192).
For better determination of mutagens, *Salmonella typhimurium* T100 and TA 98 strains were obtained by adding pkM 101 R factor plasmid carrying ampicillin resistance gene to *Salmonella typhimurium* TA1538 also TA1535 strains (193).

Plasmid-containing strains are more responsive to agents that have been shown to be mutagenic than strains without plasmids. A strain of *Salmonella typhimurium* TA97, which has the same mutagenic specificity as *Salmonella typhimurium* TA1537, but is more sensitive to frameshift mutagens because it contains the pKM 101 plasmid, has been developed. In order to detect oxidative mutagens, *Salmonella typhimurium* TA102 strain

was developed. This strain has a his G428 mutation and the pAQ1 plasmid carrying a tetracycline resistance gene (189). The genetic characteristics of Salmonella mutant strains used in mutagenicity tests are shown in Table 1.

Table 1. Genotypes of widely used bacterial strains in the Ames test

Strain	Histidine mutation	Type of mutation	pKM 101 (R Factor)	(Lipopolysaccharide)
TA98	his D3052	Frameshift mutation	+	*rfa*
TA100	his G46	Base pair substitution	+	*rfa*
TA97	his D6610	Base pair substitution	+	*rfa*
TA102	PA01 his G428/his	Base pair substitution	+	*rfa*
TA1535	his G46	Frameshift mutation	-	*rfa*
TA1537	his C3076	Frameshift mutation	-	*rfa*
TA1538	his D3052	Frameshift mutation	-	*rfa*

The Ames test has found a wide application area in determining the mutagenicity of chemicals. This test system is used to determine the mutagenic properties of chemicals as well as their antimutagenic properties. The Ames test can be applied in 2 ways; plaque incorporation and spot test. These methods can be used in two ways. The direct mutagenicity of the chemical can be measured. In addition, indirect mutagenicity of the chemical can be tested by adding an S9 mixture to the test medium. The chemical can be classified as a direct or indirect mutagen if the number of proliferating bacterial colonies exceeds the number of revertants (188).

2.8.3. Micronucleus (MN) Test

The extranuclear bodies of the damaged part of chromosome is called micronucleus (MN). MNi originating from whole chromosome or acentric chromosome fragments, which are not included in the nucleus, that occur during mitosis of the cell, usually resulting from deficiencies in the genes controlling the cell cycle, kinetochore, errors in the mitotic spindle or other parts of the mitotic apparatus, and chromosomal damage. MN is used to determine the toxicity of genotoxic substances. Investigating DNA damage at the chromosome level is an essential aspect of genotoxicity testing since chromosomal mutation is a critical event in carcinogenesis. This test is gaining importance since it can be used instead of the chromosomal aberration test. The increase in the number of MN is evaluated as an indirect indicator of chromosomal irregularities in cells caused by various agents and genomic instability in somatic cells. In studies, it has been found that the frequency of MN is significantly higher in people exposed to various physical and chemical agents, cancer and various diseases characterized by genomic dysregulation (194,195).

The MN test has become a common test used in genetic toxicology research because it can be applied *in vivo* and *in vitro* on all cell types formed via mitosis (196). In the *in vitro* MN test, cytochalasin-B substance is added to the cell cultures incubated in appropriate media. This substance inhibits cytokinesis and provides the formation of binucleate (double-nucleated) cells that complete a cell cycle. After incubation time is over, the cell culture is harvested in accordance with the protocols and the proportion of binucleate cells containing MN in the preparations is determined. In the *in vivo* MN test, the frequency of MN in mammalian erythrocyte cells whose cytokinesis is not blocked is determined (197).

The *in vitro* micronucleus test not only allows clastogens to be detected as genotoxicants, but it also allows them to be distinguished from aneugens. Chromosome loss is mostly caused by aneugenic substances interfering with the spindle apparatus (198). Whole chromatids or chromosomes form in MN due to mitotic spindle failure, centromeric DNA hypomethylation, kinetochore damage and abnormalities in the cell cycle control system (199).

2.8.4. Comet Analysis (Single Cell Gel Electrophoresis)

Ostling and Johanson invented the single cell gel electrophoresis method in 1984 to detect single cell DNA damage. Cells were placed in an agarose gel on slides, treated with salt and detergent solutions, lysed, and DNA was released by electrophoresis under neutral conditions, according to this developed procedure. The use of electric current ensured that fragmented and lighter DNA fragments to migrate quickly from the nucleus. Especially in cells with increased double-strand breaks, the migration of broken DNA from the cathode to the anode was visualized by staining it with a fluorescent dye. Because they resemble comets in appearance, these images called comets were measured and evaluated (200). Single-strand breaks could not be detected in neutral condition, which allowed for the detection of DNA double-strand breaks. Many agents that induce DNA damage cause more single-strand breaks than double-strand breaks. Furthermore, in neutral circumstances, proteins cannot be entirely removed. For these reasons, the microgel technique based on electrophoresis under alkaline conditions (pH >13) was later described by Singh et al. (201). Olive et al. developed another alkaline version of this technique (pH > 12.3) two years later (202). In the following years, modified protocols were developed by Collins et al. and Singh et al. (203,204).

The stronger lysis conditions used can destroy more than 95% of the proteins. Simple and sensitive identification of alkali labile regions and single chain breaks was achieved by alkaline electrophoresis. This version of the method has become more recommended because many genotoxic agents produce single chain breaks and/or alkali labile regions rather than double strand breaks (200,201).

At alkaline pH, DNA molecules with varying molecular weights and electrical charges move differentially in the electrical field, according to the comet method. The Comet technique, which has developed in recent years, is a sensitive, fast and reliable method used for the detection of DNA single and double strand breaks caused by various agents. Comet method is used in studies aiming to determine DNA damage and repair disorder induced by various agents in many mammalian cells. This method, based on the principle of determination of DNA breaks, is a biomonitoring test used in the determination of DNA damage caused by many physical and chemical mutagens, especially in humans, in detecting the degree and repair of DNA damage in cancer patients, in the prenatal diagnosis of some hereditary diseases, and in determining increased DNA damage in some diseases. In addition, it has a wide area of use due to its

advantages such as evaluating genotoxins in the first effect regions, applying to almost all eukaryotic cells, measuring low damage levels, and being a fast, simple and inexpensive method because it requires a small number of cell samples (205,206).

2.9. Cytotoxicity and Cytotoxicity Determination Methods

The term cytotoxic means causing cell death. To evaluate if a chemical has cytotoxic potential, cytotoxicity studies are carried out. Because of its simplicity of use and consistency with data collected from in vivo investigations, cell-based cytotoxicity studies have emerged as a viable alternative to animal experiments in toxicology laboratories (207).

Cytotoxicity is an event that damages cells to varying degrees depending on the dose and exposure time of the substance under investigation. If cells are exposed to cytotoxic substances, they may die as a result of events such as apoptosis, autophagy and necrosis, or they may lose their proliferation properties due to cytostasis (208).

To measure cell viability, a variety of techniques are used. The crucial issue is to determine the percentage of viable cells at the end of any cytotoxicity study. Possible reactions can be evaluated in vitro by using cell lines in cytotoxicity tests. The methods for determining cytotoxicity are as follows; colorimetric, dye exclusion, fluorometric, flow cytometric and luminometric assays.

2.9.1. Dye Exclusion Assays

Dye exclusion tests are the most basic methods, and they rely on the use of dyes like trypan blue, eosin, congo red and erythrosine B, that are excluded by viable cells but not by dead cells. Even though the staining method is simple in these assays, the experimental procedure might be time-consuming if the sample size is considerable. Trypan blue cannot pass through the membranes of living cells; because the membranes of living cells are whole and their structure is intact. However, it can pass through the membranes of dead cells and stain the cell (209).

2.9.2. Colorimetric Assays

Colorimetric tests measure a biochemical marker to assess the metabolic activity of cells.The colorimetric assessment of cell viability is assessed spectrophotometrically. The most commonly used colorimetric tests are MTT, MTS, XTT, crystal violet stain,

lactate dehydrogenase, sulforhodamine B (SRB), neutral red uptake, WST-8 and WST-1 assays. Measurement is based on specific staining of cells. Colorimetric cell viability assays are easy and cheap, and they may be used on both adherent cells and cell suspensions (210).

2.9.3 WST-1 Assay

WST-1 test for determination of cytotoxic effect and metabolic activity is a non-radioactive, spectrophotometric, colorimetric test based on the separation of tetrazolium salts from living cells. Tetrazolium salts are converted to formazan salts by cellular enzymes. The number of cells stained with formazan increases in direct proportion to the number of metabolically active cells in the cultured cells as enzyme activity increases. A spectrophotometer is used to measure the absorbance of cells stained at a specific wavelength, which is produced by metabolically active cells. This protocol is more preferred today, as the test procedure does not require the addition of a second reagent (alcohol or dimethyl sulfoxide) to the test medium to dissolve the formazan precipitates (211).

2.9.4. Fluorometric assays

The Alamar blue test is the most commonly used fluorometric method. Apart from Alamar blue, there are two different fluorescence methods based on protease and esterase enzyme activity used to determine cell viability. These methods are based on the principle of separating live and dead cells in terms of protease and esterase activity (212).

2.9.5. Luminometric assays

Luminometric methods, which have been frequently preferred in recent years, provide rapid and simple determination of cell proliferation and cytotoxicity. The intensity of the resulting luminescence is determined with a luminometric microplate reader. The persistence and stability of luminescence after the addition of the last reagent allows determination of both cell viability and cytotoxicity for the same well (213).

2.9.6. Flow cytometric assays

The flow cytometry method has been widely used in investigations of cell viability and toxicity. For a more detailed phenotypic cell viability study, fluorescent labeling targeting distinct characteristics of alive and healthy cells is used. Cell viability and toxicity analysis in flow cytometry can be easily combined with other phenotypic stainings to determine a cell type's differentiation state, evaluate a specific cell type from a mixed culture, and so on (210). In addition, mitochondrial membrane potential also provides information about cell viability.

2.9.7. Mitochondria assays

Mitochondria are the cells' powerhouses. The membrane potential of mitochondria is extremely important for energy production. The population of mitochondria in a live cell is maintained through a process known as biogenesis, which is a dynamic system regulated by the cell's condition and involves a balanced combination of fusion and fission events. It is possible to stain and evaluate cells with dyes that target mitochondria to determine whether they are apoptotic, have a high energy level, or have lost their mitochondrial membrane potential (214). Active mitochondria are labeled with TMRE (tetramethylrhodamine, ethyl ester). Microplate spectrophotometry, flow cytometry and fluorescence microscopy are used to measure changes in mitochondrial membrane potential in viable cells with TMRE dye (215).

3. MATERIALS and METHODS

3.1. Materials

3.1.1. Chemicals

Doxorubicin hydrochloride	Sigma Aldrich; USA
Rosmarinic acid	Sigma Aldrich; USA
(−)-Epigallocatechin gallate	Sigma Aldrich; USA
Fluorescein diacetate (FDA)	SERVA Electrophoresis GmbH; Germany
Gel Red stain	Biotium ;USA
Gel Green	Biotrend; Germany
Agarose MEEO ultra quality	Carl Roth; Germany
2-Hydroxyethylagarose	Sigma Aldrich; USA
Triton X-100	Sigma Aldrich; USA
Dimethylsulfoxid	Carl Roth; Germany
Dihydroethidium (DHE)	Merck Biosciences GmbH, Germany
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	Sigma Aldrich; USA
Sodium chloride	Carl Roth; Germany
Glycerol	Sigma Aldrich; USA
Potassium chloride	Fluka; Switzerland
Potassium dihydrogen phosphate	Merck; Germany
di-Sodium hydrogen phosphate anhydrous	Merck; Germany
Sodium dihydrogen phosphate monohydrate	Merck; Germany
Trizma base	Sigma Aldrich; USA
Methanol	Sigma Aldrich; USA
Ethanol	Sigma Aldrich; USA
1,4-Diazabicyclo [2.2.2] octane	Sigma Aldrich; USA
Dihydroethidium	Calbiochem; Germany
Agar	Sigma Aldrich; USA
L-Histidine	Fluka; Switzerland
Biotin	Sigma Aldrich; USA
β-Nicotinamide adenine	Sigma Aldrich; USA

dinucleotide phosphate (NADP)	
D-Glucose 6-phosphate disodium salt hydrate	Sigma Aldrich; USA
Potassium phosphate dibasic	Sigma Aldrich; USA
D-(+)-Glucose	Sigma Aldrich; USA
Sodium azide	Sigma Aldrich; USA
2-Aminofluorene	Sigma Aldrich; USA
2-Aminoanthracene	Sigma Aldrich; USA
4-nitro-o-phenylenediamine	Sigma Aldrich; USA
Mitomycin C	Sigma Aldrich; USA
Lyophilized Standard disc TA97a	Moltox; USA
Lyophilized Standard disc TA98	Moltox; USA
Lyophilized Standard disc TA100	Moltox; USA
Lyophilized Standard disc TA102	Moltox; USA
Lyophilized Standard disc TA1535	Moltox; USA
Nutrient Broth Oxoid	Moltox; USA
Lyophilized S9	Moltox; USA
Magnesium sulfate ($MgSO_4.H_2O$)	Sigma Aldrich; USA
Citric acid monohydrate	Sigma Aldrich; USA
Potassium phosphate (K_2HPO_4)	Sigma Aldrich; USA
Sodium Ammonium Phosphate ($NaHNH_4(PO_4.4H_2O)$)	Sigma Aldrich; USA
Ampicillin trihydrate	Sigma Aldrich; USA
Tetracycline	Sigma Aldrich; USA
Sodium hydroxide	Sigma Aldrich; USA
Hydrochloric acid	Sigma Aldrich; USA
Cell Proliferation Reagent WST-1	Roche; Germany
Tetramethylrhodamine, Ethyl Ester, Perchlorate (TMRE)	Thermo Fisher Scientific; USA
Antimycin A (AMA)	Sigma Aldrich; USA
Bovine Serum Albumin (BSA)	Sigma Aldrich; USA
Dulbecco′s Modified	Sigma Aldrich; USA

Eagle′s Medium/Ham′s Nutrient Mixture F12	
RPMI-1640 Medium	Sigma Aldrich; USA
Sodium pyruvate	Sigma Aldrich; USA
DMEM (Dulbecco's Modified Eagle Medium)	Sigma Aldrich; USA
Fetal Bovine Serum FBS	Gibco; USA
Penicillin-Streptomycin	Sigma Aldrich; USA
Acetic acid	Sigma Aldrich; USA
Giemsa solution	Sigma Aldrich; USA
Dulbecco's Phosphate-Buffered Saline	Gibco; USA
Cytochalasin B	Sigma Aldrich; USA
Trypsin/EDTA	Sigma Aldrich; USA
Phosphate buffered saline (PBS)	Gibco; USA
Horse Serum	Thermo Fisher Scientific; USA
Hydrocortisone	Sigma Aldrich; USA
Insulin	Sigma Aldrich; USA
Ethyl methanesulfonate (EMS)	Sigma Aldrich; USA
Cytochalasin B	Sigma Aldrich; USA

3.1.2. Equipment and Instruments

Eclipse 55i microscope	Nikon GmbH; Germany
Fluorescence microscope	Leitz; Germany
Fluoro Pro MP 5000 camera	Intas Science Imaging Instruments GmbH, Germany
Comet Assay Tank	Cleaver Scientific; UK
Electrophoresis power supply	Biorad; USA
Centrifuge	Sigma 3-16 PK, Germany
Incubator	Heidolph Unimax 1010, Germany
Micropipette	Eppendorf, Germany
Vortex	Heidolph Reax Top, Germany
Analytical Balance	Ohaus Explorer 214C, USA

8-Channel Multichannel Pipett	Capp, Denmark
Laminar flow cabin	Esco, Singapore
Refrigerator	Arçelik, Turkey
Deep Freezer (-20°C)	Siemens, Germany
Deep Freezer (-80°C)	New Brunswick, Canada
Phase-contrast invert microscope	Zeiss, Germany
Automatic pipettor	Hirschmann Laborgeräte, Germany
pH meter	Mettler-Toledo MP220, Germany
Waterbath	Gesellschaft Fuer Labortec, Germany
Incubator (37 °C with 5% CO_2)	Benchmark Scientific, USA
Incubator	Heal Force, China
Autoclave	Systec GmbH, Germany
Microplate reader	Thermo Multiskan Ascent, USA
Multimode microplate reader fluorescence plate reader	Thermo Scintific, Varioskan Lux, USA
Milli Q water device	Merck, Millipore, Germany
Light microscope	Zeiss, Primo Star, Germany
Inverted microscope	Zeiss, Germany
Falcon tube (15-50 ml)	Capp, Denmark
Cell Culture Flask (25 cm^2; 75 cm^2)	Corning flask, USA
Petri dishes, polystyrene	Sigma Aldrich, Germany
Cryo Vial	Isolab Laborgeräte GmbH, Germany
Eppendorf tubes	Isolab Laborgeräte GmbH, Germany
96 well plate	SPL Life science, Korea
6 well plate	TPP, Switzerland
Autoclavable schott bottle (250 ml; 500 ml; 1 l)	Isolab Laborgeräte GmbH, Germany
Neubauer counting chambers	Isolab Laborgeräte GmbH, Germany
Pipette tip (100-1000 µl)	Mettler Toledo, Germany
Pipette tip (10-100 µl)	Capp, Denmark
Microscope slides	Karl Hecht Assistent, England
Plain and frosted microscope slides	Thermofisher, USA
Cover Glass	Thorlabs, USA
Volumetric flasks (10, 25, 50, 100, 200, 500, 1000 ml)	S&H Labware, Turkey

Balance	Daihan Biomedical, China
Beaker (50, 100, 250, 1000 ml)	Isolab Laborgeräte GmbH, Germany
Hotplate stirrer	Ohaus Europe GmbH, Switzerland
Test tube heater	Hanna Instrument, USA
Staining jar	Isolab Laborgeräte GmbH, Germany
Spatulas	Isolab Laborgeräte GmbH, Germany
Magnetic stir bar	Fisher Scientific, Germany
Graduated cylinder (25, 100, 250 ml)	Isolab Laborgeräte GmbH, Germany
Test tube racks	Isolab Laborgeräte GmbH, Germany
Slide box	Isolab Laborgeräte GmbH, Germany
Parafilm	Fisher Scientific, Germany
Autoclave tape	Isolab Laborgeräte GmbH, Germany

3.2. Methods

3.2.1. Preparation of Samples

In experiments using cell lines, Dox, RA and EGCG were prepared as follows. Dox stock solution at a concentration of 1 mM was prepared. For this, 1 mg of Doxorubicin hydrochloride was dissolved in 1.7 ml of PBS (1X). It was aliquoted into sterile eppendorf tubes as 100 µL. Eppendorf tubes were covered with aluminum foil and stored at -20°C. To prepare a 2 mM RA stock solution, 0.00216 g of RA was dissolved in 3 mL of PBS. To prepare a 400 µM EGCG solution, 0.000550 g of EGCG was dissolved in 3 ml of PBS. Stock concentrations of RA and EGCG were also stored at -20°C. The working solutions for the experiment were made fresh on the day of the experiment by diluting the stock solution. For the Ames test, 10 mg of Dox was dissolved in 5 ml of PBS. Thus, when 50 µl of this solution was used, 100 µg/plate concentration was obtained, which is the highest concentration tested for the Ames test. Other concentrations were prepared by diluting this concentration with PBS. In experiments with RA and EGCG, 20 mg of RA or EGCG was weighed and dissolved with 1 ml of PBS. Thus, the highest concentrations of 1000 µg/plate of RA and EGCG were obtained when 50 µl of RA and EGCG were taken. All solutions were prepared freshly on the day of the experiment.

3.2.2. Cell lines and culture conditions

Chinese hamster ovary (CHO-K1) cells were obtained from Institute of Pharmacology and Toxicology, Würzburg. Cells were grown grown at 37 °C with 5% CO_2 in Ham's medium F12 supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% (vol/vol) antibiotics (10000 U/ml penicillin and 50 mg/ml streptomycin). The cells were routinely subcultured three times a week to keep them in exponential growth conditions.

Human lymphoblastoid (TK6) frozen cell stocks were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in exponential growth by weekly dilution in RPMI medium with 10% bovine serum, 1% sodium pyruvate, 0.4% (vol/vol) antibiotics (10000 U/ml penicillin and 50 mg/ml streptomycin) and 1% (vol/vol) L-glutamine.

The MCF-12A cell line is an epithelial cell line that is non-tumorigenic. MCF-12A was obtained from the ATCC and grown in medium containing DMEM / Ham's F12 in the presence of 2.5 % horse serum and supplemented with 500 ng/ml hydrocortisone, 20 ng/ml epidermal growth factor, 10 μg/ml insulin and antibiotic. The cells were cultivated at 37°C in a humidified incubator with 5% CO_2 at 90% humidity. The cells were sub-cultured to a new medium when they were 80 percent confluent.

MCF7 and MDA-MB-231 cells are both invasive ductal/breast cancer cells with a lot of phenotypic and genotypic differences. MDA-MB-231 and MCF-7 breast cancer cell lines were cultured adherently and maintained in DMEM containing 10% FBS, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) at 37°C in 5% CO_2 at 90% humidity.

3.2.3. Cell Viability Assay

FDA is a cytoplasmic esterase activity and membrane integrity indicator. FDA is activated by cytosolic esterases to produce green fluorescence in healthy cells, whereas GelRed can only enter cells with damaged membranes. One day prior to the experiment, the cells were seeded in 6 wells plates at a density $3x10^5$ cells/well and allowed to adhere overnight. Cells are exposed to the applied chemical for the desired amount of time. After the exposure time has elapsed, cells cultured in 6-well plates were harvested. The suspension was centrifuged for 5 min at 1000 rpm. The supernatant solution was discarded. Experiments were performed on the remaining cell suspension. 35 μl cell

suspension and 15 µl staining solution were mixed in eppendorf tube then the tube was vortexed. 15 µl of the mixture in the eppendorf tube was taken and was pipetted onto micro slide which was then covered with cover slip (21x26 mm). 2 slides were prepared for each sample. A fluorescent microscope was used with 200-fold magnification to count the percentages of green and red cells in 200 cells using FITC filter (216).

Staining Solution for Vitality Test:

2 ml PBS

12 µl FDA stock solution

2 µl Gel Red solution

3.2.4. Comet Assay

One day prior to the experiment, the cells were seeded in 6 wells plates at a density 3 x 10^5 cells/well for CHO-K1 cell line and 3.5 x 10^4 cells/well for TK6 cell line then allowed to adhere overnight. Cells were then exposed to therapeutic agent Dox, RA, EGCG and their combinations with continuous exposure for 4 h. After incubation period with compounds, cells cultured in 6-well plates were harvested. The suspension was centrifuged for 5 min at 1000 rpm. The cells were then resuspended in the remaining media after discarding the supernatant. Twenty µl of this cell solution were mixed with 180 µl of low melting point agarose that had been pre-warmed. Forty-five µl of cell-agarose was added onto a high melting point agarose pre-coated slide and was covered using a cover slip (2 replicates per treatment). The slides were dried at room temperature for 3 min and the cover slip was removed carefully. The cells on the slides were placed in pre-cooled glass cuvettes containing the lysis solution and allow the cells to undergo lysis at 4°C in the dark. After lysis, the slides were placed in the cold electrophoresis solution for 20 minutes. The electrophoresis was started and the voltage was fixed at 25V; the current was adjusted to 0.3A (300mA) by adding or removing the electrophoresis buffer and was run 20 min. After electrophoresis, the slides were removed and were washed once with distilled water, then were rinsed in Tris buffer filled in cuvettes for 5 min. The slides were placed in ice cold methanol (-20°C) for 5 min and dried. The slides were stained with Gel Red solution (20 µl per slide) and were covered with cover slip. The slides were evaluated using Komet6-software under fluorescence microscope. The

results were expressed as a percentage of DNA in the tail region for a total of 100 cells (50 each slide) (217).

Lysis Buffer (pH-10) in 1 L:

2.5 M NaCI	146.10 g/l
100 mM Na_2EDTA	37.22 g/l
10 mM TRIS	1.21/l

Lysis Solution : Freshly made and stored at 4°C under darkness

Lysis buffer	66.75 ml
DMSO (10%)	7.5 ml
Triton X (1%)	750 µl

Electrophoresis Buffer (Make up to 2l volume):

5M NaOH	200 g/l
0.2 M Na_2EDTA (pH 10)-Autoclaved	14.89 g/200ml

3.2.5. Evaluation of the cellular superoxide anion concentration

Dihydroethidium (DHE) was used to detect superoxide anion concentration in the mitochondria of living cells. DHE is blue in the cytosol until it is oxidized, at which point it intercalates into the cell's DNA, colouring the nucleus a bright fluorescent red. One day before the experiment, 2.5 x 10^5 cells were seeded on 12 mm coverslips in 6-well plates in 3 ml cell medium. The medium was removed after treatment, and the cells were washed twice with PBS. Fresh media containing 10 µM DHE was added to the cells, and they were incubated in the dark for 20 minutes at room temperature. The medium was removed at the end of the incubation time, and the cells were washed twice with PBS. After this step, an Eclipse 55i microscope and a Fluoro Pro MP 5000 camera were used to photograph the cells at 200-fold magnification. ImageJ software was used to measure the grey values of 200 cells per treatment for quantification (218).

3.2.6. Ames test

The plate incorporation method was used for the mutagenicity/antimutagenicity assays. Test tubes were placed in a 45°C water bath. Then 2 ml of top agar was put in test tubes containing histidine / biotin solution (10% of the top agar). After that 0.05 ml of test substance is placed in test tubes containing 2 ml of top agar, 0.1 ml of 12-14 hour bacterial culture. This mixture was vortexed and poured into minimal glucose agar (MGA) plates. After waiting for 15 minutes for the top agar to solidify, petri dishes were inverted and incubated in a 37°C for 48-72 hours. His^+ bacterial colonies were counted after the incubation period was over. In order to evaluate the results, solvent control and positive control experiments were also performed in parallel with the experiments with test substances. In antimutagenicity studies, 0.05 ml of a known mutagenic (positive control) substance was put in all test tubes. Possible mutagenic or antimutagenic activities of some chemicals appear after they are metabolized. S9 fraction is used to metabolize test substances. S9 is a rat liver extract containing active liver enzymes (P450 activity) that mimics hepatic metabolism in *in vitro* tests. The Ames test in the presence of S9 was performed as follows. S9 mixture was prepared fresh just before the experiment. 2 ml of top agar, histidine-biotin solution, bacterial culture and the tested substances were placed in the S9-free assay, then 0.5 ml of ice-free S9 mixture is added to mix and spread homogenously to the MGA petri dishes. After the agar was frozen, it was incubated in a 37 °C incubator for 48 hours. The colonies were manually counted and the findings were evaluated after incubation. Antimutagenicity assays were performed in the same way as mutagenicity studies. Indirect-acting mutagen 2-aminofluorene (2-AF) was used as positive mutagen for both TA98, TA100 and TA97a strains with S9 experiment and 2-Amino anthracene (2-AA) was used a positive mutagen for TA102 and TA1535 with S9 experiment. In the absence of S9 mix, direct-acting mutagens 4-nitro-o-phenylenediamine (NPD) was used as a positive direct mutagen for the TA98 and TA97a strain, sodium azide (SA) was used as positive direct mutagen for TA100 and TA1535 strains and Mitomycin C was used as a positive mutagen for TA102 strain. In this study, the antimutagenic activity of RA and EGCG against Dox was investigated (188, 219).

Preparation of *Salmonella* cultures:

Salmonella Typhimurium was streaked onto nutrient agar (histidine/biotin/ ampicillin) plates from the stock, and the plates were inoculated and incubated 48 hours at 37°C. A small portion of the subculture was transferred into a test tube containing nutrient broth (oxoid) using a sterile loop and cultured at 37°C for 12-16 hours until the growth reached log phase. The prepared liquid bacterial culture was used in the experiment.

Preparation of minimal glucose agar plates for TA98, TA100 and TA102 strains:

Agar	15 g
Distilled water	930 ml
50 X VB salt	20 ml
40% glucose	20 g in 50 ml

Preparation of minimal glucose agar plates for TA97a strain:

Agar	15 g
Distilled water	930 ml
50 X VB salt	20 ml
3% glucose	4 g in 50 ml

Vogel-Bonner (50xVB salts):

Magnesium sulfate ($MgSO_4.7H_2O$)	10 g
Citric acid monohydrate	100 g
Potassium phosphate (K_2HPO_4)	500 g
Sodium ammonium phosphate ($NaHNH_4PO_4.4H_2O$)	175 g
Distilled water (45 °C)	670 ml

The total volume is completed to 1000 ml and sterilized in an autoclave at 121 ° C for 15 minutes. Store in a dark environment at room temperature.

Top agar:

Agar	6 gr
NaCl	5 gr
Distilled water	1000 ml

Agar, salt and water were mixed and sterilized for 15 minutes at 121°C in an autoclave.

0.5 mM Histidine-Biotin Solution:

D-biotin	12.36 mg
L-histidin.HCL	9.6 mg
Distilled water	100 ml

The final volume was made up to 100 ml with distilled water. Then it was sterilized in an autoclave. This solution was added at 10 ml per 100 ml of Top agar during the experiment.

Minimal glucose agar (MGA):

Agar	15 g
Distilled water	880 ml
50xVB salt	20 ml
40% glucose	50 ml

Agar and water were mixed and dissolved and sterilized for 15 minutes at 121°C. Cool to 45 ° C and 40% glucose and 50xVB salts were added slowly with stirring, the prepared medium was divided into each petri dish as 30 ml.

Nutrient Broth Liquid Culture Media:

Nutrient Broth	5 g
Distilled water	200 ml

Nutrient broth and water are mixed and sterilized in an autoclave at 121 ° C for 15 minutes and stored at 4 ° C.

Histidine / Biotin / Ampicillin Agar (HBA Agar):

Agar	15 g
Distilled water	910 ml
50xVB salt	20 ml
40% glucose	50 ml
Sterile Histidine. HCl.H_20 (2g / 400 ml H_2O)	10 ml
Sterile 0.5 mM Biotin	6 ml
(0.8% / 0.02 M NaOH) Ampicillin	3,15 ml

Agar was added to distilled water and sterilized in an autoclave. After cooling the solution, 50x VB salts, 40% Glucose and histidine were added. Biotin was added when the solution was completely cooled. The prepared HBA agar was divided into each petri dish as 30 ml. Petri dishes can be stored at +4°C for 2 months.

(0.8% / 0.02% NaOH) Ampicillin Solution:

Ampicillin trihydrate	0,8 g
0.02 N Sodium hydroxide	100 ml

After the ampicillin solution was prepared, it was passed through a 0.22 µM pore diameter membrane filter. It was stored in the refrigerator at +4°C.

(0.13%) Biotin Solution:

D-biotin	0,65 mg
Distilled water	50 ml

Sterile Histidine.HCl.H_2O:

L-Histidine.HCl.H_2O	2 gr
Distilled water	400 ml

Preparation of S9 Microsome Enzyme (10%) S9 mixture:

S9 Microsome enzyme	1 ml
$MgCl_2$-KCl salts	0,5 ml
Glucose-6-phosphate	0,25 ml
0.1 M NADP	1 ml
0.2 M phosphate buffer (PH 7.4)	12,5 ml
Sterile distilled water	9.875 ml

All chemicals were freshly prepared. All materials were mixed in a sterile falcon tube. The tube in which the solution was prepared was placed in ice and kept cold throughout the experiment.

Salt Solution (1,65 M KCl + 0,4 M MgCl2):

Potassium chloride (KCl)	61,5 g
Magnesium chloride ($MgCl_2$)	40,7 g
Distilled water	500 ml

0.2 M Sodium-phosphate buffer (pH = 7.4):

0.2 M Sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$)	60 ml (2.76 g/100 ml)
0.2M Disodium hydrogen phosphate (Na_2HPO_4)	440 ml (14.2g/500 ml)

After the mixture is adjusted to pH 7.4, it was sterilized in an autoclave. It was stored in a glass bottle at room temperature.

1 M Glucose-6-Phosphate solution:

Glucose-6-phosphate	2,82 g
Distilled water	10 ml

Sterilization was done with a 0.22 M pore diameter filter. It was stored at -20 ° C.

0.1M β-NADP solution:

β -NADP	383 mg
Distilled water	10 ml

Sterilization was done with a 0.22 M pore diameter filter. It was stored at -20 ° C.

3.2.6.1. Mutagenic index (MI)

The mutagenic index (MI) is calculated as: MI = a/b where a is the average number of reverse mutation colonies observed on compound-treated plates and b is the average number of reverse mutation colonies per plate in the negative control. A sample is considered to have mutagenic activity when a dose-response relationship is detected and a two fold increase in the number of mutants (MI ≥ 2) is observed with at least one concentration (220).

3.2.6.2. Antimutagenicity assay

Antimutagenicity was calculated as a percentage inhibition of mutagenicity using the following formula: Inhibition rate (%) = ([A − B]/[A − C])×100 , where A is the number of revertants per plate in the presence of the positive control, B is the number of revertants per plate in the presence of mutagen and the test sample. C is the number of spontaneous revertant colonies per plate. When the inhibitory effect of the test substance was in the range of 25-40%, it was considered moderate and the inhibitory effect was >40%, it was considered strong. Inhibitory effects of less than 25% were defined as weak activity or no antimutagenicity (188,219).

3.2.7. WST-1- based cytotoxicity assay

3.2.7.1. Cytotoxicity assay for suspension cells (TK6 cells)

The WST-1 microplate test was carried out as follows, TK6 cells were seeded at a density of 5×10^4 cells/ml were seeded in the 6 well plate. After cultured overnight, cells were exposed to samples in the 6-well plate at various concentrations for 24 h and incubated at 37˚C in an incubator containing 5% CO_2. After treatment, the test chemical-containing medium was removed by centrifugation at 1000 rpm for 5 min. Phosphate-buffered saline (PBS) was used to gently wash the treated cells at least twice. After washing the cells, new RPMI medium containing 5% WST-1 dye was added. 200 µL of treated cells that mixed with dye were added to each well of 96-well plate, and incubated at 37˚C under 5% CO_2 in an incubator for 4 hours. The absorbance was measured in a microplate reader at 450 nm and the percent cytotoxicity was calculated (221).

3.2.7.2. WST-1- based cytotoxicity assay for adherent cells

Cells were seeded ($10x10^3$ cells in 100 µl of culture medium) into 96-well plates, were grown for 24 h. Then, the cell medium was aspirated and cells were exposed to 100 µl/well of freshly prepared medium containing tested substance for 24 h. The media was removed at the end of the incubation, and the cells were washed with PBS and 100 µL of medium containing 5% WST-1 was added to the wells and the 96-well plate was then incubated for another 4 hours at 37°C. A photometer was used to measure the absorbance after 4 hours at 450 nm (222).

3.2.7.3. Calculation of IC50 Value

The cell viability (%) was calculated by using equation from the raw data that we get from ELISA reader microplate. Cell survival rate (%) = (a-c)/(b-c) x 100, where a=absorbance of treated cells, b=absorbance of control cells, and c=absorbance of the blank). The half maximum inhibitory concentration, or IC50, is defined as the concentration of a chemical required to inhibit cell viability by 50%. The IC50 value was calculated from the graph as follows; cell viability, which is a percent value, is located on the y-axis of the graph, while the logarithm of the concentration of the corresponding chemical used is located on the x-axis. In this graph, for the y=50 value, the concentration on the x-axis is found from the linear (y = mx+n) formula and the IC50 value is found by taking the inverse of the log concentration (223).

3.2.7.4. Selectivity index (SI) calculation

The Selectivity Index (SI) is defined as the ratio of a sample's toxic concentration to its bioactive concentration. The ideal drug should have a toxic effect at a high concentration, but show activity at a very low concentration. The SI was calculated by dividing the cytotoxicity (IC50) on normal cells (MCF-12a) by the cytotoxicity (IC50) on cancer cells (MDA-MB-231 and MCF-7).

$$SI = \frac{IC_{50}^{\text{no cancer cell line}}}{IC_{50}^{\text{cancer cell}}}$$

A SI (<1) indicates that the sample is likely to be toxic and therefore can not be used as a drug. The studied sample must have an SI (≥ 3) to be classified as a potential anti-cancer (224,225).

3.2.7.5. *In vitro* drug interaction analysis

The drug interaction between two different drugs was analyzed using the coefficient of drug interaction (CDI). CDI was calculated by the following formula; CDI = AB/(AxB). According to each WST-1 absorbance, AB is the ratio of the two-drug combination group to the control group, and A or B is the ratio of the single drug group to the control group. As a result, CDI < 1 indicates synergism, CDI < 0.7 indicates significantly synergistic, CDI =1 indicates additivity, and CDI >1 indicates antagonism (226).

If there are three drugs, A, B and C, each with specific activity against a cell line, evaluation is made with the following criteria to determine synergy.

S_A = Percentage of viable cells after administration of Drug A compared to control cells

S_B = Percentage of viable cells after administration of Drug B compared to control cells

S_C = Percentage of viable cells after administration of Drug C compared to control cells

S_{A+B} = Percentage of viable cells after administration of both Drug A and Drug B compared to control cells

S_{A+C} = Percentage of viable cells after administration of both Drug A and Drug C compared to control cells

S_{B+C} = Percentage of viable cells after administration of both Drug B and Drug C compared to control cells

S_{A+B+C} = Percentage of viable cells after administration of Drug A and Drug B and Drug C in combination compared to control cells

If a synergistic activity is observed with the combined application of the three drugs to cancerous cells, the following equations must be met. These equations are shown in Table 2.

Table 2. The determination of Synergy

$S_{A+B+C} < [(S_A) \times (S_B) \times (S_C)]/10000$
$S_{A+B+C} < [(S_{A+B}) \times (S_C)]/100$
$S_{A+B+C} < [(S_{A+C}) \times (S_B)]/100$
$S_{A+B+C} < [(S_{B+C}) \times (S_A)]/100$
$S_{A+B+C} < [(S_{A+B}) \times (S_{A+C})]/100$
$S_{A+B+C} < [(S_{A+B}) \times (S_{B+C})]/100$
$S_{A+B+C} < [(S_{A+C}) \times (S_{B+C})]/100$
$S_{A+B+C} < [(S_{A+B}) \times (S_{B+C}) \times (S_{A+C})]/10000$

The triple combination is additive if S_{A+B+C} equals any single permutation; if S_{A+B+C} is more than any single permutation, the triple combination is antagonistic (227).

3.2.8. Mitochondrial membrane potential assay

The TK6 cells were plated at 5 x 10^4 cells/well in 3 ml of the culture medium into a 6-well plate. The 6-well plate was incubated overnight at 37 °C. The cells were treated with the test compound(s). Cells treated with the tested substance were incubated for 24 hours. The positive control (AMA, 50 µM) was applied to the cells 30 minutes before the end of the exposure time. TMRE (tetramethylrhodamine ethyl ester perchlorate) fluorescent dye (200 nM final concentration) was added to 6 well plates. The assay plates were incubated for 30 minutes at 37°C. Cells were collected in falcon tubes, washed twice with PBS-BSA (0.2%) and supernatant discarded. Cell Pellets were resuspended in 1 ml of PBS-BSA and cells were diluted 1:3 (500 µl cells + 1000 µl PBS-BSA). 200 µl cell suspension was transferred to a black 96-well plate with a clear bottom and plate was read on the plate reader. The settings were excitation about 549 nm and emission about 575 nm. The following formula was used to calculate the percentage of TMRE fluorescence signal compared to the negative control (228).

$$\text{TMRE Fluorescence Relative (\%)} = \frac{(\text{Sample RFU} - \text{Blank RFU}) \times 100}{\text{Negative control RFU} - \text{Blank RFU}}$$

Sample RFU = Fluorescence of cells exposed to a chemical substance or drug.

Blank RFU = Fluorescence of wells with no cells

Negative control RFU = Fluorescence of cells not exposed to a chemical substance or drug.

Preparation of AMA (25 mM):

Antimycin A	19.95 mg
Dimethylsulfoxide (DMSO)	1.5 ml

Preparation of TMRE (1 mM):

TMRE	2.6 mg
DMSO	5 ml

Preparation of PBS-BSA 0.2%:

Bovine serum albumin	200 mg
Phosphate buffered saline	100 ml

3.2.9. Micronucleus (MN) assay

Approximately 24 hours before to test substances treatment, TK6 cells ($2x10^5$) were seeded in 6 well plates with 3 ml medium per well. On the next day, cells were treated with solutions of the test compounds. At the end of 24 h treatment, cells were taken into the falcon tube and tubes were centrifuged for 5 min at 1000 rpm, the supernatant was discarded, and the cell pellets were washed with PBS then pellets were resuspended in 3 ml fresh cell medium with cytochalasine B (2.4 µg/ml) for 24 hours. After cytokinesis was inhibited, the cells were centrifuged and the cell pellets were washed with PBS. Five milliliters of 0.07M potassium chloride hypotonic solution (37°C), was added to the washed cell pellets. After an approximate 2 min incubation with hypotonic solution at 37°C, 0.5 mL of cold fixative solution (1:3 volume acetic acid: methanol) was placed on the hypotonic solution. The cultures were centrifuged for 5 minutes at 1000 rpm. The supernatant was decanted and 5 ml of ice-cold fixative solution was added to all tubes that were then incubated at room temperature for 5 minutes. These procedures (fixation) were repeated twice. After the last centrifugation, the cell pellets were resuspended with approximately 0.5 ml of cold fixative solution. Fixed cells were dropped onto clean, wet and cold microscope slides and allow slides to air dry. The dry slides were stained with 5 % Giemsa solution for 5 minutes, washed with water.

Micronuclei (MNi) are smaller extra nuclei visible in the interphase cell that arise when a chromosome or a fragment of it failed to be integrated into one of the daughter nuclei during cell division. MNi were counted with a light microscope at 40x-fold magnification. For each treatment, at least 1000 binucleated cells (BN) were analyzed, and the cells with micronuclei were counted.

The cytokinesis block proliferation index (CBPI) was evaluated as a criteria for assessing cytotoxicity. CBPI was calculated the using the formula $[(M1 + 2M2 + 3\,(M3 + M4)]/N$, where M1 to M4 are the numbers of cells with one to four nuclei, respectively, and N is the total number of cells scored. CBPI was determined in treated ($CBPI_T$) and control ($CBPI_C$) groups. The percentage of cytostasis was calculated as follows.

$$\% \text{ cytostasis} = 100 - 100 \left(\frac{CBPI_T - 1}{CBPI_C - 1}\right)$$

In cultured cells, the replication index (RI) is a cytokinetics index. Determining the RI in cell cultures may be beneficial in genotoxicity testing, because inhibiting DNA synthesis results in a decrease in the RI (229). RI was calculated with the formula below.

$$RI = \frac{((M2)+2\ x\ (M3+M4))/\ (\text{total number of cells})_T}{((M2)+2\ x\ (M3+M4))/\ (\text{total number of cells})_C} X100$$

Where T is the treatment group, C is the control group.

As a result, the cytostasis percentage = 100 - RI.

The nuclear division index (NDI) is a measure of the viable cell fraction's proliferative status. NDI was determined as follows.

$NDI = (M_1 + 2\,M_2 + 3\,M_3 + 4\,M_4)/N$

The CBPI, RI and NDI were calculated by counting 1000 cells, including mononuclear (M_1), binucleated (M_2), trinucleated (M_3), and tetranucleated (M_4) cells (230,231).

3.2.10. Statistical Analysis

All analyses and graphical visualization were performed using GraphPad Prism software (version 7.0). MS-Excel 2016 was used to perform the arithmetic calculations. The frequency distribution of the data was analyzed and statistical significance was considered when the p-value was less than or equal to 0.05 as determined by the T-Test, Kruskal-Wallis, or Dunnett's multiple comparison tests. At least three separate experiments were carried out for each experiment.

4. RESULTS

4.1. *In vitro* Genetic Toxicology Tests Results

4.1.1. Comet Assay

Single and double strand breaks caused by Dox and protective effect of RA and EGCG against Dox induced genotoxicity was analyzed by alkaline comet assay in CHO-K1 and TK6 cells. Cells were exposed to Dox, RA and EGCG at different concentrations alone or together for 4 hours. Typical comet images observed with GelRed staining, are shown in Figure 8.

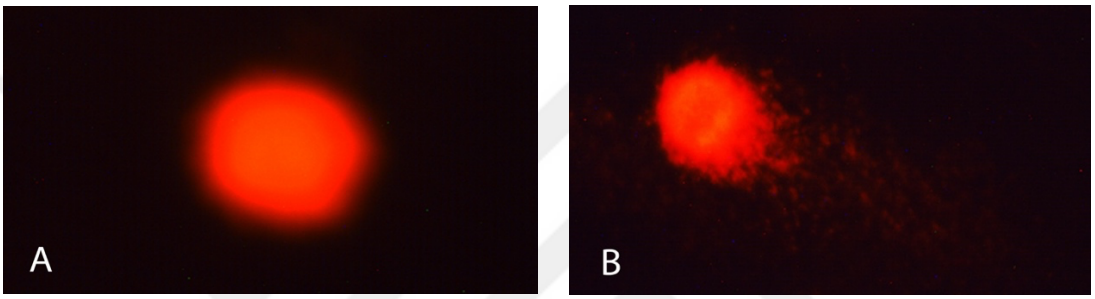

Figure 8. Examples images of comet assay on CHO-K1 cells with and without Dox. (A) Control group: shows cell without DNA fragmentation and (B) Dox 1000 nm group: shows cell with DNA fragmentation as an example of a cell with DNA damage after Dox treatment. DNA of cells were visualized under a fluorescent microscope at 200-fold magnification.

Dox was used as the DNA damaging agent. The CHO-K1 cells were treated with different concentrations (250 nM, 500 nM, 1000 nM, 2000 nM and 4000 nM) of Dox. In this study, dose response effects of Dox were observed in CHO-K1 cells. In addition, studies have been carried out to find the dose that causes DNA damage without causing apoptosis or cell death. Comet assay results are shown in Figure 9.

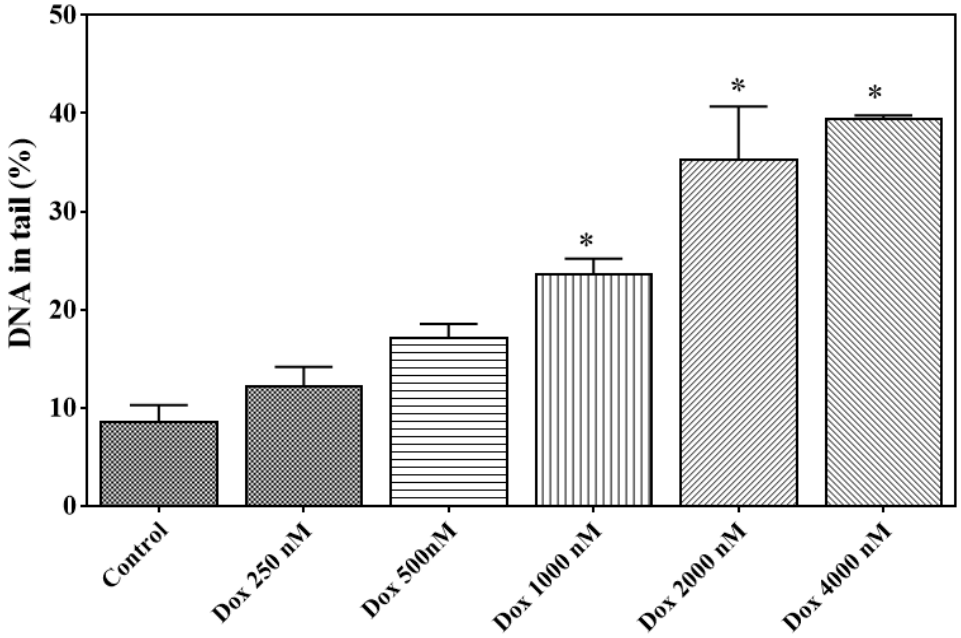

Figure 9. Concentration dependent increase in DNA damage after 4 hours Dox treatment in CHO-K1 cells. Each treatment group had 100 cells evaluated, with the findings represented as a percentage of DNA in the tail. The data are given as Mean ± Standard Error of Mean (SEM) of 3 independent experiments. Independent t-test was used to compare the control and treatment groups, *$p \leq 0.05$ vs. Control group. Dox: Doxorubicin.

The Dox concentration to be used to investigate the genoprotective effect of RA and EGCG was determined as 1000 nM. This concentration was chosen because it did not cause cytotoxicity. It has also been found to be a concentration that causes DNA damage. In this context, studies were conducted to find the protective effect dose range of RA. In the preliminary studies, 50, 100 and 200 µM concentrations of RA did not show a protective effect against Dox 1000 nM concentration. When the concentration of RA was increased, it started to show a protective effect. RA did not show any protective effect at lower doses than 0.5 mM. A statistically significant difference was observed when the amount of tail formed in the DNA of the Dox 1000 nM group and the two groups treated with Dox + 0.5 and 1 mM RA were compared. The results are shown in Figure 10.

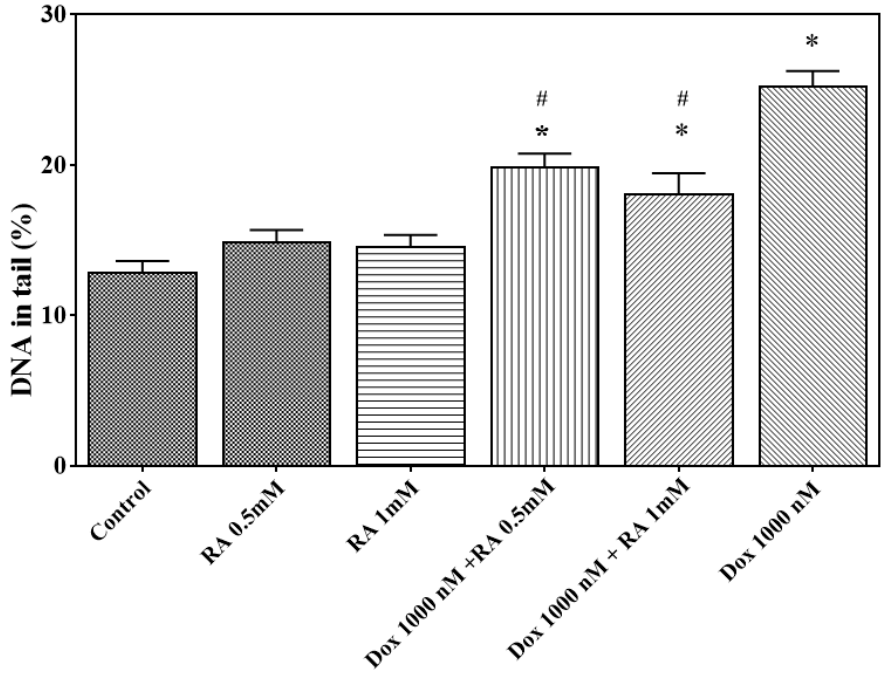

Figure 10. DNA damage induction by Dox treatment with or without RA inhibition in CHO-K1 cells. After 4 hours of Dox treatment, DNA damage was evaluated in the presence or absence of RA. The RA was added 15 minutes before the Dox treatment to provide possible RA protection. In the evaluation of comet analysis results; each treatment group had 100 cells evaluated, with the findings represented as a percentage of DNA in the tail. The data are given as Mean ± Standard Error of Mean (SEM) of 3 independent experiments. Independent t-test was used to compare means of two groups, *$p \leq 0.05$ vs. Control and #$p \leq 0.05$ vs. Dox 1000nM. Dox: Doxorubicin, RA: Rosmarinic acid.

The cultures were treated with different concentrations of EGCG (5 μM, 50 μM, and 100 μM) alone or in combination with Dox; the result was that EGCG exerted no genoprotective effect. It was observed that the amount of DNA tail formed increased when the studied EGCG concentrations increased. Therefore, low concentrations of EGCG have been studied. The results of the comet experiment using CHO –K1 cells are shown in Figure 11.

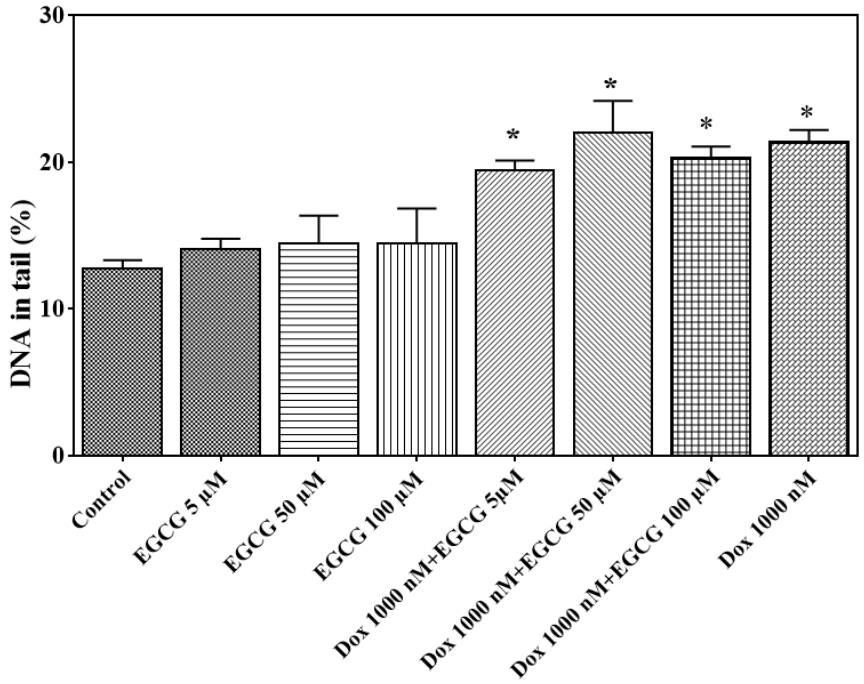

Figure 11. DNA damage induction by Dox treatment with or without EGCG in CHO-K1 cells. After 4 hours of Dox treatment, DNA damage was evaluated in the presence or absence of EGCG. The EGCG was added 15 minutes before the Dox treatment to provide possible EGCG protection. In the evaluation of comet analysis results; each treatment group had 100 cells evaluated, with the findings represented as a percentage of DNA in the tail. The data are given as Mean ± Standard Error of Mean (SEM) of 3 independent experiments. Independent t-test was used to compare means of two groups, *$p \leq 0.05$ vs. Control group. Dox: Doxorubicin, EGCG: Epigallocatechin gallate.

The possible protective effects of RA and EGCG when administered together were also investigated on CHO-K1 cells. The combination of RA 1mM and EGCG 100 µM administered together did not show a protective effect against DNA damage caused by Dox 1000 nM. Ra 1 mM showed a protective effect only when applied alone. The results of the experiment are shown in Figure 12.

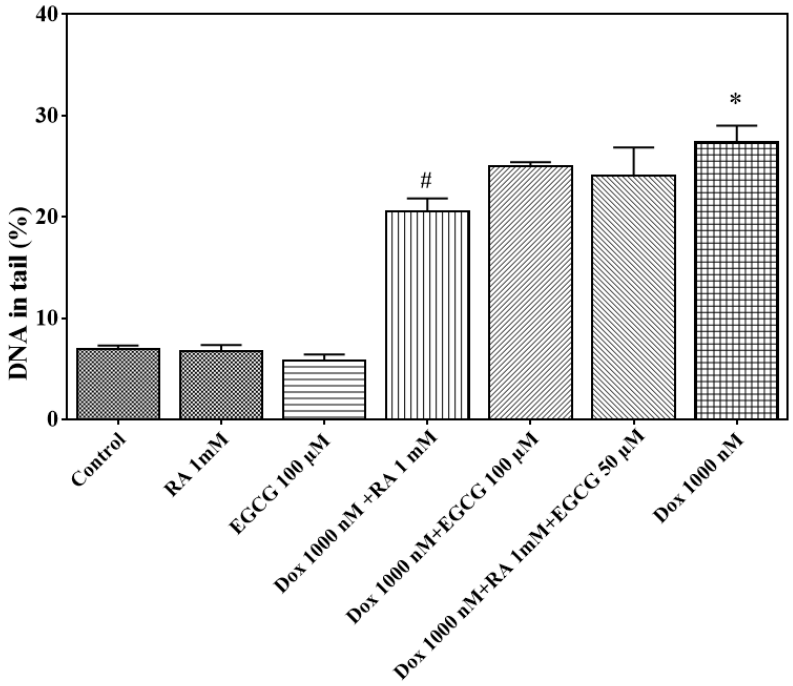

Figure 12. DNA damage induction by Dox treatment with or without RA and EGCG combination in CHO-K1 cells. After 4 hours of Dox treatment, DNA damage was evaluated. The combination of EGCG and RA was added 15 minutes before the Dox treatment. In the evaluation of comet analysis results; each treatment group had 100 cells evaluated, with the findings represented as a percentage of DNA in the tail. The data are given as Mean ± Standard Error of Mean (SEM) of 3 independent experiments. Independent t-test was used to compare means of two groups, *p ≤ 0.05 vs. Control and #p≤ 0.05 vs. Dox 1000nM. Dox: Doxorubicin, EGCG: Epigallocatechin gallate, RA: Rosmarinic acid.

The comet studies were also conducted using TK6 cells. A human origin, TK6 cells was also used because it was thought that human cells' responses to the applied chemicals would be more sensitive. Increasing concentrations of Dox were applied to the cells to decide which concentration of Dox to choose as a genotoxic agent. TK6 cells were incubated for 4 hours with increasing concentrations of Dox (125, 250, 500, 1000 and 2000 nM). As shown in Figure 13, different concentration of Dox was applied to TK6 cells.

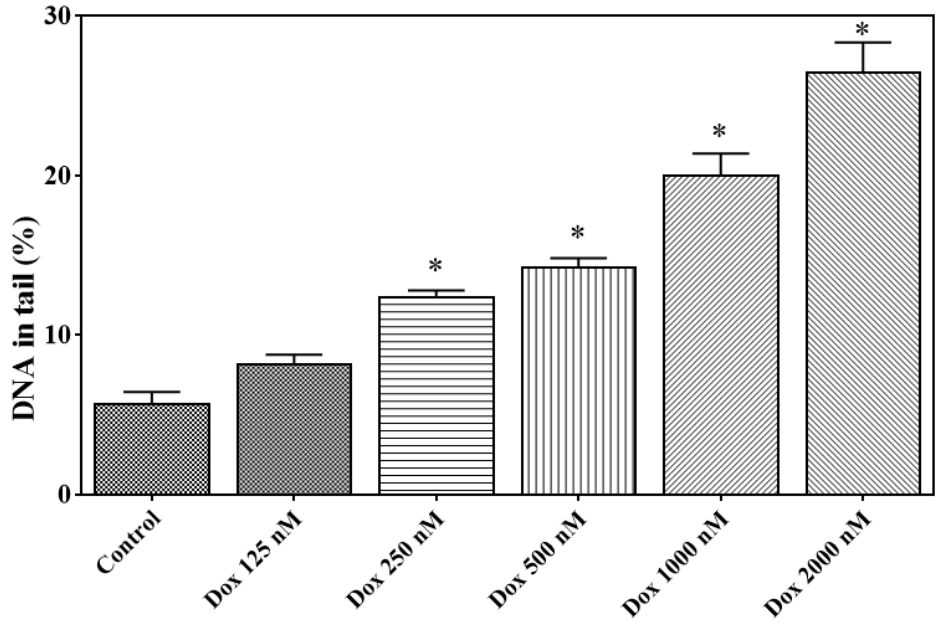

Figure 13. Concentration dependent increase in DNA damage after 4 hours Dox treatment in TK6 cells. In the evaluation of comet analysis results; each treatment group had 100 cells evaluated, with the findings represented as a percentage of DNA in the tail. The data are given as Mean ± Standard Error of Mean (SEM) of 3 independent experiments. Independent t-test was used to compare the control and treatment groups, *$p \leq 0.05$ vs. Control group. Dox: Doxorubicin.

Dox was applied to the cells in increasing concentrations starting from 125 nM concentration. The comet tail increased beginning from the lowest concentration to the highest and Dox applied groups (250nM, 500 nM,1000 nM and 2000 nM) showed statistically significant differences when compared to the control group. Increasing the Dox concentration to 2000 nM caused much more serious DNA damage. A concentration of 500 nM Dox was chosen as the DNA damage agent. The possible protective effect of RA against DNA damage induced by Dox (500 nM) in the TK6 cell line was investigated. TK6 cells were administered 50 μM and 100 μM doses of RA alone and in combination with Dox 500 nM for this study. Both concentrations of RA showed a protective effect. As the concentration of RA increased, its protective effect against Dox also increased. The protective effect of RA 100 μM showed a statistically significant difference when compared to Dox 500 nM. The results are shown in Figure 14.

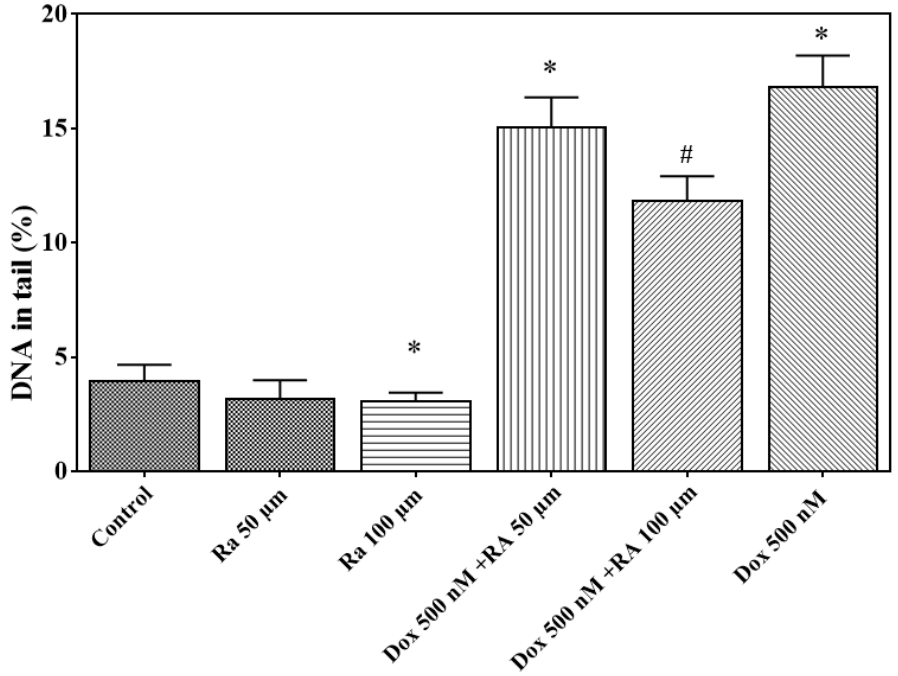

Figure 14. DNA damage induction by Dox treatment with or without RA in TK6 cells. After 4 hours of Dox treatment, DNA damage was evaluated in the presence or absence of RA. The RA was added 15 minutes before the Dox treatment to provide possible RA protection. In the evaluation of comet analysis results; each treatment group had 100 cells evaluated, with the findings represented as a percentage of DNA in the tail. The data are given as Mean ± Standard Error of Mean (SEM) of 3 independent experiments. Independent t-test was used to compare means of two groups, *p ≤ 0.05 vs. Control group, #p≤ 0.05 vs. Dox 500 nM. Dox: Doxorubicin, RA: Rosmarinic acid.

To determine whether EGCG is protective against DNA damage in TK6 cells, the comet assay was conducted, and the results are shown in Figure 15. Dox 500 nM treatment induced DNA damage 10 and 25 µM concentrations of EGCG inhibited the formation of comet tail. Two different concentrations of EGCG administered with Dox significantly reduced the percentage of mean tail DNA.

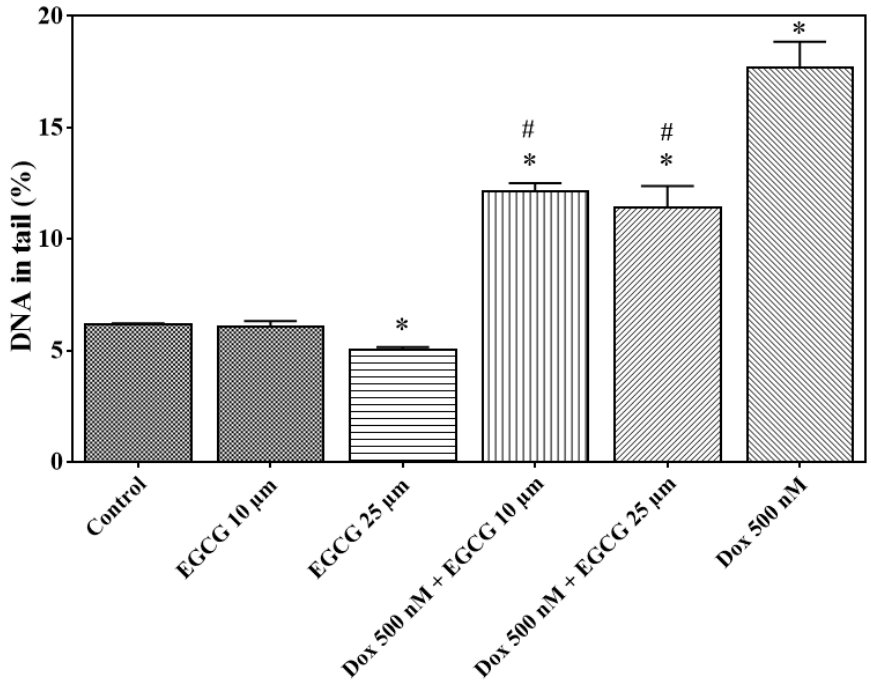

Figure 15. DNA damage induced by Dox treatment with or without EGCG in TK6 cells. After 4 hours of Dox treatment, DNA damage was evaluated in the presence or absence of EGCG. The EGCG was added 15 minutes before the Dox treatment to provide possible EGCG protection. In the evaluation of comet analysis results; each treatment group had 100 cells evaluated, with the findings represented as a percentage of DNA in the tail. The data are given as Mean ± Standard Error of Mean (SEM) of 3 independent experiments. Independent t-test was used to compare means of two groups, *$p \leq 0.05$ vs. Control group, #$p \leq 0.05$ vs. Dox 500 nM. Dox: Doxorubicin, EGCG: Epigallocatechin gallate.

In addition, the protective effect of combinations of RA and EGCG on DNA damage of TK6 cells was investigated. EGCG 25 µM and RA 100 µM were chosen for the combination. In the research, RA and EGCG showed a similar DNA protective effect when applied together or alone. The reduction of DNA tail amount did not increase with the combined application of RA and EGCG. Figure 16 shows the protective effects of RA, EGCG and combination of both against Dox induced genotoxicity.

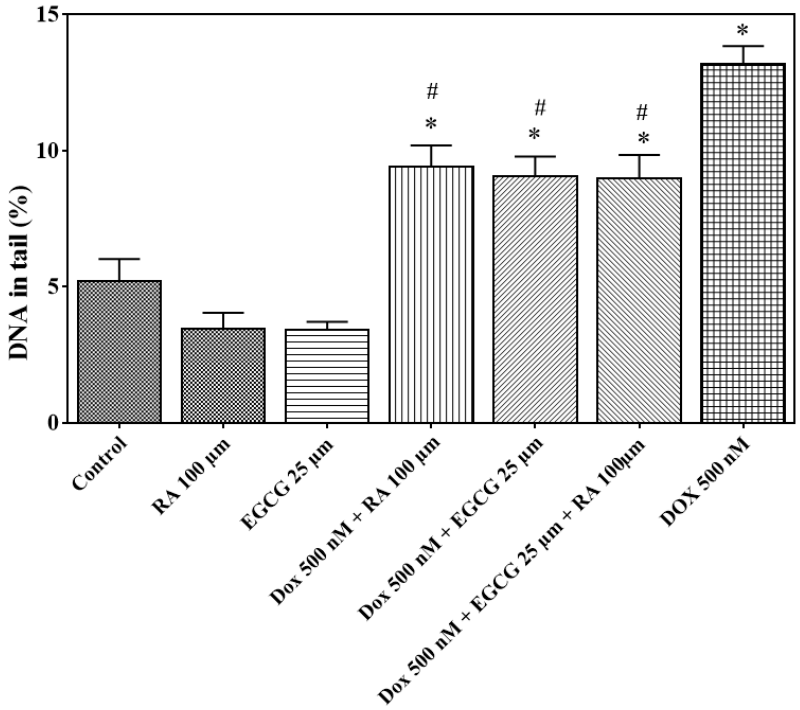

Figure 16. Dox induced DNA damage and the treated TK6 cells were incubated with RA and EGCG for 4 h. The RA and EGCG were added 15 minutes before the Dox treatment to provide possible EGCG protection. In the evaluation of comet analysis results; each treatment group had 100 cells evaluated, with the findings represented as a percentage of DNA in the tail. The data are given as Mean ± Standard Error of Mean (SEM) of 3 independent experiments. Independent t-test was used to compare means of two groups, *p ≤ 0.05 vs. Control group, #p≤ 0.05 vs. Dox 500 nM. Dox: Doxorubicin, EGCG: Epigallocatechin gallate, RA: Rosmarinic acid.

4.1.1.1. Results of vitality test

Mammalian cells' viability was assessed using fluorescence-based live-dead assays. The fluorescent diacetate (FDA) and GelRed, which stain living and dead cells, were used in a vitality test. The appearance of cells under the microscope was shown in Figure 17.

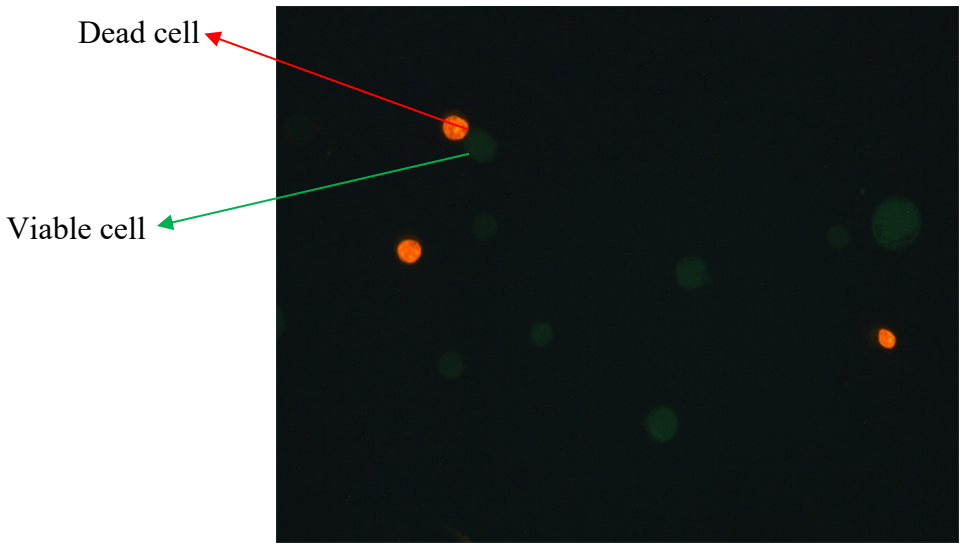

Figure 17. Representative pictures of viable and dead cells under the microscope. Cells were photographed under a fluorescent microscope using FITC filter at 200-fold magnification.

CHO-K1 cells were treated with different concentrations of Dox (250–4000 nM) and TK6 cells were treated from 125 to 2000 nM of Dox for 4 hr and percentages of viable and dead cells were calculated (Table 3 and 4).

Table 3. Percentage of the living cells that was determined on CHO-K1 cells after 4 hours of Dox incubation.

Treatment	CHO cell viability
Control	94,75 ± 4,0
250 nM Dox	95,20 ± 2,9
500 nM Dox	95,50 ± 0,7
1000 nM Dox	95,67 ± 1,5
2000 nM Dox	93,67 ± 2,52
4000 nM Dox	93,67 ± 4,04

Data are presented as mean ± SD of three independent experiments.

Table 4. Percentage of the living cells that was determined on TK6 cells after 4 hours of Dox incubation.

Treatment	TK6 cell viability
Control	95,58 ± 1,01
125 nM Dox	93,58 ± 2,96
250 nM Dox	93,00 ± 3,78
500 nM Dox	96,08 ± 0,59
1000 nM Dox	96,33 ± 0,31
2000 nM Dox	95,50 ± 1,77

Data are presented as mean ± SD of three independent experiments.

After the cells were treated with DOX, cell viability analysis was performed to determine the percentage of dead cells. The results obtained from cell viability analysis showed that viability greater than 50%. No decrease in cell viability was observed in CHO and TK6 cell lines when different Dox concentrations were applied. In compared to the control group, there was no significant effect in cell viability. Cell viability of CHO and TK6 cells to which RA and Dox were applied together are shown in Table (5 and 6).

Table 5. Percentage of the living cells that was determined on CHO-K1 cells after 4 hours of Dox, RA and combination of both incubation.

Treatment	CHO cell viability
Control	94,00 ± 2,56
1000 nM Dox	95,75 ± 2,67
1 mM RA	96,92 ± 1,94
0.5 mM RA	96,83 ± 0,59
1000 nM Dox + 1 mM RA	96,00 ± 0,41
1000 nM Dox + 0.5 mM RA	94,83 ± 5,20

Data are presented as mean ± SD of three independent experiments.

Table 6. Percentage of the living cells that was determined on TK6 cells after 4 hours of Dox, RA, combination of Dox and RA incubation.

Treatment	TK6 cell viability
Control	96,67 ± 1,01
500 nM Dox	95,58 ± 0,31
50 μM RA	97,00 ± 1,47
100 μM RA	96,83 ± 0,92
500 nM Dox + 50 μM RA	95,08 ± 2,06
500 nM Dox + 100 μM RA	96,25 ± 1,43

Data are presented as mean ± SD of three independent experiments.

When cell viability was compared to the control group, no decrease in cell viability was observed in both applied cell lines. Cell viability of CHO-K1 and TK6 cells treated with EGCG, Dox and their combinations are shown in Table (7 and 8).

Table 7. Percentage of the living cells that was determined on CHO-K1 cells after 4 hours of Dox, EGCG and combination of Dox and EGCG incubation.

Treatment	CHO cell viability
Control	97,75 ± 0,41
1000 nM Dox	97,42 ± 1,03
5 μM EGCG	96,58 ± 2,18
50 μM EGCG	94,75 ± 3,88
1000 nM Dox + 5 μM EGCG	96,42 ± 3,70
1000 nM Dox + 50 μM EGCG	95,83 ± 4,31
1000 nM Dox + 100 μM EGCG	94,50 ± 4,61

Data are presented as mean ± SD of three independent experiments.

Table 8. Percentage of the living cells that was determined on TK6 cells after 4 hours of Dox, EGCG and combination of Dox and EGCG incubation.

Treatment	TK6 cell viability
Control	96,50 ± 0,54
500 nM Dox	93,17 ± 1,33
10 μM EGCG	97,08 ± 0,62
25 μM EGCG	96,08 ± 1,16
500 nM Dox + 10 μM EGCG	94,58 ± 2,44
500 nM Dox + 25 μM EGCG	95,33 ± 2,29

Data are presented as mean ± SD of three independent experiments.

The cell viability results of the cells of the comet test performed by applying the triple combination of RA, EGCG and Dox are shown in table (9 and 10).

Table 9. Percentage of the living cells that was determined on CHO-K1 cells after 4 hours of Dox, EGCG,RA and combination of Dox, RA and EGCG incubation.

Treatment	CHO cell viability
Control	99,08 ± 0,12
1000 nM Dox	99,00 ± 0,20
1 mM RA	97,75 ± 0,74
100 μM EGCG	95,67 ± 2,55
1 mM RA + 1000 nM DOX	98,25 ± 0,74
100 μM EGCG + 1000 nM DOX	98,83 ± 0,42
1 mM RA + 100 μM EGCG + 1000 nM DOX	96,42 ± 1,05

Data are presented as mean ± SD of three independent experiments.

Table 10. Percentage of the living cells that was determined on TK6 cells after 4 hours of Dox, EGCG,RA and combination of Dox, RA and EGCG incubation.

Treatment	TK6 cell viability
Control	97,50 ± 0,41
500 nM Dox	97,00 ± 0,41
100 μM RA	97,42 ± 0,12
25 μM EGCG	98,00 ± 0,94
100 μM RA + 500 nM DOX	96,33 ± 0,51
25 μM EGCG + 500 nM DOX	95,83 ± 1,03
100 μM RA + 25 μM EGCG + 500 nM DOX	95,67 ± 3,14

Data are presented as mean ± SD of three independent experiments.

The genotoxicity/antigenotoxicity studies must be evaluated at the concentration levels below the IC50 value of tested substances, hence the cytotoxicity assessment must be done prior to genotoxicity study. In our study, cell viability experiments were carried out simultaneously with the comet test. The cytotoxicity of the substances used was assessed using a fluorescent dye (GelRed/fluorescein diacetate). According to the results, there was no significant decrease in cell viability in any group evaluated in the comet test compared to the control group. Cell viability did not reach below 90% in all experiments with CHO-K1 and TK6 cells. A vitality test was used to differentiate between cytotoxicity and genotoxicity of the Dox, RA and EGCG concentrations used in the comet assay. This test was carried out simultaneously with the comet assay. Before the comet test, the cell viability of the treated cells was examined. The cell viability results were close to each other and there was no statistically significant difference between the control group. No decrease in cell viability was observed in any group.

4.1.2. Micronucleus Assay Results

First of all, in this study, the induction of DNA damage by Dox was evaluated with the micronucleus (MN), also known as cytokinesis block micronucleus test, which detects the induction of chromosomal damage. MN test is an another genotoxic endpoint detection assay. It was used to identify possible DNA damage. A cell line TK6 was used for the MN assay. TK6 was chosen due to its widespread use and application in genotoxicity testing, as well as the benefits and suggestions indicated. The dose of Dox that caused genotoxicity but did not affect cell viability was chosen. In the next step, EGCG, RA and their combinations were applied to the cells and their potential genoprotective effects were investigated. A typical appearance of the cells scored in the MN assay can be seen in Figure 18.

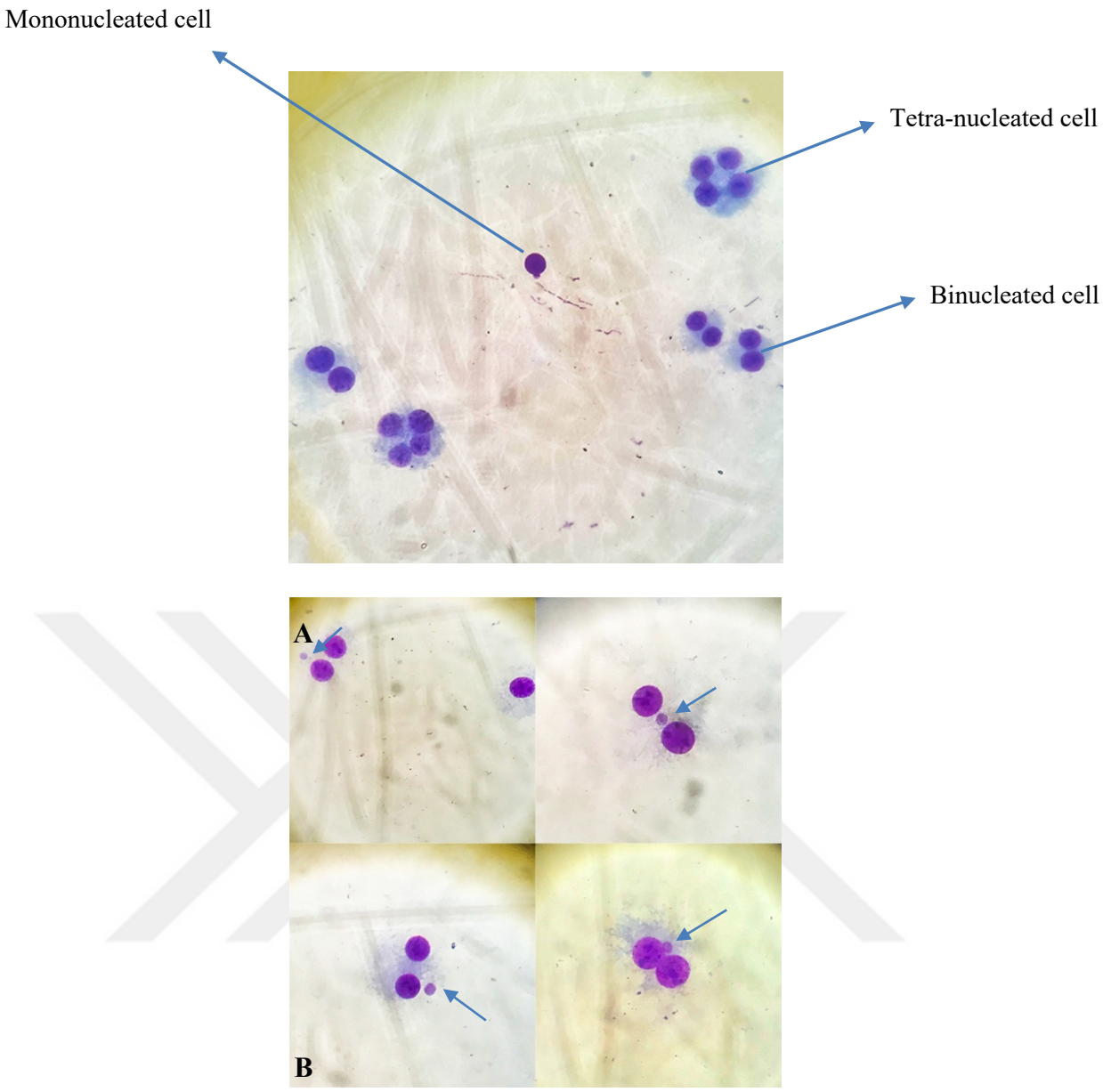

Figure 18. Representative images showing TK6 cells stained by Giemsa under a light microscope. A) Examples of observed mononucleated, binucleated and multinucleated cells with clear cytoplasm under light microscope B) Examples of binucleated cells with micronuclei in the presence of cytochalasin-B. The arrows show micronuclei. The nuclei were stained purple, while the cytoplasm was stained blue by Giemsa.

WST-1 cell viability assay was performed to determine the appropriate Dox concentration for this study. Concentration-response curves of Dox to TK6 cells were obtained. Dox showed a moderate toxicity with a IC50 172.95 ± 9.33 nM after 24 h treatment. Based on these results, Dox at concentrations of 10, 25, 50 and 100 nM were chosen for the MN experiments. The MN test could not be evaluated at 100 nM concentration of Dox. Because the number of binucleated cells was not sufficient for MN evaluation. The results showed that Dox significantly increased the number of MN in TK6 cells in a dose-dependent manner.

Dox increased the number of MN in the cells, ranging from 8 to 32 MN per 1000 cells. While MNi numbers (±SD) observed in 1000 binucleated cells were 7.5 ± 2.12, 11.5 ± 0.71 and 31.5 ± 6.36 for 10, 25 and 50 nM, respectively, 1 MN was found for the negative control, PBS. The number of MN observed at Dox 50 nM concentration was statistically different compared to the control group. From the data from the MN test, four more parameters were calculated. To assess cytotoxicity and cellular proliferation progression. These calculated parameters are cytokinesis block proliferation index (CBPI), nuclear division index (NDI), replication index (RI) and cytostasis percentage. CBPI values were found to be 1.83 ± 0.01, 1.73 ± 0.02, and 1.37 ± 0.09 for 10, 25, and 50 nM, respectively, and 1.87 for negative control, PBS. The CBPI value decreased as the applied Dox concentration increased. A statistically significant decrease was found when the Dox 50 nM concentration group was compared with the control group. The calculated NDI data are as follows; 1.93 ± 0.005, 1.88 ± 0.0018, 1.76 ± 0.013 and 1.38 ± 0.096 for control, 10, 25, and 50 nM, respectively. There was a significant decrease trend in NDI with increasing dose of Dox, a statistically significant difference was found between Dox 50 nM and control group. In addition, all mean NDI values were between 1.93 and 1.38, indicating that the TK6 culture method was successful. The RI value decreased as the Dox concentration increased. On the contrary, the cytostasis value increased with the increase in concentration. RI values are between 95 and 40. Accordingly, cytostasis is over 50% at Dox 50 nM concentration. The cytostasis induced by Dox clearly demonstrates that Dox significantly affects cell division. The results of Dox-induced MN number also CBPI, NDI and RI values in TK6 cells are shown in Figure 19.

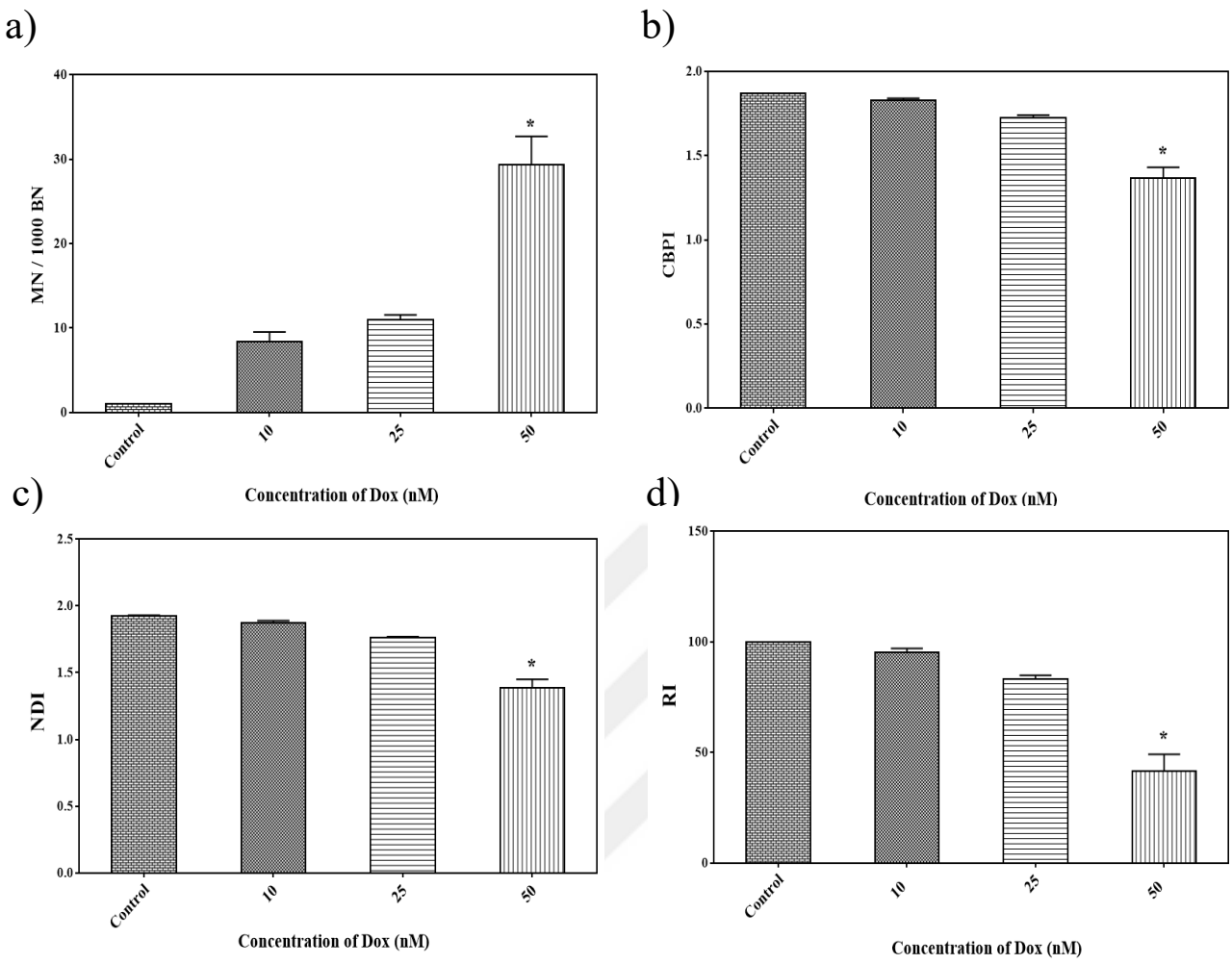

Figure 19. Micronucleus and cell viability tests of Dox in TK6 cells. Cell were treated with different doses (10, 25, 50 nM) of Dox without S9 mix for 24 h a) Mean number of MNi was analysed after treatment with Dox. The results are expressed as the mean of MN-containing cells in 1000 binucleated cells (BN) ± SEM of three separate tests b) Cytokinesis-block proliferation index (CBPI): (number of mononucleated cells + 2xnumber binucleated cells + 3xnumber multinucleated cells)/(sum of mononucleated, binucleated and multinucleated cells) c) nuclear division index (NDI): (number of mononucleated cells + 2xnumber binucleated cells + 3x number of tri-nucleated cells + 4x number of tetra-nucleated cells)/(sum of mononucleated, binucleated, tri-nucleated and tetra-nucleated cells) d) replication index (RI): (100 - %cytostasis). The Kruskal-Wallis nonparametric analysis was used; the bars show the mean ± SEM of three independent experiments (the p-value is displayed on the top of each bar). Compared to negative control group, * indicates statistically significant differences ($p \leq 0.05$). Dox: Doxorubicin.

In order to test the protective effect of RA against Dox-induced genotoxicity, the Dox concentration with maximum MN induction was chosen. The IC50 value of RA was found to be 0.22 ± 0.05 mM according to the WST-1 cell viability test. In the MN assay, 50 and 100 µM concentrations of RA were studied. The increase in the number of MN that induced by Dox was reduced by RA. The MNi (±SD) number of Dox at a

concentration of 50 nM was found to be 22.50 ± 0.71. When Dox 50 nM and RA 50 µM or RA 100 µM were applied together, the number of MNi observed in binucleated cells decreased. However, the decrease in the number of MN formation was not directly related to the concentration of RA. The observed MNi (±SD) number was determined to be 12 ± 1.41 when Dox 50 nM and RA 50 nM were applied together. This number was found to be 14 ± 1.41 when Dox 50 nM and RA 100 µM were applied together. Under this concentration range, there was no change in cytostasis and RI values when compared to the cells treated with Dox alone and the group treated with Dox and RA together. The effect of increasing doses of RA alone and combination with Dox on the number of MN in TK6 cells after 24 h is shown in Figure 20.

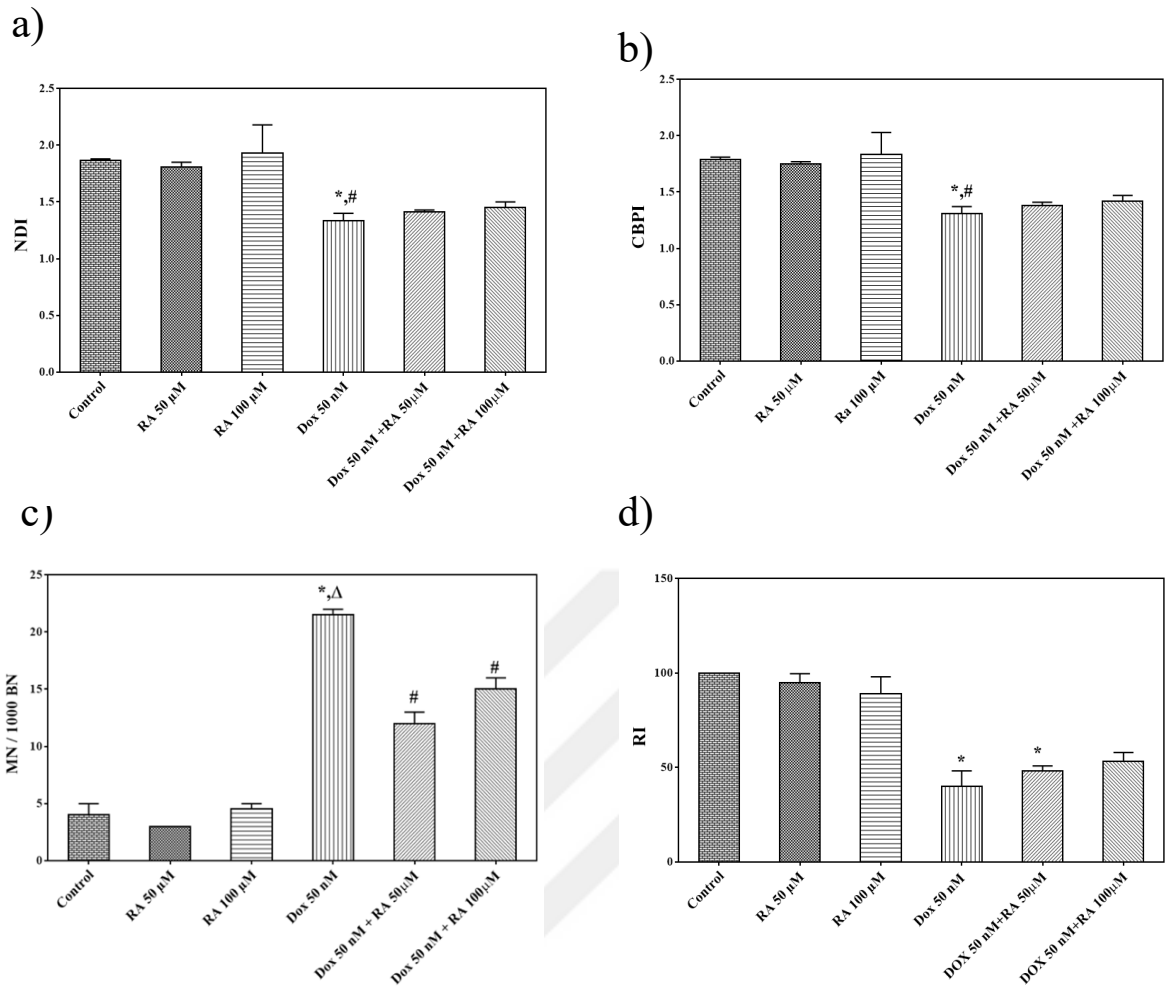

Figure 20. Number of MNi and cytotoxicity biomarkers in TK6 cells treated with Dox, RA and their combinations. Cell were treated with 50 nM of Dox, RA 50,100 µM and combinations of Dox and RA without S9 mix for 24 h. a) Mean number of MNi was analysed after treatment with samples. * significantly different from negative control group, Δ significantly different from RA 50 µM group, # significantly different from Dox 50 nM group ($p \leq 0.05$). The results are expressed as the mean of MN-containing cells in 1000 BN ± SEM of three separate tests. b) Cytokinesis-block proliferation index (CBPI): (number of mononucleated cells + 2x number binucleated cells + 3x number multinucleated cells)/(sum of mononucleated, binucleated and multinucleated cells), * significantly different from negative control group, # significantly different from RA 100 µM group ($p \leq 0.05$). c) nuclear division index (NDI): (number of mononucleated cells + 2xnumber binucleated cells + 3x number of tri-nucleated cells + 4x number of tetra-nucleated cells)/(sum of mononucleated, binucleated, tri-nucleated and tetra-nucleated cells), * significantly different from negative control group, # significantly different from RA 100 µM group ($p \leq 0.05$). d) replication index (RI): (100 - %cytostasis), * significantly different from negative control group. The Kruskal-Wallis nonparametric analysis was used; the bars show the mean ± SEM of three independent experiments (the p-value is displayed on the top of each bar). Dox: Doxorubicin, RA: Rosmarinic acid.

We also evaluated the chemoprotective effects of the EGCG against the genotoxicity induced by Dox. The protective effects of EGCG were dose-dependent, as it decreased the number of MN caused by Dox and increased the proliferation of Dox-affected TK6 cells. The protective effects of EGCG 10 and 25 µM concentrations were investigated. These concentrations were chosen to be lower than the IC50 value found in the WST-1 cell viability assay. In this experiment, the number of MNi (±SD) was 21.5 ± 2.12 when Dox 50 nM was applied alone. Dox 50 nM and EGCG 10 µM were applied together, the observed MNi (±SD) number was 11.5 ± 0.71, when EGCG 25 µM was applied together with Dox 50 nM, the observed MNi number was 8 ± 2.83. The results of the evaluations are shown in Figure 21.

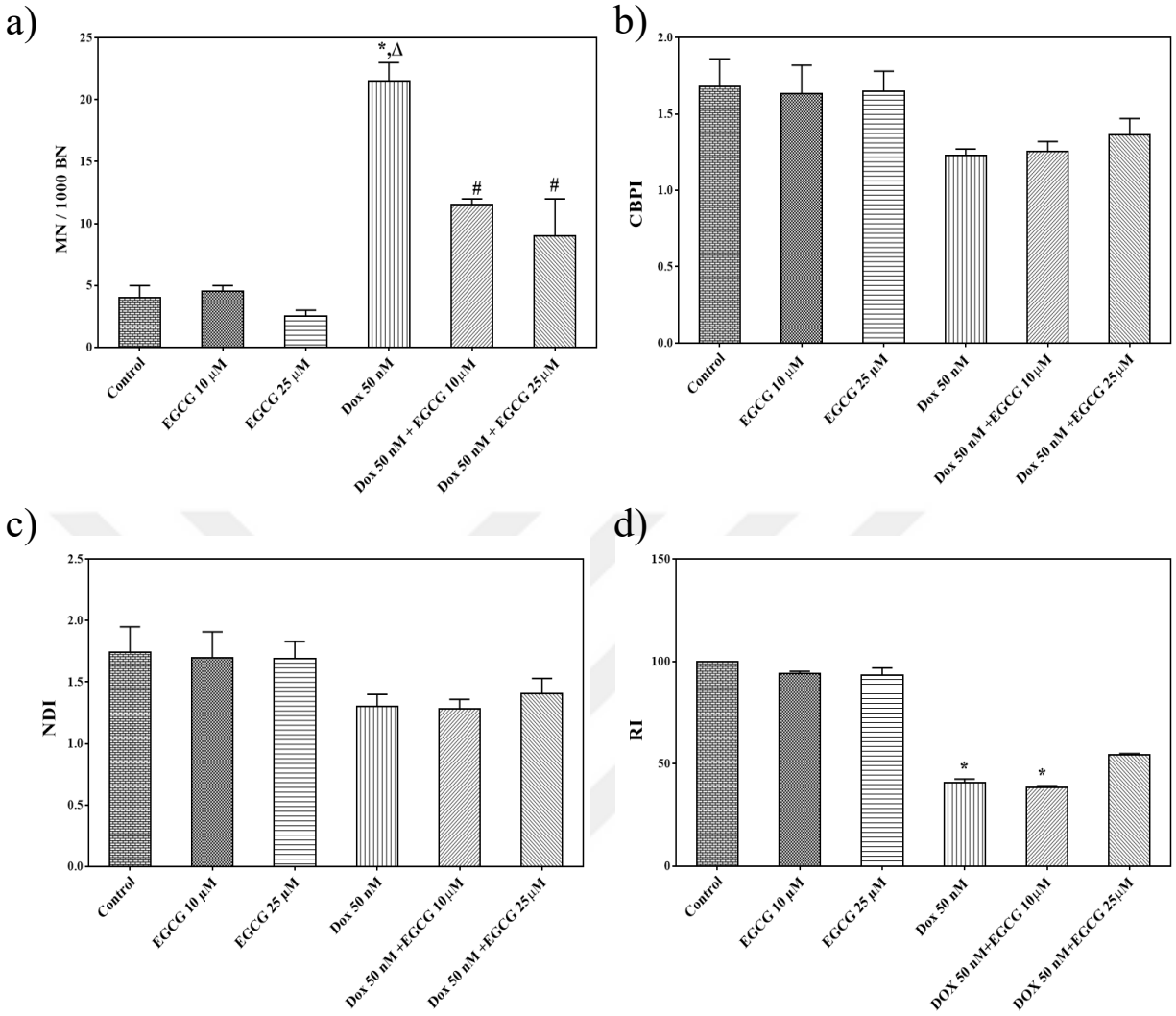

Figure 21. Number of MNi and cytotoxicity biomarkers in TK6 cells treated with Dox, EGCG and their combinations. Cell were treated with 50 nM of Dox, EGCG 10, 25 μM and combinations of Dox and EGCG without S9 mix for 24 h. a) Mean number of MNi was analysed after treatment with samples. * significantly different from negative control group, Δ significantly different from EGCG 25 μM group, # significantly different from Dox 50 nM group ($p \leq 0.05$). The results are expressed as the mean of MN-containing cells in 1000 BN ± SEM of three separate tests. b) Cytokinesis-block proliferation index (CBPI): (number of mononucleated cells + 2xnumber binucleated cells + 3xnumber multinucleated cells)/(sum of mononucleated, binucleated and multinucleated cells), * significantly different from negative control group. c) nuclear division index (NDI): (number of mononucleated cells + 2xnumber binucleated cells + 3x number of tri-nucleated cells + 4x number of tetra-nucleated cells)/(sum of mononucleated, binucleated, tri-nucleated and tetra-nucleated cells). d) replication index (RI): (100 - %cytostasis), * significantly different from negative control group. The Kruskal-Wallis nonparametric analysis was used; the bars show the mean ± SEM of three independent experiments (the p-value is displayed on the top of each bar). Dox: Doxorubicin, EGCG: Epigallocatechin gallate.

In order to analyze the genoprotective effect of phenolic compounds combinations, RA and EGCG were combined at concentrations in which a maximum reduction of MN was observed from their individual effect against Dox induced genotoxicity. The co-administration of RA and EGCG did not result in a significant decrease in the MN number when compare with the application of RA and EGCG alone. When RA and EGCG were applied alone, they showed a statistically significant protective effect against genotoxicity caused by Dox 50 nM. Only Dox caused a reduction in NDI; this was not reversed when Dox was combined with RA, EGCG, or a combination of both in any of the protocols used. The changes in the investigated parameters when RA and EGCG are applied together or alone are shown in Figure 22.

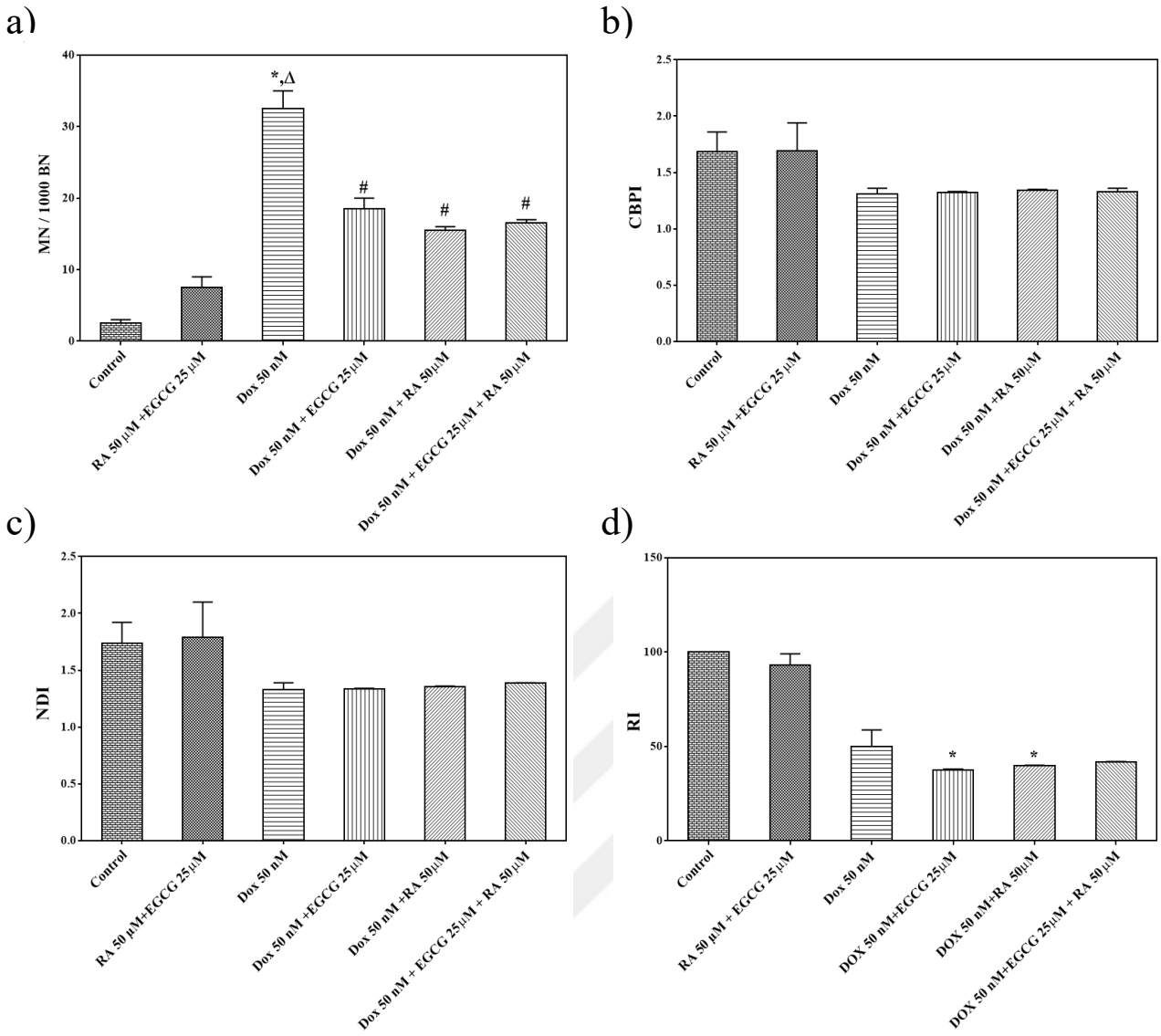

Figure 22. Number of MNi and cytotoxicity biomarkers in TK6 cells treated with Dox, RA,EGCG and their combinations. The groups applied to the cells are as follows: Dox 50 nM, RA 50 µM, EGCG 25 µM, Dox 50nM +RA 50 µM, Dox 50 nM + EGCG 25 µM and Dox 50nM +RA 50 µM + EGCG 25 µM without S9 mix for 24 h. a) Mean number of MNi was analysed after treatment with samples. * significantly different from negative control group, # significantly different from Dox 50 nM group, Δ significantly different from RA 50 µM +EGCG 25 µM group ($p \leq 0.05$). The results are expressed as the mean of MN-containing cells in 1000 BN ± SEM of three separate tests. b) Cytokinesis-block proliferation index (CBPI): (number of mononucleated cells + 2 x number binucleated cells + 3 x number multinucleated cells)/(sum of mononucleated, binucleated and multinucleated cells) c) nuclear division index (NDI): (number of mononucleated cells + 2xnumber binucleated cells + 3x number of tri-nucleated cells + 4x number of tetra-nucleated cells)/(sum of mononucleated, binucleated, tri-nucleated and tetra-nucleated cells) d) replication index (RI): (100 - %cytostasis), * significantly different from negative control group. The Kruskal-Wallis nonparametric analysis was used; the bars show the mean ± SEM of three independent experiments (the p-value is displayed on the top of each bar). Dox: Doxorubicin, RA: Rosmarinic acid, EGCG: Epigallocatechin gallate.

4.1.2.1. WST-1-based cell cytotoxicity assay

The concentrations used in the MN assay were determined based on data obtained from WST-1 cell viability tests performed prior to the MN experiment. TK6 cells were treated with various concentrations of Dox (62.5, 125, 250 and 500 nM) for 24 h. Figure 23 shows the cell viability for TK6 cells after exposure to Dox for 24 h. The maximum cytotoxic activity was detected at a concentration of 500 nM. Following Dox exposure, TK6 cells showed a dose-dependent decrease in cell viability. The IC50 value of Dox was 172.95 ± 9.33 nM for 24 h.

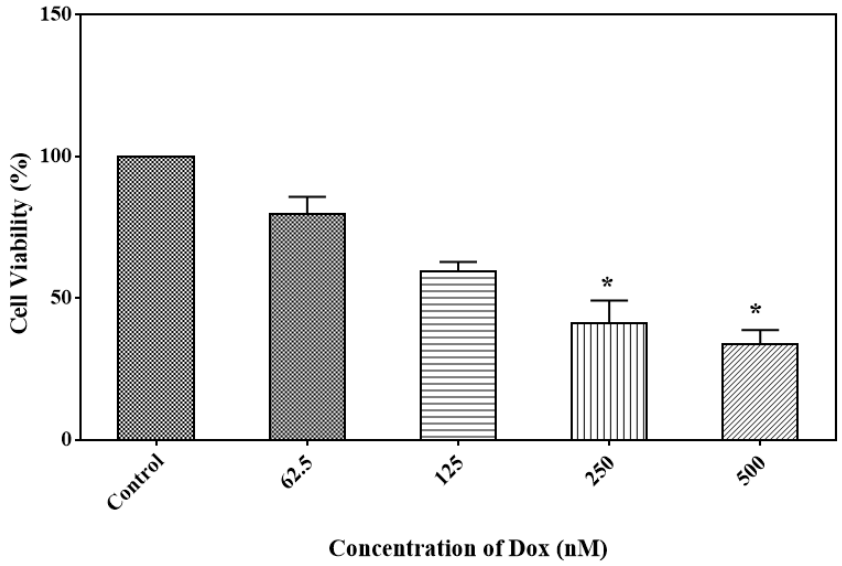

Figure 23. TK6 cells were treated with different concentrations of Dox for 24 h, and viability was assessed by WST-1. Dox reduced cell proliferation in TK6 cells in a dose-dependent manner. The results (mean ± SEM) were expressed as a percentage of the control values. Data were analysed by Kruskal Wallis test.*$p \leq 0.05$ vs. Control group.

The effects of different EGCG concentrations at 24 hours on cell viability in TK6 cells were also investigated. Different doses of EGCG (5, 10, 25, 50 and 100 M) were applied to TK6 cells. The IC50 value of EGCG was found as 49.01 ± 8.85 μM. Figure 24 shows the cell viability on TK6 cells after 24 h treatment with EGCG.

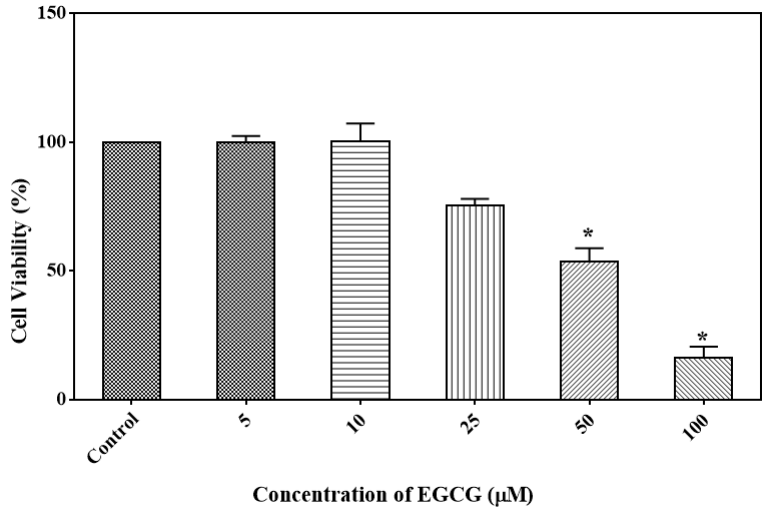

Figure 24. The effects of EGCG on cell viability of TK6. Data are represented of three separate repeats tests and presented as mean ± SEM. Data were analysed by Kruskal Wallis test, *p ≤ 0.05 vs. Control group.

RA was applied to TK6 cells at concentrations of 0.0625, 0.125, 0.25 and 0.35 mM for 24 hours. With the increasing treatments of RA, the cell viability of TK6 cells decreased. In the calculation based on the percent viability of the control group, the IC50 value of RA was found as 0.22 ± 0.05 mM. Figure 25 shows the effect of RA on cell viability.

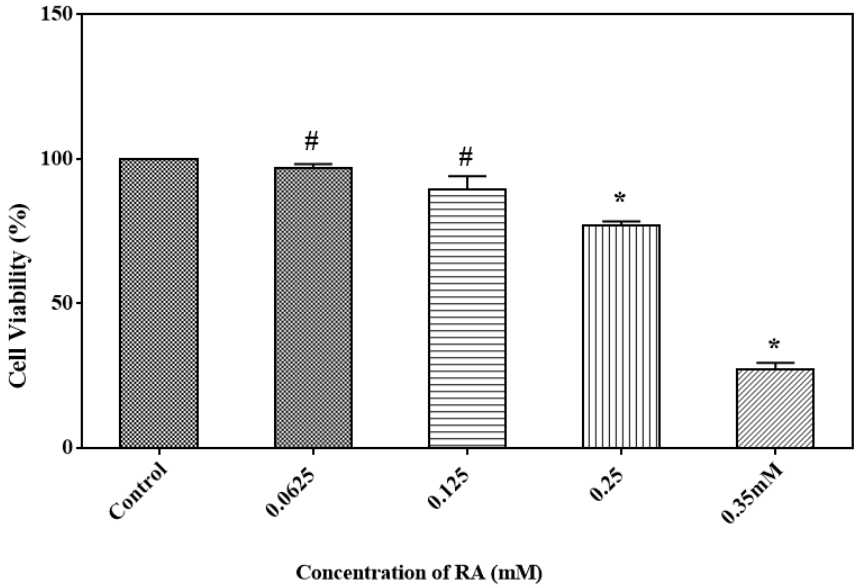

Figure 25. The effects of RA on cell viability of TK6. Each bar represents the mean ± SEM of three independent experiments. Data were analysed by Kruskal Wallis test, *p ≤ 0.05 vs. Control group and #p ≤ 0.05 vs. RA 0.35 mM group.

In addition, the possible protective effects of RA and EGCG alone or in combination against the cytotoxic effect caused by Dox on TK6 cells were investigated with the WST-1 assay. Considering Dox's IC50 value, the Dox 100 nM concentration was chosen. The possible protective effects of RA and EGCG against Dox 100 nM were investigated. TK6 cells were treated with various doses of EGCG, RA and their combination for 24 h. Figure 26 shows the effect of EGCG, RA and their combinations against Dox induced cytotoxicity**.**

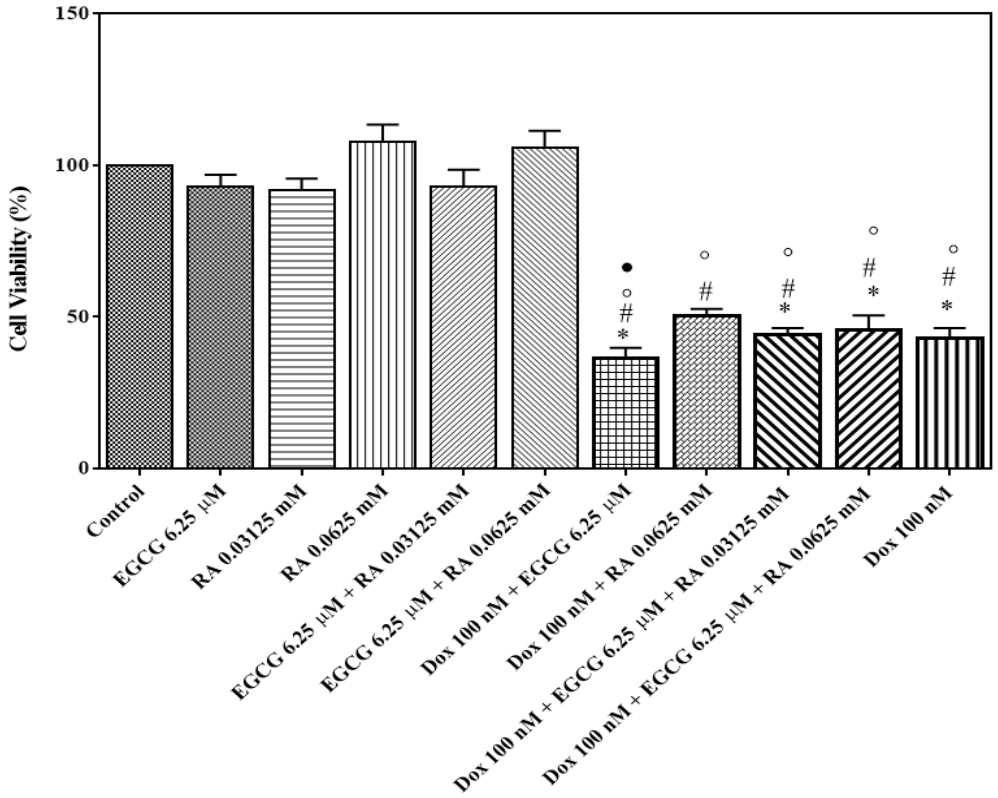

Figure 26. The protective effect of the combination of EGCG and RA against Dox induced cell viability. *P ≤ 0.05 indicates a statistically significant reduction in cell viability compared to the corresponding untreated control. #P ≤ 0.05 vs. EGCG 6.25 μM + RA 0.0625 mM, °P≤ 0.05 vs. RA 0.0625 mM, • P ≤ 0.05 vs. RA 0.03125 mM and EGCG 6.25 μM.

When EGCG 6.25 μM, RA 0.03125 mM, and RA 0.0625 mM administered alone or in combination, they did not show a statistically significant protective effect against cytotoxicity caused by Dox 100 nM. Although not statistically significant, 0.0625 mM concentration of RA showed the most protective effect against cytotoxicity caused by Dox.

4.2 Microscopic analysis of ROS production

The oxidative stress status of various groups was assessed by DHE staining. The amount of cellular superoxide at the concentration of Dox causing DNA damage was determined by this test. The amount of ROS formed after Dox was applied to the cells were assessed in a time-dependent manner. To clarify the antioxidant effect of RA and EGCG, Dox treated to provide a similar condition for oxidative stress. Dox treatment increased the intensity of red color, as shown in Figure 27.

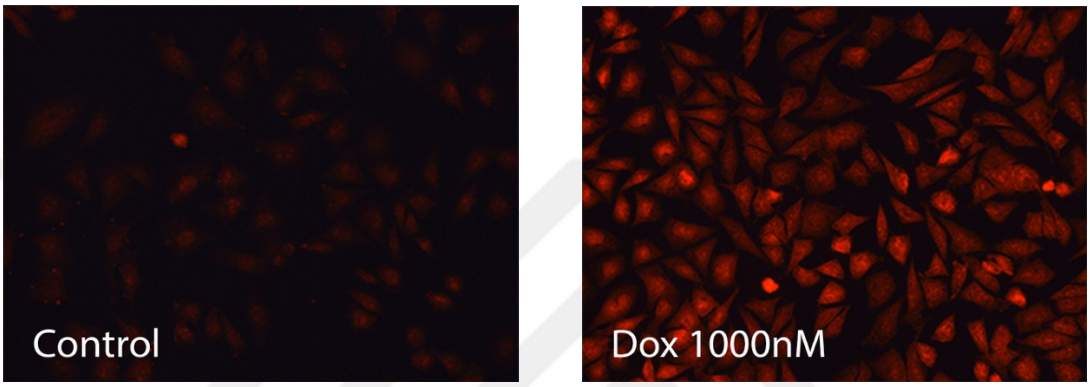

Figure 27. Representative pictures of DHE stained CHO-K1 cells are shown. The photo on the left shows the control group, whereas the one on the right shows the Dox concentration of 1000 nM after application. Pictures were taken at 200-fold magnification.

The amount of intracellular ROS production after Dox administration in CHO cells was investigated. Dox was applied to the cells for different time periods, 0.5, 1, 2, and 4 hours, as shown in Figure 28. Superoxide production was increased in cells that were treated with Dox 1000 nM for 0.5 hours or longer.

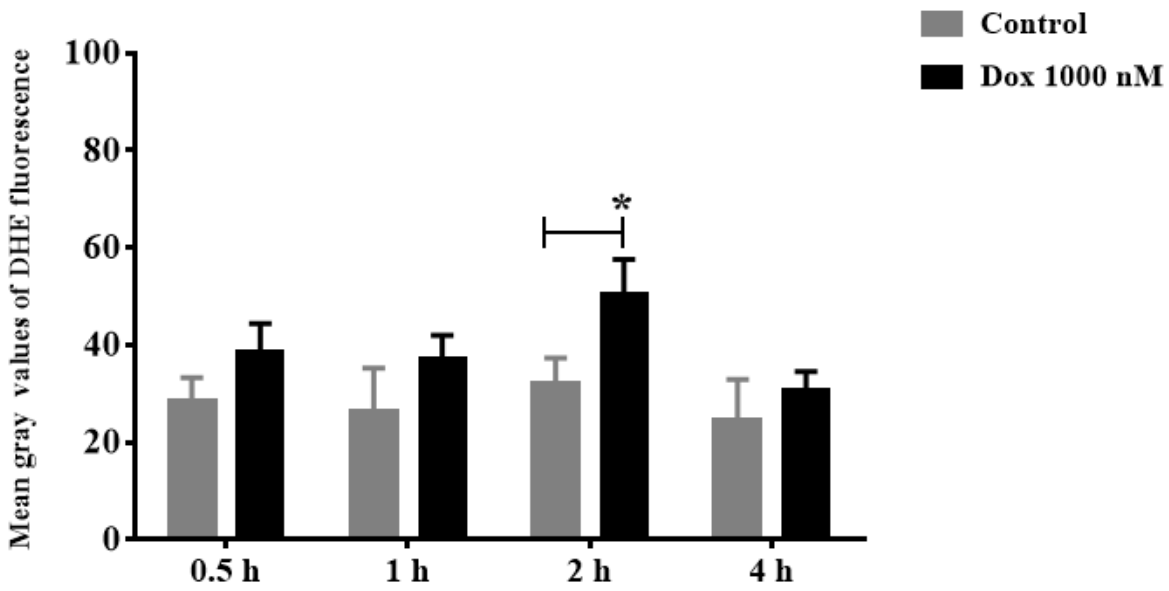

Figure 28. Superoxide production was detected using the dye DHE in CHO-K1 cells treated with 1000 nM Dox for different time (0.5 h to 2 hr). DHE fluorescence was quantified using image j software, which measured the mean grey value of 200 cells. Results are shown as mean ± SEM of three separate tests. The mean comparison of two independent groups was analyzed using the independent sample t-test. *p ≤ 0.05 vs. Control group. Dox: Doxorubicin.

As a result of the time-response experiment, the highest amount of ROS production was observed at 2 hours exposure of Dox 1000 nM. When the Dox group was compared to the control group after 2 hours application, statistical significance was found. The effect of RA and EGCG on intracellular oxidative stress was investigated after 2 hours of administration. In addition, the effect of RA and EGCG against the oxidative stress caused by Dox applied to the cells for 2 hours was also investigated. The cells were treated with Dox and RA for 2 hours to investigate the potential antioxidant activity of RA in a cellular system. The effect of RA on Dox-produced superoxide in CHO cells was shown in Figure 29.

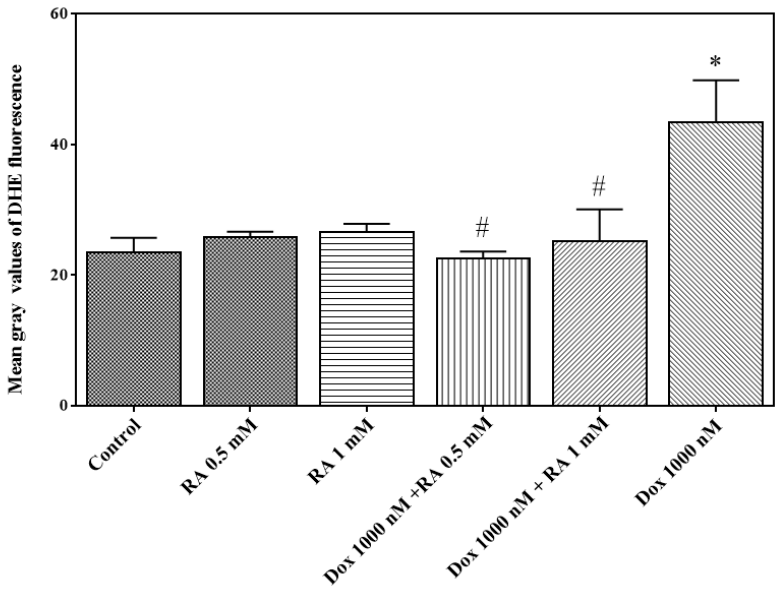

Figure 29. Superoxide production and its inhibition by RA in CHO-K1 cells. DHE fluorescence was quantified using image j software, which measured the mean grey value of 200 cells Results are shown as mean ± SEM of three independent tests. Kruskal Wallis test was used for analysis, *p ≤ 0.05 vs. Control and #p≤ 0.05 vs. Dox 1000 nM. Dox: Doxorubicin, RA: Rosmarinic acid.

Ra 1mM and Ra 0.5mM reduced the amount of ROS produced by Dox. The decrease in the amount of ROS formed was statistically significant. When the concentration of RA was doubled, there was no tragic concentration-dependent reduction in the amount of Dox-induced ROS.

The effects of EGCG and RA and their combinations on ROS produced by Dox in CHO-K1 cells were also studied. The cells were treated with Dox 1000 nM and different concentrations of EGCG (50 and 100 μM) and Ra 1 mM alone or in combination for 2 hours to investigate the potential antioxidant activity in a cellular system. The results are shown in Figure 30.

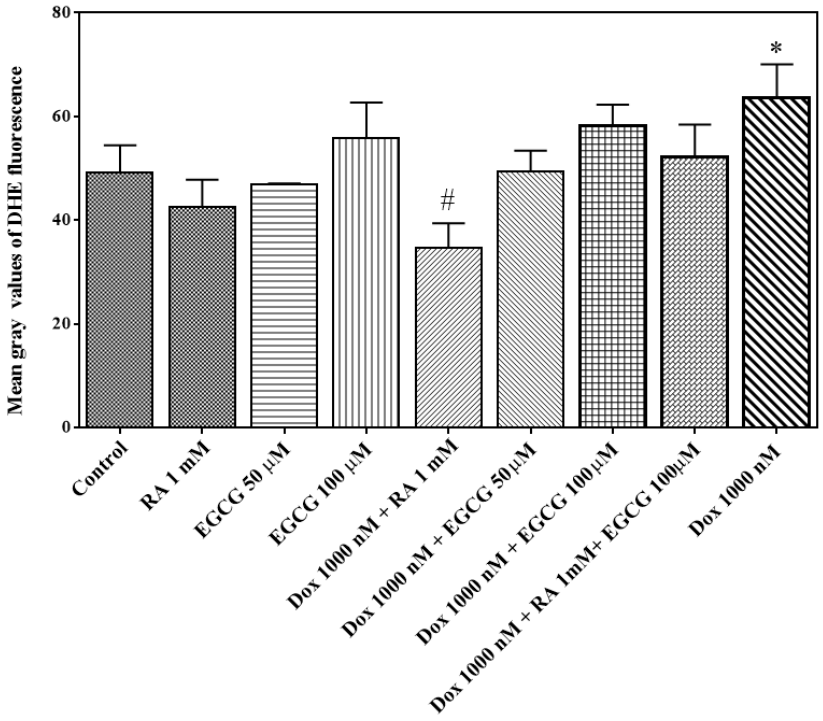

Figure 30. Intracellular ROS after treating the cells with RA 1mM and EGCG (50, 100 µM) for 2 h with and without the addition of Dox. DHE fluorescence was quantified using image j software, which measured the mean grey value of 200 cells. Results are shown as mean ± SEM of three independent tests. Kruskal Wallis test was used for analysis, *p ≤ 0.05 vs. Control and #p≤ 0.05 vs. Dox 1000 nM. Dox: Doxorubicin, RA: Rosmarinic acid, EGCG: Epigallocatechin gallate.

When RA 1mM and EGCG 50 µM were administered to CHO-K1 cells alone with Dox, they reduced the amount of ROS produced by Dox. When RA 1mM was administered with Dox, it was found that reduced the amount of ROS formed compared to the Dox 1000 nM group statistically significant. No reduction in ROS to the expected degree was observed with the combined administration of EGCG 100 µM and RA 1 mM. Surprisingly, there was an increase in the amount of ROS when 100 µM EGCG was applied to CHO-K1 cells.

4.3 Mitochondrial Membrane Potential Assay

Mitochondrial membrane potential was detected using cationic fluorescent dye (TMRE) by fluorescence plate reader. TMRE is a cationic red-orange dye that accumulates in active mitochondria. Mitochondria that are depolarized or inactive have a lower membrane potential and are unable to bind TMRE. In this study, firstly, Dox, EGCG and RA were applied to TK6 cells alone. According to the results obtained from these experiments, the concentration was determined for each sample. TK6 cells were

exposed to different (10, 25, 50, 100 and 200 nM) concentrations of Dox for 24 h, the mitochondrial membrane potential of TK6 cells changed with dose as assessed by the TMRE assay, is shown in Figure 31.

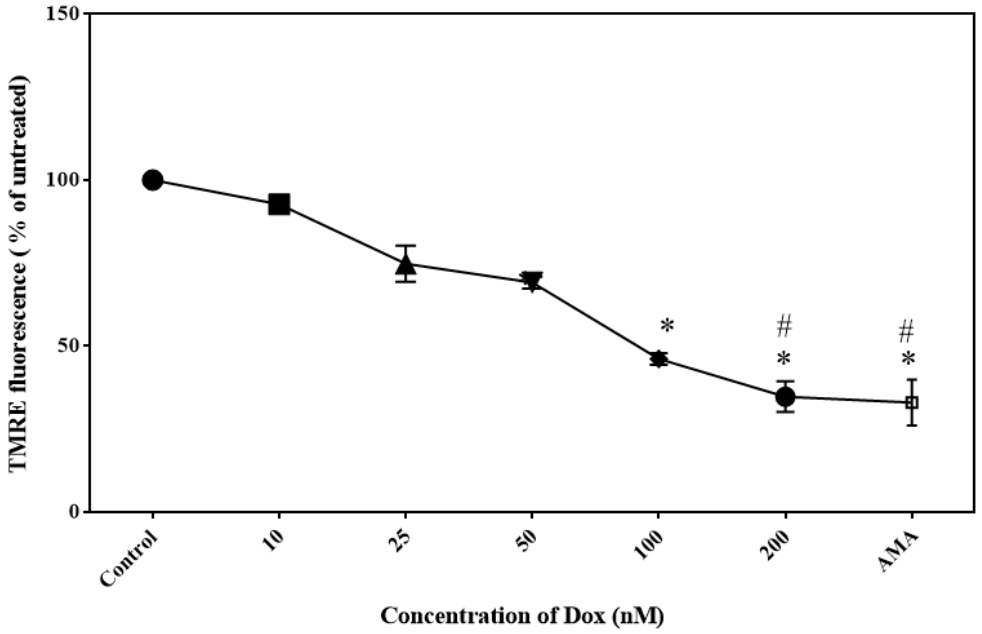

Figure 31. Dose-dependent effects of Dox on mitochondrial membrane potential. Antimycin A (AMA) was used as a positive control, is an inhibitor of cellular respiration, was applied at the concentration of 50 μM for 30 min. The cells were incubated with 200 nM TMRE for 30 min at 37°C, 5% CO2. Data are expressed as mean values ± SEM (n=3). Kruskal Wallis test was used for analysis, *P ≤ 0.05 vs. Control group, #P ≤ 0.05 vs Dox 10 nM. Dox: Doxorubicin.

A statistically significant difference was found when TK6 cells treated with Dox 100 and 200 nM concentrations were compared with the negative control group treated with PBS.

Mitochondrial membrane potential of TK6 cells after 24 h of treatment with EGCG (5, 10, 25 and 50 μM). The mitochondrial membrane potential, which changes depending on the EGCG concentration, is shown in Figure 32.

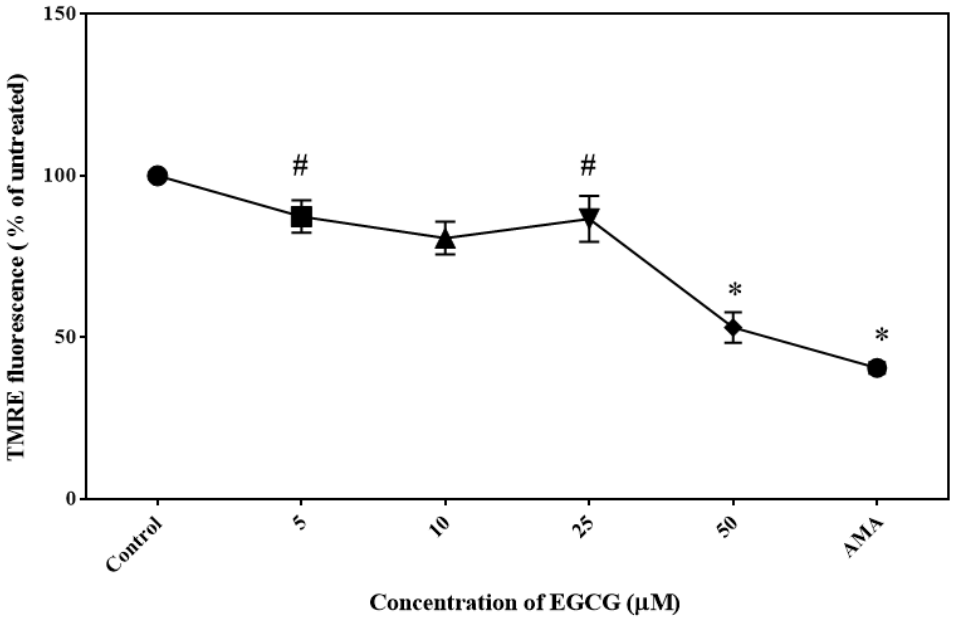

Figure 32. Dose-dependent effects of EGCG on mitochondrial membrane potential. Antimycin A (AMA) was used as a positive control, is an inhibitor of cellular respiration, was applied at the concentration of 50 µM for 30 min. The cells were incubated with 200 nM TMRE for 30 min at 37°C, 5% CO2. Data are expressed as mean values ± SEM (n=3). Kruskal Wallis test was used for analysis, *P ≤ 0.05 vs. Control group and #P ≤ 0.05 vs AMA 50 µM. EGCG: Epigallocatechin gallate.

EGCG 50 µM concentration decreased the mitochondrial membrane potential statistically significantly when compared to the negative control group.

Then, the effect of RA on the mitochondrial membrane potential was investigated. The mitochondrial membrane potential of TK6 cells after 24 h of treatment with RA (0.03125, 0.0625, 0.125 and 0.25 mM) is shown in Figure 33.

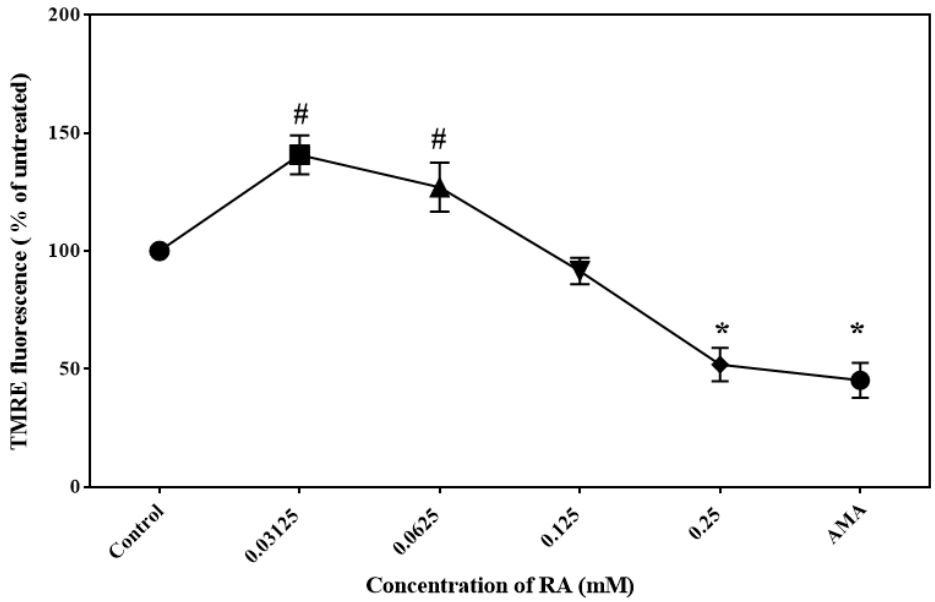

Figure 33. Dose-dependent effects of RA on mitochondrial membrane potential. Antimycin A (AMA) was used as a positive control, is an inhibitor of cellular respiration, was applied at the concentration of 50 μM for 30 min. The cells were incubated with 200 nM TMRE for 30 min at 37°C, 5% CO2. Data are expressed as mean values ± SEM (n=3). Kruskal Wallis test was used for analysis, *P ≤ 0.05 vs. Control group and #P ≤ 0.05 vs AMA 50 μM. RA: Rosmarinic acid.

Different doses of RA (0.03125, 0.0625 and 0.125 mM) were administered to TK6 cells to investigate the alteration of Dox (100 nM)-induced depolarization of mitochondrial membrane potential. Figure 34 shows the effect of RA on mitochondrial membrane potential.

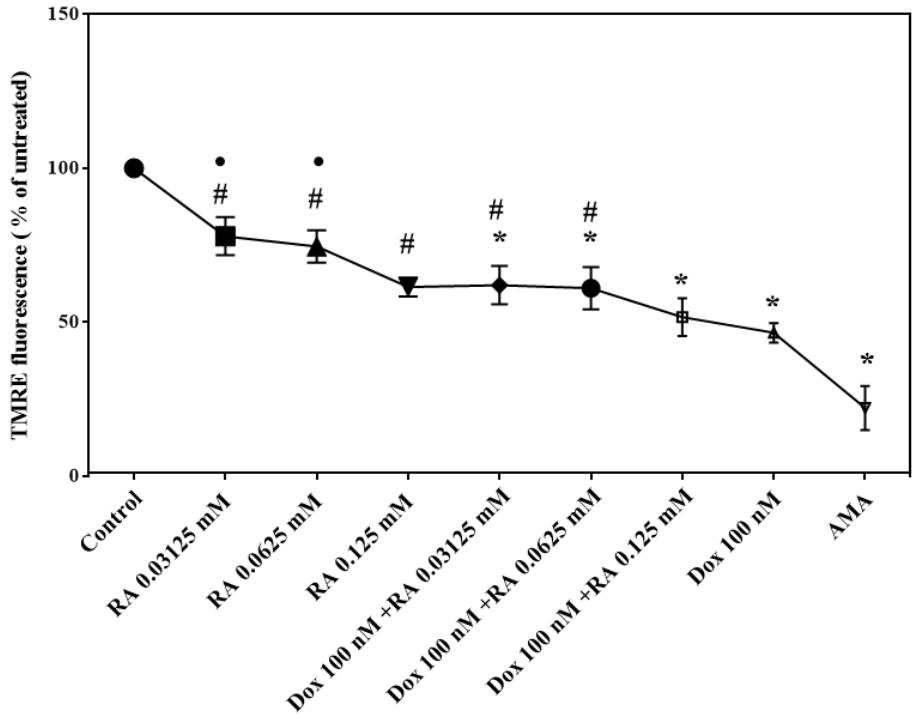

Figure 34. Mitochondrial membrane potential of TK6 cells after 24 h of treatment with 100 nM Dox and/or RA. Mitochondria were labeled with the fluorescent probe 200 nM TMRE for 30 min. and 50 µM AMA was used as a positive control. Data are expressed as mean values ± SEM (n=3). Kruskal Wallis test was used for analysis, *P ≤ 0.05 vs. Control group, #P ≤ 0.05 vs AMA 50 µM and • P ≤ 0.05 vs. Dox 100 nM. Dox: Doxorubicin, RA: Rosmarinic acid.

There was no statistically significant difference when the group in which RA was applied together with Dox 100 nM compared with the group in which only Dox 100 nM was applied. RA showed weak protective effect against Dox-induced oxidative stress. The membrane potential is higher at low doses of RA, implying that cell viability is also higher.

Different doses of EGCG (6.25 and 12.5 µM) were administered to TK6 cells to investigate the alteration of Dox (100 nM)-induced depolarization of mitochondrial membrane potential. Figure 35 shows the effects of combinations of Dox and EGCG on mitochondrial membrane potential.

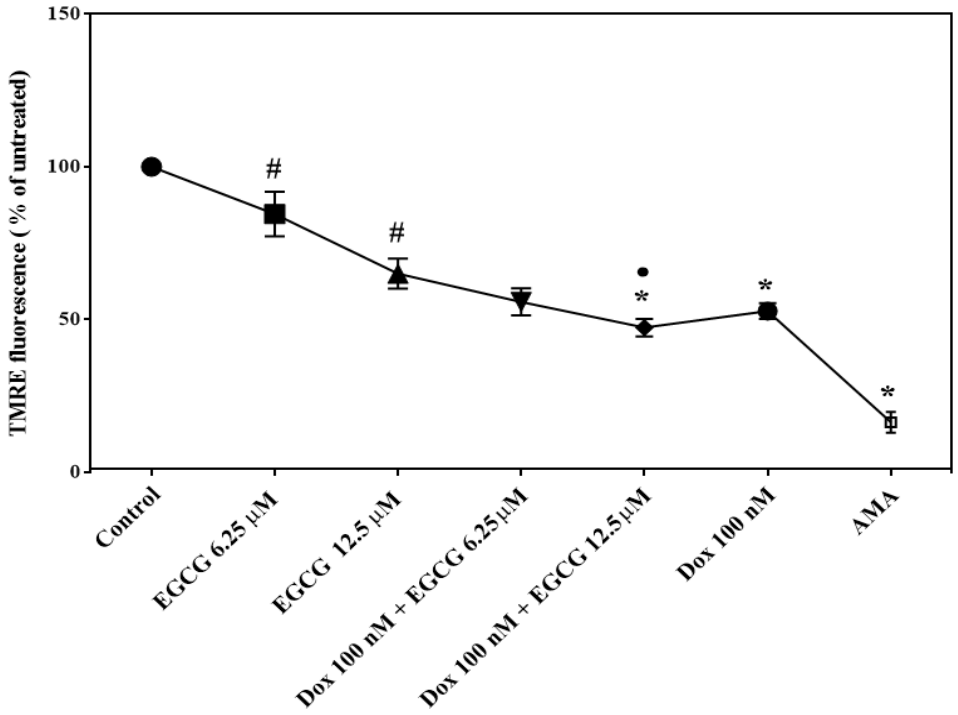

Figure 35. Mitochondrial membrane potential of TK6 cells after 24 h of treatment with 100 nM Dox and/or EGCG (6.25 and 12.5µM). Mitochondria were labeled with the fluorescent probe 200 nM TMRE for 30 min. and 50 µM AMA was used as a positive control. Data are expressed as mean values ± SEM (n=3). Kruskal Wallis test was used for analysis, *P ≤ 0.05 vs. Control group, #P ≤ 0.05 vs AMA 50 µM and • P ≤ 0.05 vs. EGCG 6.25 µM. Dox: Doxorubicin, EGCG: Epigallocatechin gallate.

Results revealed that EGCG did not showed any protective effect. In fact, as the EGCG dose increased, the mitochondrial membrane potential decreased.

For the next study, it was investigated how Dox 100 nM-induced reduction in membrane potential changed when EGCG and RA were applied simultaneously. Doses of EGCG and RA that did not reduce mitochondrial membrane potential were chosen. Cells were treated with different concentrations of both drugs either alone or in combination and incubated for 24 h. For this experiment, the EGCG concentration was 6.25 µM, and the RA concentrations were 0.03125 and 0.0625 mM.

When RA and EGCG were applied in combination, they did not significantly affect the membrane potential reduction caused by Dox. Figure 36 shows the effects of combinations of RA and EGCG on Dox-induced reduction of mitochondrial membrane potential

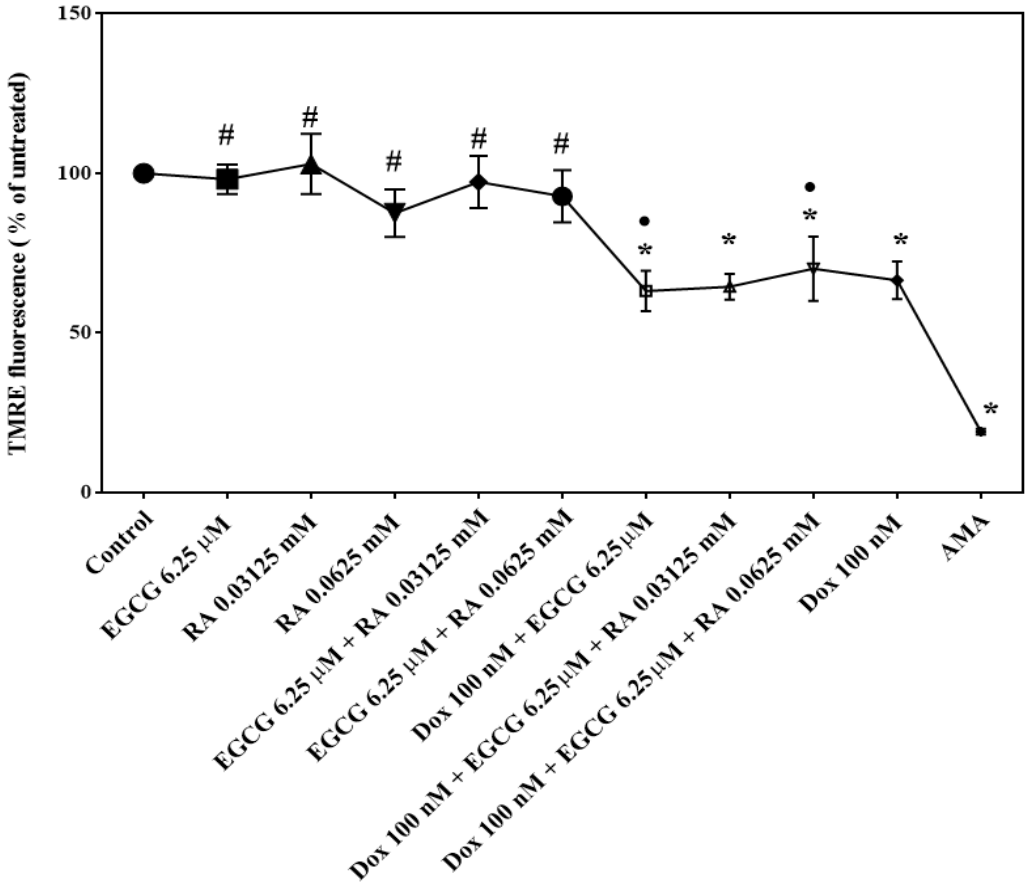

Figure 36. Mitochondrial membrane potential of TK6 cells after 24 h of treatment with 100 nM Dox and combination of EGCG and RA. Mitochondria were labeled with the fluorescent probe 200 nM TMRE for 30 min. and 50 µM AMA was used as a positive control. Data are expressed as mean values ± SEM (n=3). Kruskal Wallis test was used for analysis, *P ≤ 0.05 vs. Control group, #P ≤ 0.05 vs AMA 50 µM and • P ≤ 0.05 vs. EGCG 6.25 µM and RA 0.03125 mM.

4.4 Mutagenicity and Antimutagenicity Assays

4.4.1 Bacterial Reverse Mutation Test Results

A standard plate incorporation Ames test was used for the mutagenicity assay. The Ames assays were performed in the presence and absence of metabolic activation system (S9). The mutant strains of *Salmonella typhimurium* TA98, TA97a, TA100, TA1535, and TA102 were used in the study. First of all, it was investigated in which bacteria strains showed mutagenic activity for different concentrations of Dox. Test results were given in Table 11.

Table 11. Mutagenic activity expressed as the mean and standard deviation of the number of revertants/plate in *Salmonella typhimurium* TA98, TA97a, TA100, TA102, and TA1535 strains exposed to Dox at various concentrations, with and without S9 metabolic activation.

In the absence of S9		**Number of colony/plate**				
		TA98	**TA97a**	**TA100**	**TA102**	**TA1535**
Negative Control		25.67 ± 6.4	161.75 ± 7.72	181.17± 13.15	472.17± 83.60	23,50 ± 7,85
Positive Control		922.3 ±32.6*	610.5 ± 19.1*	896.8 ±36.8*	979.5 ± 28.9*	420.0 ± 28.4*
	0.5 µg/plate	79.00 ± 9.5	198.00 ± 1.73	209.50 ± 24.95	436.67± 75.78	29.00 ± 7.18
	1 µg/plate	209.33 ± 55.01*	179.00 ± 18.36	197.17 ± 21.12	462.00± 99,26	20.60 ± 11.89
	5 µg/plate	705.33 ± 17.47*	279.00 ± 11.79*	341.67 ± 40.20*	538.50± 77.36	26.20 ± 9.15
	10 µg/plate	744.67± 29.69*	297.33 ± 19.01*	317.67 ± 64.89*	550.17± 51.30	22.50 ± 4.20
	25 µg/plate	162.33± 78.74	274.67 ± 29.74	141.33 ± 20.55	678.17± 117.78*	16.00 ± 3.74
	50 µg/plate	18.33± 5.86	17.67± 6.43	107.83 ± 71.32	720.83± 77.95*	3.60 ± 0.89*
	100 µg/plate	1.33± 0.58	11.67 ± 1.15	12.00± 8.83*	974.33± 33.26*	1.00 ± 0.45*
In the presence of S9						
Negative Control		29.67± 3.79	389.33 ± 78.56	146.50 ± 27.31	537.67 ± 18.42	12,67 ± 1,53
Positive Control		657.7 ±20.6*	593.3 ± 36.7*	618.8 ±50.4*	1124.0 ± 53.40*	98.3 ± 9.9 *
	0.5 µg/plate	52.67 ± 15.18	292.33 ± 12,66	144.50 ± 34.65	510.67± 47.35	13,00 ± 2,00
	1 µg/plate	58.67 ± 3.06	348,33 ± 63,00	179.00 ± 21.79	611.50 ± 57.17	16,00 ± 4,58
	5 µg/plate	170.00 ± 8.72	436,00 ± 78,43	272.00 ± 27.84	680.33 ± 69.77*	16,67 ± 3.06
	10 µg/plate	362.00 ± 12.77*	265,67 ± 75,59	286.75 ± 28.92*	794.67 ± 53.32*	13,67 ± 3.06
	25 µg/plate	482.33 ± 57.74*	109,67 ± 10,41*	116.25 ± 35.61	786.33 ± 126.28*	9,33 ± 2.08
	50 µg/plate	218.33 ± 18.01*	60,00 ± 10,00*	48.25 ± 38.16	798.33 ± 174.14*	4,67 ± 1,53
	100 µg/plate	12.67 ± 4.51	30,67 ± 7,37*	4.00 ± 2.94	1255.83 ± 325.54*	2,33 ± 1,15*

Dunnett's multiple comparison test was carried out for statistical analysis. Control vs Treatment, *$p \leq 0.05$. PBS (50 µL/plate) was used as a negative control. With the S9 experiments, 2-aminofluorene (2-AF - 5 µg/plate) was used as a positive mutagen for TA98, TA100 and TA97a strains, 2-Amino anthracene (2-AA – 10 µg/plate) was used a positive mutagen for TA102 and TA1535. In the absence of S9 mix, 4-nitro-o-phenylenediamine (NPD - 20 µg/plate) was used as a positive direct mutagen for the TA98 and TA97a strain, sodium azide (SA - 1 µg/plate) was used as positive direct mutagen for TA100 and TA1535 strains and mitomycin C (0.5 µg/plate) was used as a positive mutagen for TA102 strain.

In strain TA 98, in the presence and absence of metabolic activation, the concentration of 100 μg/plate resulted in a reduction in the number of revertant colonies as a result of Dox-induced toxicity. In the absence of metabolic activation in TA98 bacterial strain, Dox showed mutagenic activity from the lowest applied concentration to 50 μg/plate concentration. The number of bacteria began to decrease at a dosage of 50 μg/plate. This indicates that at this concentration, toxicity has begun. According to the Dox concentrations applied to the plates at 0.5, 1, 5, 10, and 25 μg/plate, the mutagenic index (MI) were 3.08, 8.15, 27.48, 29 and 6.32, respectively. These concentrations more than doubled the number of colonies compared to the control groups. In this case, Dox showed high mutagenic activity.

The MI value was less than 2 in the presence of metabolic activity at 0.5 and 1 μg/plate concentrations. Dox did not show mutagenicity at these low concentrations. MI values for 10, 25 and 50 μg/plate concentrations were 5.73, 12.20, 16.25 and 7.35, respectively. In the presence and absence of S9, none of the Dox concentrations tested in strain TA97a had a MI value higher than 2. The number of revertant bacterial colonies in TA97a strain without S9 mix was statistically significantly higher than the control group at only 5 and 10 μg/plate concentrations. Dox showed mild mutagenic effects at these doses. In addition, mean number of colonies were reduced in strain TA97a in the presence and absence of metabolic activation at the two highest doses of Dox. This decrease in the number of bacterial colonies is due to toxicity caused by Dox. *S. typhimurium* TA100 strain exposed to different concentrations of Dox, did not show a two-fold or greater increase in the mean number of revertants compared to the negative control group. The toxicity of Dox induced a reduction in the number of revertants in the TA 100 strain with and without S9 mix at a concentration of 100 μg/plate. Mild mutagenic activity was observed at 5 and 10 g/plate concentrations of Dox in the absence of metabolic activation in the TA100 strain, while mild mutagenic activity was observed at 10 μg/plate concentration in the presence of metabolic activation. Mutagenic activity was observed in TA102 strain at 100 μg/plate with 2.34 and 2.06 MI, respectively, in the presence and absence of metabolic activation. In TA102 strain, no toxicity was observed to reduce bacterial colony growth at any concentration where Dox was tested. Dox did not show mutagenicity in TA1535. In the experiments performed without metabolic activation in TA1535 strain, Dox showed a toxic effect at doses of 50 and 100 μg/plate, and Dox showed a toxic effect at a dose of 100 μg/plate in the presence of metabolic activation.

These results indicate that Dox has a mutagenic activity in strains TA 98 and TA102, in the presence and the absence of metabolic activation. Dox showed dose-response effect in TA98 and TA102 strains, and MI was found greater than 2 in these strains.

Mutagenicity studies of EGCG and RA were also performed on these two strains. Table 12 shows average the number of revertants bacteria colonies /plate and the standard deviation after the treatments with the RA, in the two different strains of *Salmonella typhimurium* TA 98 and TA102, with or without metabolic activation.

Table 12. Mutagenicity results of RA in *Salmonella typhimurium* TA98 and TA102 strains exposed to RA at various concentrations (1-1000 µg/plate), with and without S9 metabolic activation.

In the absence of S9		**Number of colony/plate**	
		TA98	**TA102**
Negative Control		36.67 ± 5.51	474,00 ± 88,43
Positive Control		976.5 ±19.5*	989.5 ± 18.9*
	1 µg/plate	26,00 ± 7,07	429,33± 67,14
	10 µg/plate	31,00 ± 4,24	404,00 ± 49,51
	100 µg/plate	34,50 ± 9,19	339,00 ± 59,91
	500 µg/plate	31,67 ± 8,08	421,00 ± 37,24
	1000 µg/plate	32,67 ± 7,09	378,67 ± 49,94
In the presence of S9			
Negative Control		32.50 ± 0.71	383.00 ± 13.53
Positive Control		609.7 ±10.6*	1124.0 ± 153.0*
	1 µg/plate	38.00 ± 4.24	412,50 ± 45,96
	10 µg/plate	31.00 ± 2.83	427,00 ± 24,04
	100 µg/plate	26.50 ± 2.12	423,00 ± 21,21
	500 µg/plate	36.00 ± 8.49	357,50 ± 17,68
	1000 µg/plate	27.50 ± 2.12	339,00 ± 1,41

Dunnett's multiple comparison test was carried out for statistical analysis. In the assay without metabolic activation, 4-nitro-o-phenylenediamine (NPD – 20 µg/plate) was used as a positive direct mutagen for the TA98 strain and mitomycin C (0.5 µg/plate) was used as a positive mutagen for TA102 strain. With the S9 experiments, 2-aminofluorene (2-AF - 5 µg/plate) was used as a positive mutagen for TA98 and 2-Amino anthracene (2-AA – 10 µg/plate) was used a positive mutagen for TA102.

Table 13 shows the mutagenicity of EGCG using *Salmonella typhimurium* TA98 and TA102 strains in presence and absence of S9 mix. The mutagenicity of EGCG was tested with doses ranging from 1 to 1000 g/plate.

Table 13. Mutagenicity results of EGCG in *Salmonella typhimurium* TA98 and TA102 strains exposed to EGCG at various concentrations (1-1000 µg/plate), with and without S9 metabolic activation

In the absence of S9		**Number of colony/plate**	
		TA98	**TA102**
Negative Control		28.50 ± 0.71	455.5 ± 21.92
Positive Control		976.5 ±19.5*	989.5 ± 18.9*
	1 µg/plate	39.00 ± 8.49	378.0 ± 19.80
	10 µg/plate	23.50 ± 2.12	392.0 ± 8.49
	100 µg/plate	27.00 ± 1.41	458.5 ± 9.19
	500 µg/plate	30.00 ± 11.31	422.0 ± 77.78
	1000 µg/plate	27.00 ± 1.41	420.5 ± 41.72
In the presence of S9			
Negative Control		34.00 ± 1.73	383.00 ± 13.53
Positive Control		609.7 ±10.6*	1124.0 ± 153.0*
	1 µg/plate	35.00 ± 2.83	417.50 ± 61,52
	10 µg/plate	25.50 ± 2.12	358.5 ± 50.20
	100 µg/plate	25.50 ± 4.95	331.0 ± 16.97
	500 µg/plate	33.00 ± 4.24	416.0 ± 42.43
	1000 µg/plate	30.50 ± 0.71	360.5 ± 81.32

Dunnett's multiple comparison test was carried out for statistical analysis. In the assay without metabolic activation, 4-nitro-o-phenylenediamine (NPD – 20 µg/plate) was used as a positive direct mutagen for the TA98 strain and mitomycin C (0.5 µg/plate) was used as a positive mutagen for TA102 strain. With the S9 experiments, 2-aminofluorene (2-AF - 5 µg/plate) was used as a positive mutagen for TA98 and 2-Amino anthracene (2-AA – 10 µg/plate) was used a positive mutagen for TA102.

The mutagenic potential of RA and EGCG were determined at different concentration levels by using TA98 and TA102 strains with and without metabolic activation. The results showed that RA and EGCG, at concentrations ranging from 1 to 1000 µg/plate, did not influence growth of bacteria, suggesting no toxicity in the tested

strains TA98 and TA102 at concentrations up to ≤1000 µg/plate with or without S9 mix. The results also showed that in the presence of varying doses of RA and EGCG, the mutation frequencies for the examined strains did not alter considerably when compared to spontaneous mutation frequencies in TA98 and TA102 strains with and without metabolic activation.

4.4.2 Antimutagenicity Assay Results

The Ames test is a commonly used method used to determine whether or not a substance is antimutagenic. Considering the increase in the number of bacterial colonies after Dox was applied to TA 98 and TA 102 bacterial strains, antimutagenicity tests against Dox were continued with these strains. In this study, Dox was used as direct and indirect mutagen for TA98 and TA102 strains. RA was found to reduce the number of revertants produced by Dox (5 µg/plate) to TA98 strain with metabolic activation. For 100 µg/plate, 500 µg/plate, and 1000 µg/plate the percentage of inhibition produced by RA was 26.14, 32.98, and 45.2% respectively. However, the inhibition rate of RA was found below 25% in antimutagenicity studies performed on TA98 strain without S9 mixture. EGCG exhibited strong antimutagenic activity at dose of 1000 µg/plate (60.27 % and 45.07 % inhibition, respectively) against Dox in the presence and absence of S9 mix in TA98 strain. Possible protective effects against mutagenicity caused by Dox 5 µg/plate were also investigated in combination. RA and EGCG were combined using the same concentrations. These concentrations were chosen as 250, 500 and 1000 µg/plate. In the experiment performed without metabolic activation in TA98 strain, the inhibition rate was found to be 35.74 % at 250 µg/plate and 42.22 % at 500 µg/plate concentration of both RA and EGCG. When RA and EGCG were performed using 1000 µg/plate concentrations, inhibition was found to be 65.57%. Strong antimutagenic activity was observed in the two highest combinations. Moderate antimutagenic activity was observed in the study using 250 µg/plate concentration of EGCG and RA. However, in the presence of metabolic activation, the inhibition rate was not that high in TA98 strain. In the antimutagenicity experiment using 250 µg/plate doses of both RA and EGCG, inhibition was found to be 5.06%, while it was 29.80% at 500 µg/plate concentration and 56% at 1000µg/plate concentration. Strong antimutagenic activity was observed at high concentration. Figure 37 shows the results of studies on the antimutagenic potential of

RA, EGCG and a combination of RA and EGCG in the presence and absence of metabolic activity in strain TA98.

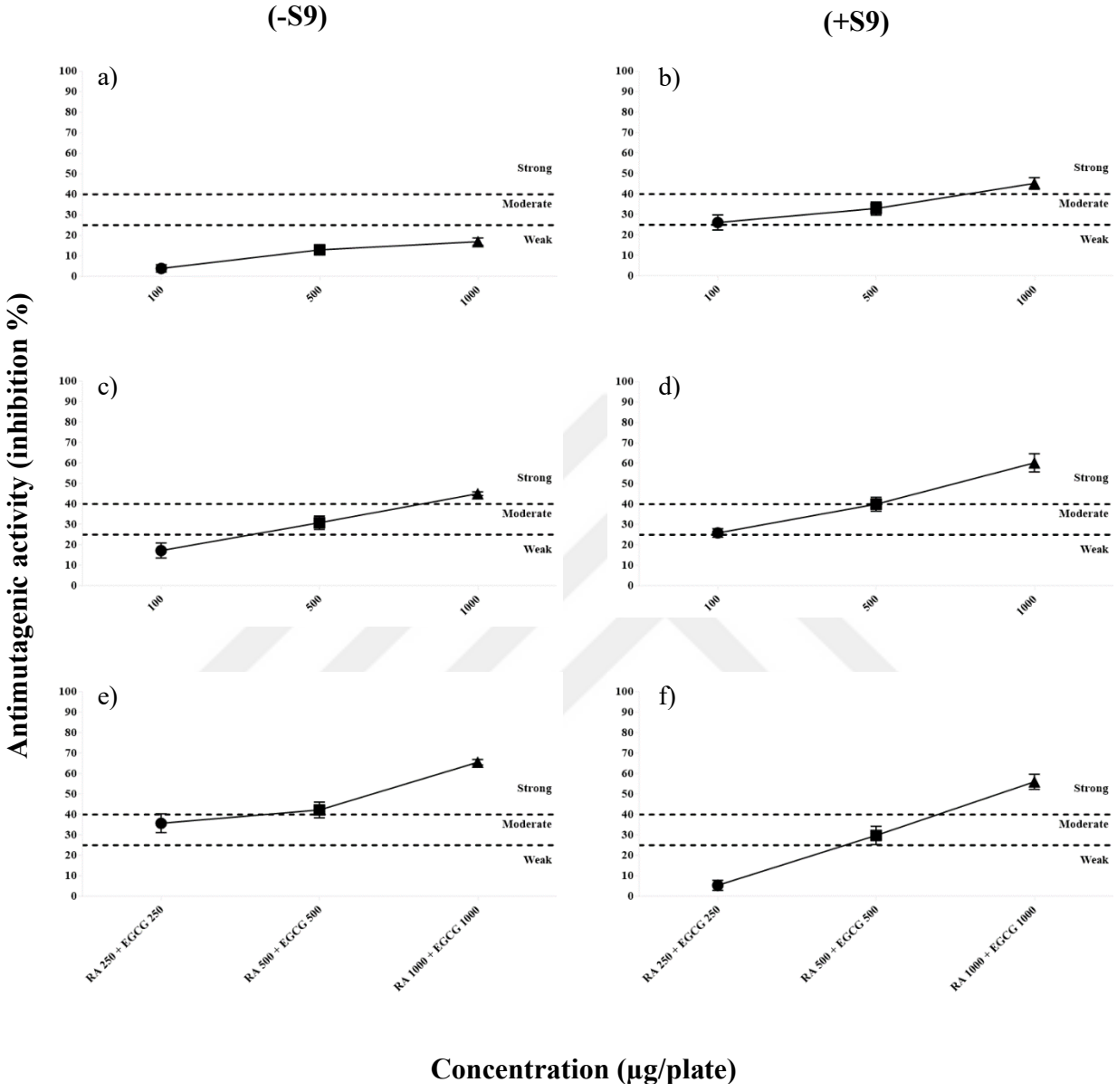

Figure 37. Inhibitory effect of RA, EGCG also combination of RA and EGCG against the mutagenicity of Dox (5µg/plate) to *Salmonella typhimurium* TA98 strain. (a) Antimutagenic effect of RA against Dox-induced mutagenicity in the absence of metabolic activation in strain TA98. (b) Antimutagenic effect of RA against Dox-induced mutagenicity in the presence of metabolic activation in strain TA98. (c) Antimutagenic effect of EGCG against Dox-induced mutagenicity in the absence of metabolic activation in strain TA98. (d) Antimutagenic effect of EGCG against Dox-induced mutagenicity in the presence of metabolic activation in strain TA98. (e) Antimutagenic effect of combination of RA and EGCG against Dox-induced mutagenicity in the absence of metabolic activation in strain TA98. (f) Antimutagenic effect of combination of RA and EGCG against Dox-induced mutagenicity in the presence of metabolic activation in strain TA98. PBS (50 µL) was used as a negative control. Each value is expressed as mean ± SEM.

In TA102 strain, all concentrations of RA showed weak antimutagenicity against Dox-induced mutagenicity without S9 mix, in the presence of metabolic activation, RA showed a weak antimutagenic effect up to 1000 μg/plate concentration, while it showed moderate inhibitory effect at 1000 μg/plate concentration. The antimutagenicity of EGCG increased from 37.86% to 50.51% in a dose-dependent manner without metabolic activation and inhibition increased from 25.45% to 62.91% in the presence of metabolic activation. At a concentration of 1000 μg/plate, EGCG showed strong antimutagenic activity in the presence and absence of S9 in strain TA102 strain. In antimutagenicity studies where RA and EGCG were used together, inhibition increased as the RA and EGCG concentrations increased. In the case of TA102 with and without S9 mix activation, a weak inhibitory effect was shown by combination of RA and EGCG at 250–500 μg/plate also a moderate inhibitory effect was shown at 1000 μg/ plate. Figure 38 shows the results of studies on the antimutagenic potential of RA, EGCG and a combination of RA and EGCG in the presence and absence of metabolic activity in strain TA102.

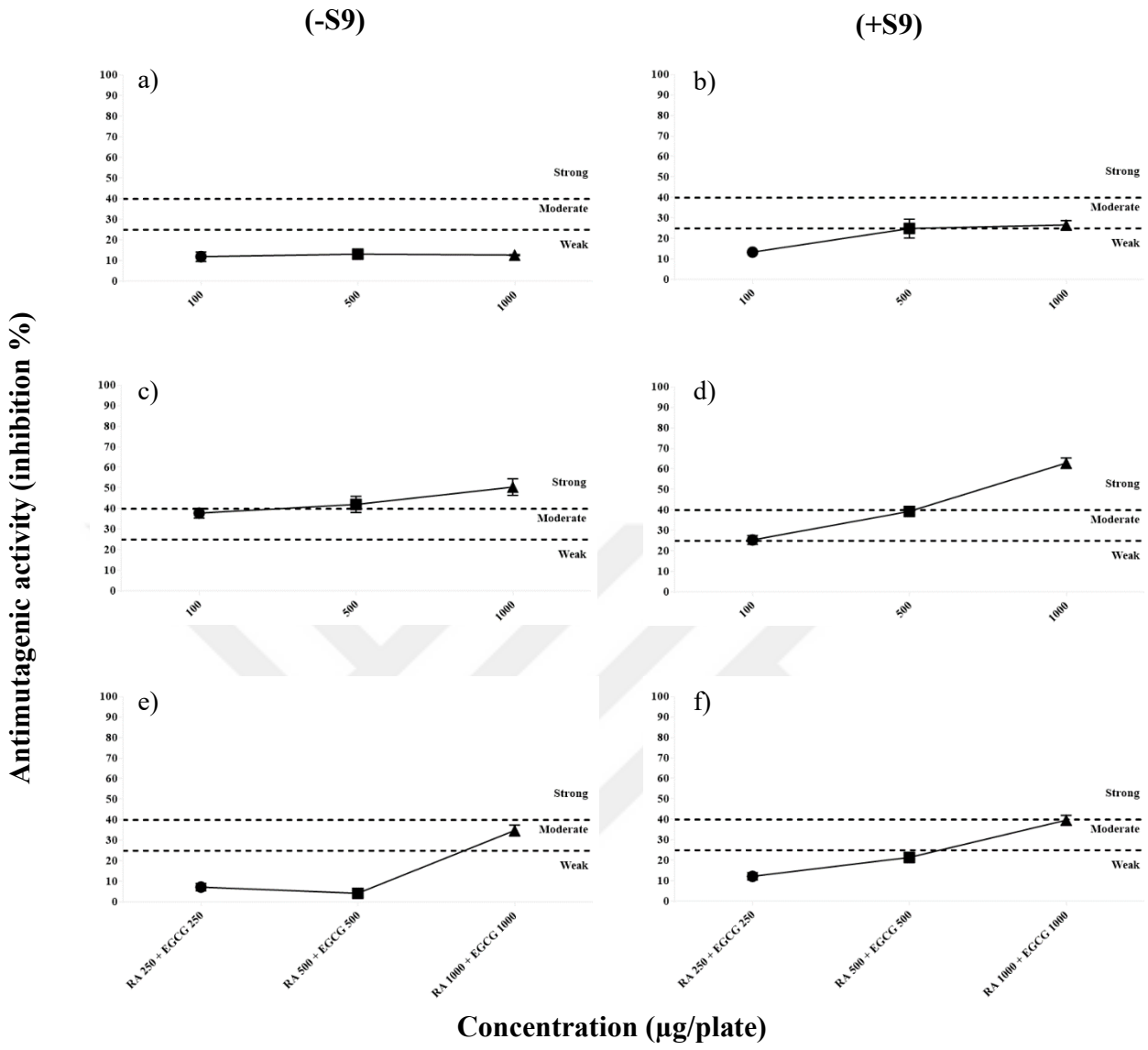

Figure 38. Inhibitory effect of RA, EGCG also combination of RA and EGCG against the mutagenicity of Dox (5 µg/plate) to *Salmonella typhimurium* TA102 strain. (a) Antimutagenic effect of RA against Dox-induced mutagenicity in the absence of metabolic activation in TA102 strain. (b) Antimutagenic effect of RA against Dox-induced mutagenicity in the presence of metabolic activation in TA102 strain. (c) Antimutagenic effect of EGCG against Dox-induced mutagenicity in the absence of metabolic activation in TA102 strain. (d) Antimutagenic effect of EGCG against Dox-induced mutagenicity in the presence of metabolic activation in TA102 strain. (e) Antimutagenic effect of combination of RA and EGCG against Dox-induced mutagenicity in the absence of metabolic activation in TA102 strain. (f) Antimutagenic effect of combination of RA and EGCG against Dox-induced mutagenicity in the presence of metabolic activation in TA102 strain. PBS (50 µL) was used as a negative control. Each value is expressed as mean ± SEM.

4.5 Anticancer Activity

4.5.1 Determination of IC50 value by WST-1 Assay

Cytotoxic effect of Dox, RA and EGCG on MCF-7, MDA-MB-231 and MCF12A cells were evaluated using WST-1 assay. The estimated IC50 values of all chemicals analyzed in this study on cancer cell lines MCF-7, MDA-MB-231, and on the non-tumorigenic immortalized cell line MCF-12A are summarized in Table 14.

Table 14. The concentrations of the studied compounds that inhibit 50% of cell survival (IC50).

Compounds	Cancerous cell line		Normal cell line
	MCF-7	MDA-MB-231	MCF12A
DOX (μM)	1.71 ± 0.88	20.64 ± 5.87	31.99 ± 5.31
EGCG (μM)	108.41 ± 6.90	40.16 ± 4.76	191.77 ± 6.10
RA (mM)	0.33 ± 0.11	0,16 ± 0.04	0.58 ± 0.14

For three independent observations, all substances were tested in triplicate. Values are given as mean ± S.D.

Dox, RA and EGCG were applied to cell lines for 24 hours. The highest activity was displayed by Dox in MCF-7 and MDA-MB-231 cancer cells (IC50 = 1.71 and 20.64 μM, respectively). The IC50 of DOX for MCF12A was higher than that of cancer cells. While the IC50 value of EGCG was found 40.16 μM in the MDA-MB-231 cell line, but the IC50 value increased in MCF-7, another breast cancer cell line, and was found 108.41 μM. The IC50 value was found 191.77 μM in the normal breast epithelial cell line. RA was found to have cytotoxic activity in the cancer cell lines studied, but IC50 values (range 0.33 – 0.16 mM) were higher than Dox and EGCG. The IC50 value of RA found in normal cells (0.58 mM) is higher than the IC50 value found in MCF-7 and MDA-MB-231 cell lines.

4.5.2 Selectivity Indexes (SI) of Samples

To determine the toxicity of these compounds, the cytotoxic effects were investigated in normal breast cell line (MCF12A). The SI value is the ratio of the IC50 value found in the normal cell line to the IC50 value found in the tumor cell line. A higher SI value indicates higher anticancer specificity, and compounds exhibiting SI values greater than 3 are considered highly selective. The SI values of RA,EGCG and Dox are shown in Table 15.

Table 15. Calculated SI of compounds using MCF12A cells as reference

Compounds	SI	
	MCF-7	MDA-MB-231
DOX	18.71	1.55
EGCG	1.77	4.78
RA	1.76	3.63

SI: selective index, IC50 for normal cell line/IC50 for cancerous cell line

The SI > 3 indicates that the cytotoxic effect of the samples is selective against MCF-7 cells and MDA-MB-231. Dox showed SI= 18.71 and SI= 1.55, respectively, against MCF-7 and MDA-MB-231 cells while EGCG exhibited SI = 1.77 and SI = 4.78, respectively, against the same cell lines. The SI values of RA in MCF-7 and MDA-MB-231 cancer cells were 1.76 and 3.63, respectively. In MCF-7 cancer cells, the SI value of Dox was as high as 18.71, indicating greater anticancer specificity. However, RA and EGCG did not show selectivity in MCF-7 cells, but showed selectivity in MDA-MB-231 cell line. A SI (<1) indicates that the sample is likely to be toxic and therefore cannot be used as a drug. The SI values of the substances used in the study were not found less than 1.

4.5.3 Evaluation of Combination Effect by Coefficient of drug interaction (CDI)

Following the cell viability results, CDI was investigated, as shown in Table 15-18. MDA-MB-231 and MCF-7 cells were treated for 24 h with increasing concentrations of RA and EGCG in the presence of a fixed concentration of Dox to assess potential synergy between Dox and RA or EGCG. To investigate the possible synergistic inhibitory effects of the combination of EGCG and RA with Dox, they were mixed at a fixed ratio (1:1, v/v) and applied to the cells in combination at different concentrations. The selected Dox concentration is close to the IC50 value obtained from the WST-1 cell viability assay. A constant concentration of Dox was chosen in both cell lines. On the other hand, different concentrations of EGCG and RA which were lower and higher than the IC50 values, were chosen and combined with Dox. The viability of MDA-MB-231 cells were decreased in the presence of Dox and EGCG. EGCG potentiated the inhibitory effects of Dox on MDA-MB-231 cells. CDI was calculated using results from the WST-1 cell

viability assay. CDI values calculated after administration of combined doses of Dox 12.5 μM and EGCG (10, 20, 40 and 80 μM) to MDA-MB-231 cells are shown in Table 16.

Table 16. Determination of CDI in MDA-MB-231cells by co-administration of Dox and EGCG

Co-Administration Combination	CDI
Dox 12.5 μM + EGCG 10 μM	0.014
Dox 12.5 μM + EGCG 20 μM	0.014
Dox 12.5 μM + EGCG 40 μM	0.013
Dox 12.5 μM + EGCG 80 μM	0.013

CDI: coefficient of drug interaction (CDI value < 0.7 significantly synergistic effect, CDI value < 1 synergistic effect, CDI value = 1 additive effect, CDI value > 1 antagonistic effect). CDI = AB/(A B), where AB is the ratio of the absorbancy values of the combination (A + B) and control groups, and A or B is the ratio of the single agent and control group absorbancy values. Dox: Doxorubicin, EGCG: Epigallocatechin gallate.

The CDI value of all Dox and EGCG combinations were less than 0.7. When the CDI is less than 0.7, the drugs are significantly synergistic. In the MDA-MB-231 cell line, concentrations lower and higher than the IC50 value of RA were studied to evaluate the possible synergistic effect of RA. Concentrations of RA 0.03125, 0.0625, 0.125, 0.25 and 0.5 mM were chosen and combined with Dox 12.5 μM concentration.

The combination of Dox and RA produced synergistic effects depending on the range of cell viability level. All combinations applied to cells showed a significantly synergistic effect.The calculated CDI values are shown in Table 17.

Table 17. Determination of CDI in MDA-MB-231cells by co-administration of Dox and RA

Co-Administration Combination	CDI
Dox 12.5 μM + RA 0.03125 mM	0.014
Dox 12.5 μM + RA 0.0625 mM	0.014
Dox 12.5 μM + RA 0.125 mM	0.014
Dox 12.5 μM + RA 0.25 mM	0.015
Dox 12.5 μM + RA 0.5 mM	0.012

CDI: coefficient of drug interaction (CDI value < 0.7 significantly synergistic effect, CDI value < 1 synergistic effect, CDI value = 1 additive effect, CDI value > 1 antagonistic effect). CDI = AB/(A B), where AB is the ratio of the absorbancy values of the combination (A + B) and control groups, and A or B is the ratio of the single agent and control group absorbancy values. Dox: Doxorubicin, RA: Rosmarinic acid.

The possible synergistic effect of EGCG and RA when co-administered with Dox was also investigated in another breast cancer cell line, MCF7. EGCG 40,80 and 200 μM concentrations were combined with Dox 2.5 μM concentration and applied to the cells. The calculated CDI values were found to be less than 0.7. This result shows us that EGCG enhances the anticancer effect of Dox. The calculated CDI results are shown in Table 18.

Table 18. Determination of CDI in MCF-7 cells by co-administration of Dox and EGCG

Co-Administration Combination	CDI
Dox 2.5 μM + EGCG 40 μM	0.009
Dox 2.5 μM + EGCG 80 μM	0.010
Dox 2.5 μM + EGCG 200 μM	0.019

CDI: coefficient of drug interaction (CDI value < 0.7 significantly synergistic effect, CDI value < 1 synergistic effect, CDI value = 1 additive effect, CDI value > 1 antagonistic effect). CDI = AB/(A B), where AB is the ratio of the absorbancy values of the combination (A + B) and control groups, and A or B is the ratio of the single agent and control group absorbancy values. Dox: Doxorubicin, EGCG: Epigallocatechin gallate.

The effects of combinations of RA and Dox on MCF-7 cells were also investigated. RA 0.125, 0.25 and 0.5 mM concentrations were combined with Dox 2.5 μM concentration and applied to the cells. As shown in Table 19, the CDI of RA in

combination with Dox in MCF-7 breast cancer cells was less than 0.7, which means the drugs acted significantly synergistic.

Table 19. Determination of CDI in MCF-7 cells by co-administration of Dox and RA

Co-Administration Combination	CDI
Dox 2.5 μM + RA 0.125 mM	0.010
Dox 2.5 μM + RA 0.25 mM	0.017
Dox 2.5 μM + RA 0.5 mM	0.047

CDI: coefficient of drug interaction (CDI value < 0.7 significantly synergistic effect, CDI value < 1 synergistic effect, CDI value = 1 additive effect, CDI value > 1 antagonistic effect). CDI = AB/(A B), where AB is the ratio of the absorbancy values of the combination (A + B) and control groups, and A or B is the ratio of the single agent and control group absorbancy values. Dox: Doxorubicin, RA: Rosmarinic acid.

The CDI value was less than 0.7 in all studied combinations. As a result, RA and EGCG increased Dox- mediated cell death. In addition, the triple combination of RA, EGCG and Dox was applied to MCF-7 and MDA-MB-231 cells. Inhibition of breast cancer cells using the WST-1 assay was determined by applying different concentrations of three substances (Dox+RA+EGCG). These combination effects were further evaluated on the basis of the synergistic equations for triple combination of drug. The effect of the triple combination of substances on MCF-7 cells was investigated with different concentrations of Dox 2.5 μM, EGCG 40 μM and RA (0.0625, 0.125 and 0.25 mM). All of the triple combinations made showed a synergistic effect in the MCF-7 cell line. When EGCG and RA were applied to MCF-7 cells together, DOX-induced antitumor effects were increased. There was a significant decrease in cell proliferation with the combination treatment. Dox 12.5 μM concentration, EGCG 40 μM concentration and different concentrations of RA (0.125, 0.25 and 0.5 mM) were selected in MDA-MB-231 cells. The results were evaluated according to the triple drug combination equation. According to the results, after the application of RA and EGCG with Dox to MDA-MB-231 cells, it showed an antagonistic effect according to triple drug equation. While synergistic effect was observed in MCF-7, it was thought that the reason for the antagonist effect in MDA-MB-231 cells was due to the different physiological characteristics of the two cell lines.

5. DISCUSSION

Dox commonly used to treat breast, ovarian, uterine, lung and cervical cancers, as well as Hodgkin's disease, primary soft tissue and bone sarcomas, and a number of other cancers. However, Dox treatment is associated to dose-dependent cardiotoxicity and its clinical use is limited. Dox appeared to cause membrane changes via lipid peroxidation, free radical formation, decreased myocardial DNA and RNA synthesis and cardiomyocyte apoptosis. Not only cardiomyocytes are a target of Dox toxicity, but also endothelial cells are affected. Dox, like other topoisomerase II inhibitors, has been showed to cause genotoxicity in both in vivo and in vitro studies. Also, Dox-therapy patients may experience myelosuppression and genotoxicity, which can cause secondary cancer. The formation of ROS / RNS as a result of redox-cycling drugs is one of the most prevalent causes of drug-induced toxicity, which is a complex process. As a result, it has been proposed that some phytochemicals with strong antioxidant capacity, when combined with antitumour agents, can decrease the toxic side effects of chemotherapy caused by oxidative stress, and thereby reduce the risk of cardiotoxicity (232–234).

Antioxidants, general blocking agents and free radical scavenging agents, and are present in plant-derived foods. Natural compounds are considered to have the ability to minimize chemotherapy genotoxicity on normal cells as well as physiological adverse effects (235). Natural substances have been shown to protect against the mutagenic activities of a variety of compounds (2,236,237). Polyphenols are a class of phytochemicals that includes various compounds found naturally in mostly edible plants, and the most studied group is the flavonoids. These compounds contribute to the prevention of diseases and the preservation of genomic instability by showing antioxidant, antimutagenic and anticarcinogenic effects. Polyphenols increase cell viability with their antioxidant effects, and inhibit tumor growth by stimulating apoptosis with their pro-oxidant effects (238–240). Although both EGCG and RA are phenolic compounds, they belong to different chemical classes. EGCG is tannin with flavan-3-ol structure esterified with gallic acid, while RA is a phenolic acid. Since EGCG contains more phenolic –OH groups than RA, it was shown to have higher scavenging activity when compared to RA in a study conducted by Soobrattee et al. (11). Therefore, we aimed to investigate whether EGCG has a stronger antioxidant and therefore antigenotoxic activity against Dox than RA. To our knowledge, the protective effect of the EGCG and RA together against Dox-induced genotoxicity in normal cells has not been investigated

before. In addition, the effect of this triple combination on breast cancer cells has not been investigated before. For these reasons, EGCG and RA which are polyphenolic antioxidant plant extracts were studied in this thesis.

The effects of Dox on DNA damage, cell viability and oxidative stress were investigated. The ability of RA and EGCG to protect cells from the toxicity caused by Dox, or to reduce the toxicity, was also studied. In addition, anticancer activities were investigated when they are used alone or together using breast cancer cells. The rationale behind this study was to link genotoxicity of Dox and protective effect of phytochemicals.

Three well known genotoxicity tests (micronucleus, Ames and comet tests) were used to examine the genotoxic effect induced by Dox, due to their capability to evaluate diverse endpoints that would assist appropriate conclusion of an agent's function[241]. In TK6 and CHO-K1 cells, the alkaline comet test was applied to detect Dox-induced DNA fragmentation. Because of their widespread use and applicability in genotoxicity testing, the TK6 and CHO-K1 cell lines were selected in this study[242]. Overall, the DNA damaging capacity of Dox was investigated for TK6 and CHO-K1, and the effect of RA and EGCG in protecting against Dox-induced damage was examined.

According to results, the increase in the amount of DNA tail from 1000 nM concentration to 4000 nM concentration was statistically significant, when comparing to the negative control group. Dox caused DNA damage in CHO-K1 cells in a dose dependent manner (243,244). Similarly, earlier studies have shown that Dox causes DNA strand breaks in V79 cells (Chinese hamster lung fibroblasts) in a concentration-dependent manner. In another study in V79 cells, different concentrations of Dox 0, 1, 2.5, 5, 10, 25 or 50 μg/ml were applied to the cells. Dox increased DNA damage in a concentration-dependent manner. Also, at different times, cells were exposed to Dox. As a result of this study, maximum DNA damage was detected in cells exposed to Dox at a concentration of 10 μg/ml for 1 hour (245). In our study, the number of ghost cells were increased significantly in a dose-dependent manner. At the highest dose of 4000 nM there was an increase in the number of ghost cells. Hedgehog cells (ghost cells) are apoptotic cells that arise from heavily damaged cells. Despite the fact that there are strong arguments against associating such comets with apoptotic cells, they are frequently used as indicators of cytotoxicity that results in cell death (246).

When most of the DNA is in the tail, the comet assay's dynamic range is limited, and it becomes saturated (247). The saturation limit of the assay is reached at the highest

concentration of 4000 nM of Dox in our study. The comet tail length values increase between 0–2000 nM and then reach a plateau. For these reasons, the Dox concentration of 1000 nM was selected to investigated the protective effect of phenolic compounds against the genotoxicity of Dox in CHO cell line.

The experiments were also carried out with TK6 cells. A human cell, TK6, was used because it was thought that the response of human cells to applied chemicals would be more sensitive than animal cell lines. Some laboratories choose TK6 cells because they are human-derived and have a functioning p53 gene, as opposed to rodent cell lines with a deficient p53 function (248). TK6 cell was exposed to different concentrations of Dox for 4 hours and the amount of DNA tail formed was evaluated. The increase in the amount of DNA tail from 250 nM concentration to 2000 nM concentration was statistically significant, when comparing to the negative control group. As a DNA damaging agent, concentration of Dox 500 nM was used in TK6 cells. TK6 cells were more sensitive to Dox compared to CHO-K1 cells. Therefore, the concentration of Dox causing DNA damage was used at a lower concentration in the TK6 cells compared to the CHO-K1 cell line. These concentrations were chosen because they did not cause cytotoxicity and did not cause a significant increase in the number of ghost cells. The optimum genotoxic effect was also observed at these concentration levels. The protective effects of phenolic compounds against the genotoxic effects of Dox at these concentrations were investigated by the comet test. Studies have been conducted using different concentrations to find the dose range showing the protective effect of RA. Cells were pre-incubated with RA for 15 minutes before exposure with Dox and RA for 4 hours. Many phenols have chemical structures and solubility properties that make it easy for them to enter the cell and oxidize through numerous metabolic pathways. Antioxidants are first treated into cells to exert protective effects and enter the cell before positive control (249). Dietary phenolic compounds are usually consumed in diluted doses of less than hundreds of miligrams. As a result, human bioavailability of phenols obtained from diet is very low (250). Therefore, possible protective effects were investigated by using low concentrations of RA and EGCG first. In our initial experiments, different concentrations of RA (1 μM, 5 μM, and 10 μM) did not show protective effect against 1000 nM Dox in the CHO-K1 cell. No protective effect was observed in CHO-K1 cells at RA concentrations less than 0.5 mM. RA 0.5 mM and 1 mM concentrations reduced Dox-induced DNA damage. Despite the fact that the concentration was doubled, the amount of DNA damage reduction was not changed. The absence of a major dose-dependent protective effect of RA suggests that

there is an optimum concentration for the maximum protective effect of RA. The comet assay showed that increasing the concentration of RA above the optimum concentration had no effect. In a similar study on the protective effects of RA, an optimum concentration was found at which RA had a protective effect against Dox, and the effect did not change at concentrations higher than this optimum level (9). In our study, co-administration of 100 μM RA with Dox 500 nM showed an optimum protective effect by decreasing DNA tail length in TK6 cell line. Possible protective effects of EGCG against Dox-induced genotoxicity in CHO-K1 cells were investigated using 3 different concentrations (5, 50 and 100 μM). However, no protective effect was observed. Surprisingly, when we applied higher concentrations of EGCG, it caused DNA damage. Similarly, as a result of the comet assay performed on TK6 cells, it was found that EGCG caused DNA damage above a certain concentration. EGCG alone was found to induce DNA damage at concentrations above 25 μM. In contrast, at the concentrations between10 and 25 μM, EGCG significantly reduced oxidative DNA damage induced by Dox in TK6 cells. Previous studies revealed that EGCG has both anti and pro-oxidant properties (251). EGCG is the most effective tea catechin in reacting with most reactive oxygen species. Catechins, on the other hand, can undergo auto-oxidation and function like pro-oxidants under certain circumstances (252). It is stated that the effect of killing cancer cells and directing them to apoptosis is due to this pro-oxidant characteristic (253).

In a few studies looking at the protective effect of EGCG against oxidative DNA damage in various cell lines, it was found that EGCG prevented DNA damage at low doses, while it behaved as a pro-oxidant at high concentrations. It was observed that 200 μM of EGCG elevated oxidative DNA damage in human lymphocyte DNA caused by H_2O_2 (254). Another study found that EGCG at concentrations above 100 μM induced oxidative DNA damage in Jurkat T-lymphocytes and 10 μM EGCG inhibited DNA damage induced by 3-morpholinosidnonimin and H_2O_2 in the same cell line (255). Moreover, EGCG has been shown to induce DNA damage, mutagenicity and cell death in human lung and normal skin cells. Low concentrations of EGCG were considered to scavenge free radicals, preventing oxidative damage to cellular DNA, whereas high concentrations of EGCG alone were considered to cause DNA damage (256). In a similar study, the effect of EGCG on DNA damage induced by H_2O_2 in human lymphocytes was investigated by comet assay. DNA damage was observed at all concentrations studied above 5 μM EGCG alone. However, EGCG at concentrations of 0.01-10 μM significantly reduced oxidative DNA damage caused by H_2O_2 (257). Kanadzu et al. found that low

concentrations of EGCG prevented DNA strand breaks caused by mutagens in human lymphocytes, but high concentrations of EGCG caused DNA breakage in human lymphocytes (251). Glei et al., reported that EGCG reduced strand breaks induced by another chemotherapeutic drug, Bleomycin, in human peripheral blood lymphocytes *in vitro* as measured by the comet assay. They concluded that this effect was probably due to the free radical scavenging activity of EGCG (258).

The comet test has been used in a number of studies to evaluate the genoprotective effect and genotoxicity of RA. The previous results on protective effect of RA are similar to those in our study. In a study conducted by Furtado el al. (2010), 0.28, 0.56, and 1.12 mM of RA were used in V79 cells alone or in combination with Dox for 3 hours. When the comet test was performed to evaluate the results, it was observed that RA had no genotoxic effects and significantly reduced Dox-induced DNA damage at all three concentrations tested (9). Similarly, 0.5 and 1 mM concentratiıns of RA showed protective effect against Dox-induced genotoxicity in CHO-K1 cells in our study.

Similar to our *in vitro* results, intraperitoneal administration of RA did not cause genotoxicity *in vivo* in rats. Its genotoxic effect on brain tissue was evaluated using the comet assay and it was found that RA did not cause genotoxicity (153). In another study demonstrating the protective effect of RA, oxidative DNA damage was induced by H_2O_2 in human peripheral lymphocytes and the genotoxicity potential of rosemary at various levels determined by the comet assay. The findings showed that rosemary ethanolic extract at various concentrations do not cause DNA damage in human lymphocyte. It also protected the cells against DNA damage induced by H_2O_2 (157).

The MN assay was used in this study to further investigate the genotoxic potential of Dox, RA and EGCG, as well as the antigenotoxic effect of EGCG and RA. The TK6 cell was preferred because it is a human derived and p53-competent cells and its response to the applied substances is more sensitive. In the MN experiment, different concentrations of Dox (10, 25 and 50 nM) were applied for 24 hours. The increase in MN frequency observed in our study depending on the dose of Dox indicates that this compound is a potential genotoxic compound. Because, an increase in the frequency of MNi is an indication of chromosomal damage (259). The number of MN formed at Dox 50 nM concentration was found to be statistically different compared to the control group. In addition, CBPI, NDI and RI values, which are indicators of cytotoxicity, were calculated for each group. These values decreased as the Dox concentration increased. Similarly in a previous study, administration of various concentrations of Dox to HeLa

cells for 2 h resulted in a significant and dose-dependent reduction in cell survival. It was found that the number of MNi increased depending on the concentration in that study. Dox concentrations used in that study are higher than the concentrations in our study. Because the application time of Dox and the applied cell type are different (260). In another study, Dox was applied to HL-60 human leukemia cells at 0.01, 0.025 and 0.05 µg/ml concentrations for 6 hours. The frequency of MN-containing cells was statistically significantly increased at 0.05 µg/ml Dox. A reduction in CBPI was also observed in that study (261). In two studies, Dox's ability to cause DNA strand breaks and chromosomal abnormalities was considered to be the primary reason of MN stimulation. Dhawan et al. (2003) have also shown an increase in MNi formation in human lymphocytes administered 20 hours of Dox at a similar dose range (262). In a study conducted in cultured human lymphocytes, it was observed that the MNi frequency increased in a dose-dependent manner after 24 h in the concentration range of 0.01 µg/ml - 2 µg/ml of Dox. When the Dox concentrations applied in this study are converted to nM units, they are close and higher than the concentrations we used in our study. In addition, as in our study, a decrease in NDI was observed in cells as the applied Dox concentration increased (263). A research was conducted with TK6 cells that were treated with Dox for 4 hours at concentrations ranging from 5-500 nM. MNi frequencies was increased from 5 nM Dox concentrations. The highest MNi number was found at 200 nM concentration. Insufficient binuclear cells were observed for MN assessment due to toxicity at the highest concentration of 500 nM of Dox (264). In our study, the highest MNi number was observed at 50 nM after administering Dox at various concentrations for 24 hours. As a result, the protective effects of RA and EGCG at this concentration were studied. Administration of EGCG alone did not cause an increase in MN frequency compared to the solvent control. MN frequency was reduced significantly when EGCG (10 and 25 M) was given together with Dox. EGCG has antioxidant properties and has prevented the genotoxic effect of chemicals in other studies as well as in our study (265).

In the literature review, similar findings were found. The MN test was used to assess the protective effect of EGCG against H_2O_2, tert-butyl hydroperoxide, and superoxide in WIL2-NS cells, a human B lymphoblastoid cell line. It has been reported that EGCG causes an increase in MN frequency at high concentrations (100 µmol/L). The induction of chromosomal damage by EGCG at high concentrations is thought to be due to H_2O_2 production. On the other hand, EGCG at < 10 µmol/L did not produce H_2O_2 and did not induce chromosomal damage. In addition, EGCG at < 10 µmol/L dose-

dependently prevented chromosomal damage induced by positive controls. In line with the results found, it has been reported that the inhibitory effect of EGCG against H_2O_2-induced chromosomal damage is related to the inhibition of hydroxyl radical formation in cells through the iron chelating activity of EGCG (266). The effect of EGCG was studied in rats against hepatotoxicity induced by N-nitrosodiethylamine, a well-known hepatocarcinogen. In rats treated N nitrosodiethylamine in combination with EGCG, there was a significant dose-dependent decrease in MNi frequency (267). In a study evaluating the protective role of EGCG against malathion-induced genotoxicity using the human lymphocyte model, malathion showed significant genotoxic damage, but treatment with EGCG reduced the genotoxicity of malathion. EGCG 20 and 50 µM were administered with malathion (24 µM) for 24 hours. At both concentrations, EGCG significantly reduced the oxidative and genotoxic effects of malathion on human lymphocytes (268). One of the EGCG concentrations in this study is similar to the concentration we studied, while the other studied EGCG concentration is higher than the concentration we studied.

In another study, EGCG was applied to the surface of the EpiDerm^{TM} system and normal human epidermal keratinocytes at different concentrations (0.625 mg/mL-10 mg/mL) for 24 hours. The effect of EGCG on MN induction was evaluated using EpiDerm^{TM} and normal human epidermal keratinocytes. EGCG had no effect on MN induction in EpiDermTM, but it caused dose-dependent cytotoxicity. After treatment with EGCG, normal human epidermal keratinocytes showed a significant increase in MN induction compared to the control group at a concentration of 10 mg/ml (269). This concentration is far higher than the EGCG studied in our research. The increase in the number of MN is due to the fact that EGCG exhibits oxidative properties *in vitro*, especially at higher concentrations. It is thought that especially EGCG produces H_2O_2 and gives positive results in *in vitro* genotoxicity tests. This is consistent with our findings in a induction of DNA damage from 25 µM EGCG in comet assay (270). In the MN assay using RA 50 and 100 µM concentrations, non of the investigated concentrations caused genotoxicity when RA was administered alone. RA 50 µM and RA 100 µM showed a statistically significant protective effect against genotoxicity caused by Dox 50 nM. The amount of protection did not increase as the concentration of RA increased. In the literature, similar results were obtained in genotoxicity and antigenotoxicity studies with RA. In a study investigating the antigenotoxic effect of RA, V79 cell cultures were treated with different concentrations of RA (0.28, 0.56 and 1.12 mM), alone or in combination with Dox. The results showed that RA did not cause

genotoxicity, but significantly reduced the frequency of MNi. However, no significant difference was observed between the groups in which 0.56 mM and 1.12 mM RA were administered together with Dox in terms of MNi frequency (9). RA reduced the frequency of MNi induced in human lymphocytes by gamma irradiation, according to similar findings. Ionizing radiation is known to produce ROS and cause cellular DNA damage, resulting in chromosomal aberrations and mutations. It was concluded that RA protects human lymphocyte cells thanks to its ability to scavenge free radicals (271). This finding is in accordance with our results of microscopic analysis, indicating ROS scavenging activity of RA in CHO cell line. The protective effect of EGCG 25 μM and RA 50 μM against genotoxicity caused by Dox 50 nM when applied together were also investigated in our study. EGCG and RA used in combination showed a similar protective effect when applied alone. This observation may be explained by saturation of ROS scavenging activity of phenolic compounds investigated in this study. This result supports previous findings that the increase in the concentration of antioxidants results in a plateau, indicating saturation in protective efficacy (272).

In a cytotoxicitystudy conducted by Almeida et al. (2018), TK6 cells were treated with Dox at the concentrations ranging from 0 to 1 μM using trypan blue assay. Dox increased cytotoxicity in a dose-dependent manner, with 86 percent of cells dead at the highest concentration of 1 μM in that study (273).

In our study, the highest cytotoxicity was observed at 500 nM concentration of Dox in TK6 cell line. Following Dox exposure, TK6 cells showed a dose-dependent decrease in cell viability and the IC50 value of Dox was found to be approximately 172.95 nM.

The IC50 value of RA and EGCG were found to be approximately 0.22 mM and 49.01 μM, respectively. Previous studies have found the similar IC50 value of EGCG to be 69.73 μM in prostate cancer PC3 cells (274), 131.6 μM in Hs578T breast cancer cells, 288.31 μM in LO2 normal human hepatic cell (275), 191.03 μM in Huh-7 hepatocellular carcinoma cells, 74.04 μM in HepG2 hepatocellular carcinoma cells and 65.81 μM in HCC-LM3 hepatocellular carcinoma cells (276). In a study similar to ours, using the rat pheochromocytoma-derived cell line (PC12), different concentrations of EGCG were applied for 24 hours. At concentrations higher than 50 μM, as a result of pro-oxidant activity, it showed cytotoxicity. Pro-oxidant effects increased and cell viability decreased with increasing concentration and time (277). To estimate the protecting property of RA and EGCG and their combination on Dox toxicity, TK6 cells were exposed to Dox (100

nM) for 24 h. The concentrations of RA and EGCG applied to the cells were chosen to be lower than the IC50 values. RA, EGCG and their combinations did not show evidence of protection against Dox's cytotoxicity. As in our study, other *in vitro* studies revealed that RA had no effect on the cytotoxic effect of Dox (278). In EGCG, it did not showed a protective effect when applied to TK6 cells together with Dox. This finding may be explained by the induction of apoptosis by EGCG in damaged cells (279). Effective therapies against Dox-induced oxidative stress and DNA damage should be predicted to maintain or increase Dox's anticancer therapeutic effects. In this sense, we hypothesized that the formation of ROS is prevented by an antioxidants. Several pharmacologic treatments, including hematopoietic cytokines, iron-chelating agents and antioxidants have been studied thus far to reduce the adverse affects of Dox (280,281). Many of these agents have shown beneficial effects in reducing oxidative stress. Therefore, in this thesis, the protective effects of EGCG and RA, which have strong antioxidant activity, against oxidative stress caused by Dox were investigated. For this, a fluorescent dye-based detection method was applied for the detection of superoxide in CHO-K1 cells. DHE was used as a fluorescent probe to determine the formation of ROS in this study. Time-course measurements were performed to optimize the incubation time of Dox. In CHO-K1 cells, ROS was measured using the same concentration of Dox (1000 nM), RA and EGCG as in the comet assay. At the end of the 2 hour incubation period, maximum ROS generation was observed at 1000 nM concentration of Dox. RA 0.5 and 1 mM concentrations significantly reduced Dox-induced ROS formation, while EGCG 50 and 100 µM concentrations did not. When RA and EGCG were applied together, no significant reduction in ROS formation was observed. EGCG did not show a protective effect against Dox-induced genotoxicity in the comet assay in CHO-K1 cells, but RA showed a significant protective effect. The results of the two experiments are similar when evaluated in this respect. This finding suggests that the protective effect in DNA damage caused by Dox would be related to the decrease in the amount of ROS. Considering that free radical production is one of the main mechanisms responsible for Dox's genetic toxicity, it can be predicted that DNA damage will decrease if free radical formation is reduced (126). In similar studies in the literature, it has been shown that substances with antioxidant properties protect against ROS production and genotoxicity caused by Dox. In one of these studies, thymoquinone reduced DNA damage and oxidative stress caused by Dox in human leukocytes (282). Thymoquinone also partly protected cardiac tissue from Dox-induced oxidative damage in rats feeding a thymoquinone-rich diet (283). The

protective action of thymoquinone against Dox-induced cardiotoxicity could be explained in part by superoxide scavenging and anti-lipid peroxidation activity. In an *in vivo* study, green tea extracts were given orally to rats. The effect on Dox-induced cardiovascular abnormalities was investigated. There was a significant increase in lipid peroxidation in the Dox-treated group when compared with the negative control group. A significant reduction in serum markers of cardiotoxicity such as lipid peroxide and significant increase in antioxidant enzyme activity as well as improvements in hemodynamic parameters was observed when green tea extracts and Dox were administered in combination. As a result, it was concluded that green tea extracts had antioxidant activity and can protect against Dox-induced cardiovascular abnormalities (284). In an *in vivo* study evaluating the protective effect of RA, a single intraperitoneal injection of Dox (15mg/kg) was administered to induce Dox-induced cardiotoxicity in mice. The effects of RA were assessed by histological investigation, molecular markers and echocardiography. DOX-induced cardiomyocyte apoptosis and cardiac dysfunction were significantly reduced by RA. This research also shown that RA has strong anti-apoptotic properties and can protect cells from DNA damage and apoptosis caused by hydrogen peroxide (285). A study was conducted to investigate the mechanisms of the inhibitory effect of RA on Dox-induced apoptosis in H9c2 cardiac muscle cells. By reducing intracellular ROS production and recovering mitochondria membrane potential, RA has been shown to significantly reduce apoptotic properties. Furthermore, RA has been observed to reverse Dox-induced antioxidant enzyme downregulation (286). Depending on the experimental method, we could not perform this experiment on the TK6 cell line, since our attempts to perform this experiment on a suspending cell line were not successful.

Mitochondrial membrane potential, one of the oxidative stress parameters, was measured in TK6 cells. Depending on the pH, fluorescent dyes accumulate in healthy mitochondria. The fluorescence intensity of TMRE, which we used in our study, accumulates in the electronegative interior of mitochondria in live cells and depicts the mitochondrial state. Fluorescence imaging and measurement are both used to characterize the cytotoxicity of a compound. In the mitochondrial membrane potential assay, compounds were applied to TK6 cells for 24 hours. A significant reduction was seen at Dox 100 and 200 nM concentrations. Likewise, mitochondrial membrane potential showed a statistically significant decrease at EGCG 50 µM and RA 25 mM concentrations compared to the control group. RA, EGCG and combinations used to prevent potential

reduction caused by Dox did not show any effect on membrane potential. RA showed a slight protective effect, but this was not statistically significant. Despite RA's significant free radical scavenging abilities, a research found that due to its physicochemical properties, it can't be targeted to mitochondria, so its protection of mitochondrial membrane potential is limited (287). These findings were similar to the results obtained in the WST-1 experiment. A decrease in mitochondrial membrane potential ($\Delta\Psi m$) versus normal levels in Dox treated cells indicates decreased cell viability as well as increased apoptosis. Depending on the oxidative stress, the amount of ROS increases and a decrease in mitochondrial membrane potential occurs with the induction of apoptosis in cells (288).

The mutagenic potential of Dox was assessed in our study using five different *Salmonella* strains with and without metabolic activation, as recommended by the OECD (289). The concentrations used in the experiments were 0.5, 1, 5, 10, 25, 50 and 100 μg/plate. The number of revertant colonies increased as the Dox concentration increased in the TA98 and TA102 strains with and without metabolic activation. Dox was found to be mutagenic in the TA98 and TA102 strains, with and without S9 activation. The TA102 strain has been linked to DNA mutations caused by oxidative stress. Due to the quinone structure of Dox's metabolites, it was expected that when they were metabolized in the presence of S9, ROS generation would be induced, resulting in an increase in the number of bacterial colonies. When the results from *S. typhimurium* TA98 are analyzed, it can be concluded that increasing frameshift mutation is caused as the concentration is increased. TA97a and TA100 strains showed weak mutagenic activity in the presence and absence of S9 at Dox 5 and 10 μg/plate concentrations. No mutagenic effect was observed in TA1535 strain. Based on these results, it can be said that Dox causes frameshift and oxidative stress-based mutagenicity. In a study conducted by Seino et al. (1978), Dox showed mutagenicity in TA98 and TA100 strains without metabolic activation (S9). Similar to our results TA98 strain was more sensitive to mutagenicity of Dox than TA100 strain. In addition, the mutagenicity of Dox was more apparent in the experiment without S9 activation (290). In another similar study, mutagenicity of Dox was tested in *Salmonella typhimurium* TA1535, TA1538, TA100, and TA98 bacterial strains. TA98 showed higher sensitivity than TA100 strain. Addition of the S9 mix for metabolic activation did not alter the mutagenic response of any of these strains (130). Therefore, TA98 and TA102 were selected for antimutagenicity tests in this study. The possible antimutagenic potential of RA and EGCG alone and in combinations was studied against Dox in the same strains. RA and EGCG did not show mutagenicity in Ames tests

performed in the presence and absence of S9 mixture in TA98 and TA100 strains. Similarly, in studies in the literature, RA and EGCG did not cause mutagenicity in Ames tests. No genotoxicity was found in the Ames test with extracts of rosemary, a plant with high RA content. On the contrary, rosemary showed antimutagenic activity in an Ames test with *Salmonella typhimurium* (291). RA inhibited the mutagenic potential caused by sodium azide and N-Methyl-N′-nitro-N-nitrosoguanidine. The researchers concluded that the antimutagenic activity of RA may be due to modulation of the redox environment (292). EGCG was not found mutagenic in *Salmonella typhimurium* TA98, TA102, TA100, TA97a, TA1535 and TA1537 strains up to 1000 g/plate concentration, whether metabolic activity is present or not (293). Contraversely, EGCG inhibited the direct-acting mutagenicity of Trp-P-2(NHOH) and Glu-P-1(NHOH) in *S. typhimurium* TA98 strain (294). The protection of EGCG against cisplatin-induced genotoxicity was evaluated by the Ames test. It was observed that EGCG significantly reduced the number of returning colonies induced by cisplatin in the Ames assay in the presence and absence of S9 in strains TA98 and TA100 (295). EGCG showed dose-dependent antimutagenic activity against N-nitroso-N-methylurea, N-methyl-N-nitro-N-nitrosoguanidine, 4-nitroquinoline-N-oxide, 9-aminoacridine, and aflatoxin B1-induced mutagenicity in TA98 and TA100 strains assessed by Ames test (296). In our study, RA showed weak antimutagenic activity without S9 activation, while it showed a strong antimutagenic effect with S9 mix in TA98 strain. EGCG, on the other hand, showed strong antimutagenic activity in the presence and absence of metabolic activation. Strong antimutagenic activity was observed when RA and EGCG were applied in combination, but this effect was not more than the effect observed when they applied alone in TA98 strain. In TA102 strain, EGCG showed strong antimutagenic activity, while RA showed weak and moderate antimutagenicity in the absence and presence of metabolic activation. Likewise, the combined application of RA and EGCG showed moderate and strong protective effects in the absence and presence of S9, respectively. EGCG showed a protective effect in both strains, suggesting that EGCG inhibits both frameshift and oxidative stress mutations. However, RA showed strong antimutagenic activity in the presence of S9 mix only in TA98 strain. This finding may be explained by the protective properties of RA metabolites against frameshift mutations.

In this thesis, the selectivity index and combination effect of EGCG and RA on breast cancer cells was also investigated. The synergistic or antagonistic effects of RA and EGCG on the anticancer activity of Dox in breast cancer cells were examined after

dosage levels of RA and EGCG alone and in combination were determined. In order to calculate the selectivity index, IC50 values of Dox, EGCG and RA were found in MCF7, MDA-MB-231 and MCF12A cell lines. Tumor cell lines are commonly used in cancer research as basic models that give important tools for biological analysis. The most often used cell lines in breast cancer studies are MCF-7 and MDA-MB-231. The MCF-7 cell line expresses the estrogen receptor and sensitizes these cells to estrogen. Hormone receptors and human epidermal growth factor receptor-2 are not expressed in the MDA-MB-231 cell line. MDA-MB-231 cells invade aggressively, whereas MCF-7 cells are noninvasive tumor cells (297,298).

The IC50 values found for Dox were approximately 1.71, 20.64 and 31.99 μM for MCF7, MDA-MB-231 and MCF12A cells, respectively. MCF-7 and MDA-MB-231 differ in sensitivity to Dox treatment. MDA-MB-231 cells are more sensitive than MCF-7 cells, according to most studies (299–301). However, some study groups reported that MCF-7 cells were more sensitive than MDA-MB-231 cells (225,302,303). The estrogen receptor status of the two cell lines explains the differences in their responses to Dox (304). This differences in susceptibility may also be explained by the status of p53 and caspase-3 which are important in cell cycle and/or apoptosis control. MDA-MB-231 cells have mutant p53 and functional caspase-3, whereas MCF-7 cells have wild type p53 but no caspase-3. Previous research has found caspase-3 to be involved in Dox-induced apoptosis. However, it is not clear if and how much caspase-3 activity influences the Dox response (305–307). Dox had a higher IC50 value in MCF12A than MDA-MB-231 and MCF-7. IC50 values of RA and EGCG were higher than Dox. The IC50 value of EGCG in the MDA-MB-231 cell line clearly indicated that EGCG was more sensitive to this cell line. After the IC50 value of each compound in different cell lines was found, the SI value was calculated. SI was calculated by dividing the IC50 value found in the non-cancerous breast cell line by the IC50 value found in the cancerous cell line (308). As an indication of a compound's potential as a cancer chemotherapeutic agent, the SI ought to be greater than three (309). The SI value of Dox is higher than RA and EGCG in the MCF-7 cell line. However, the SI value of EGCG and RA were higher than Dox in MDA-MB-231 cell line. These results suggest that RA and EGCG are cytotoxic towards MDA-MB-231cancer cell lines and is more selective than Dox. We hypothesize that there are two types of breast cancer with different p53 status and estrogen receptor. Therefore, they reacted differently to the applied chemicals. As a result, EGCG and RA show promise in treating triple negative breast cancer with mutant p53.

Our findings are also supported by existing literature. EGCG has been proven to inhibit breast tumor growth via the estrogen receptor signaling pathway in previous studies (310). Furthermore, EGCG increases chemotherapeutic-induced cellular apoptosis in MDA-MB-231 breast cancer cells, indicating that EGCG acts on estrogen signal transduction to exert its anticancer activity (311). Apoptosis can be triggered by a variety of mechanisms, which are unknown. According to research, EGCG inhibits MDA-MB-231 cell growth via inactivating the β-catenin signaling pathway (312). RA has been identified as a powerful agent in many cancer cell lines, including breast cancer, in cancer research (313). A recent study evaluated the effects and mechanisms of RA in two breast cancer cells MDA-MB-231 and MDA-MB-468 which are triple-negative breast cancer cell lines. The findings show that RA has cytotoxic and antiproliferative effects in these cell lines that are dose and time dependent. RA was induced cell cycle arrest-associated apoptosis and altered the expression of numerous apoptosis-related genes. RA was also arrested MDA-MB-231 cells in the G0/G1 phase (314).

Another aim of our study with breast cancer cells was to find the synergistic cytotoxic effect of EGCG and RA when administered with Dox. Our results indicated that EGCG and RA had significant synergistic effect in MDA-MB-231 and MCF-7 cells when combined with Dox. However, When EGCG added to the Dox-RA combination or RA added to Dox-EGCG combination in MDA-MB-231 cell line , the synergistic interactions of the Dox-EGCG or Dox-RA binary combination decreased, while the antagonistic interactions increased. On the other hand, in the MCF-7 cell line the synergistic effect increased when the third compound was added to the dual combination. Previous studies have shown that the combination of plant-based substances with Dox has a synergistic effect on its anticancer activity. Synergistic growth inhibition potentials in pancreatic, breast (MCF-7) and prostate cancer cell lines were investigated with red beetroot extract and Dox. The combination of red beetroot extract and Dox showed synergistic cytotoxicity in cancer cell lines (315). Similar to our observation, the combination of EGCG and Dox increased the cytotoxicity of Dox in osteosarcoma cell lines (316). Combination effect by coefficient of drug interaction obtained from this study, clearly indicated the synergistic effects of RA and EGCG on inhibiting the growth of breast cancer cells.

6. CONCLUSION

In conclusion, this study showed that EGCG and RA application protect against Dox-induced DNA damage by using different methods. However, when the two phenolic compounds were applied in combination, the protective effect against Dox-induced DNA damage was not as much as we expected. Dual behavior was observed with EGCG, which exhibited both pro-oxidant and antioxidative activities. Dox produced oxidative stress, which was decreased by RA and EGCG. RA, in particular, provided significant protection. Our findings revealed that EGCG and RA selectively targeting MDA-MB-231 cells that are more aggressive, more difficult to treat and more likely to recur than MCF-7 cells Dox, on the other hand, was found to be more selective on MCF-7 cells when the selective index value was calculated. The combined application of EGCG or RA with Dox increased the cytotoxicity induced by Dox, and a synergistic effect was observed in both breast cancer cells. Dox and EGCG or RA co-therapy can be used in breast cancer treatment to reduce the prescribed dose of Dox and avoid Dox's adverse effects. EGCG and RA have also been found to be promising agents in reducing Dox-induced genotoxicity without reducing the antitumor effect of Dox. Identifying plants that protect against genotoxic agents and secondary cancers caused by treatment with chemotherapy may be used in the near future to reduce the side effects of chemotherapeutic treatment. In this context, EGCG and RA are promising plant-derived active components. Another important point is the evaluation of the safety of herbal products. It should be taken into account that herbal products may increase the toxicity of chemotherapeutic agents. Both EGCG and RA were safe in terms of genotoxicity, and in fact, they had a protective effects against Dox induced genotoxicity. Since EGCG and RA reduce DNA damage and free radical production, it may be expected that co-administration of RA or EGCG with Dox decrease the adverse effects of Dox therapy. EGCG and RA may be a candidate compounds that can be used as an adjuvant during chemotherapy in the future, but further *in vitro* and *in vivo* studies are required to elucidate their effectiveness, safety, and molecular mechanism.

7. REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394-424.
2. Furtado MA, de Almeida LCF, Furtado RA, Cunha WR, Tavares DC. Antimutagenicity of rosmarinic acid in Swiss mice evaluated by the micronucleus assay. *Mutat Res - Genet Toxicol Environ Mutagen*. 2008;657(2):150-154.
3. Thorn CF, Oshiro C, Marsh S, et al. Doxorubicin pathways: pharmacodynamics and adverse effects. *Pharmacogenet Genomics*. 2011;21(7):440-446.
4. Alexander JF, Aguirre-Villarreal D, Godin B. Liposomal encapsulation masks genotoxicity of a chemotherapeutic agent in regulatory toxicology assessments. *Nanomedicine Nanotechnology, Biol Med*. 2017;13(3):829-833.
5. Venkatesh P, Shantala B, Jagetia GC, Rao KK, Baliga MS. Modulation of doxorubicin-induced genotoxicity by Aegle marmelos in mouse bone marrow: A micronucleus study. *Integr Cancer Ther*. 2007;6(1):42-53.
6. Efferth T, Li PCH, Konkimalla VSB, Kaina B. From traditional Chinese medicine to rational cancer therapy. *Trends Mol Med*. 2007;13(8):353-361.
7. Ferguson LR. Role of plant polyphenols in genomic stability. *Mutat Res - Fundam Mol Mech Mutagen*. 2001;475(1-2):89-111.
8. Huang S sheng, Zheng R liang. Rosmarinic acid inhibits angiogenesis and its mechanism of action in vitro. *Cancer Lett*. 2006;239(2):271-280.
9. Furtado RA, De Araújo FRR, Resende FA, Cunha WR, Tavares DC. Protective effect of rosmarinic acid on V79 cells evaluated by the micronucleus and comet assays. *J Appl Toxicol*. 2010;30(3):254-259.
10. Higdon J V, Frei B, Blumberg J. Critical Reviews in Food Science and Nutrition Tea Catechins and Polyphenols: Health Effects, Metabolism, and Antioxidant Functions Tea Catechins and Polyphenols: Health Effects, Metabolism, and Antioxidant Functions. *Crit Rev Food Sci Nutr*. 2003;43(431):37-41.
11. Soobrattee MA, Neergheen VS, Luximon-Ramma A, Aruoma OI, Bahorun T. Phenolics as potential antioxidant therapeutic agents: Mechanism and actions. *Mutat Res - Fundam Mol Mech Mutagen*. 2005;579(1-2):200-213.
12. Sadzuka Y, Sugiyama T, Sonobe T. Efficacies of tea components on doxorubicin induced antitumor activity and reversal of multidrug resistance. *Toxicol Lett*. 2000;114(1-3):155-162.
13. Stammler G, Volm M. Green tea catechins (EGCG and EGC) have modulating effects on the activity of doxorubicin in drug-resistant cell lines. *Anticancer Drugs*. 1997;8:265–268.
14. Venkatachalam K, Gunasekaran S, Namasivayam N. Biochemical and molecular mechanisms underlying the chemopreventive efficacy of rosmarinic acid in a rat colon cancer. *Eur J Pharmacol*. 2016;791:37-50.
15. Hausman DM. What is cancer? *Perspect Biol Med*. 2007;62(4):1-16.
16. Alfano RR, Das BB, Cleary J, Prudente R, Celmer EJ. Light sheds light on cancer - Distinguishing malignant tumors from benign tissues and tumors. *Bull New York Acad Med J Urban Heal*. 1991;67(2):143-150.
17. Hajdu SI. A note from history: Landmarks in history of cancer, part 3. *Cancer*. 2012;118(4):1155-1168.
18. Faguet GB. A brief history of cancer: Age-old milestones underlying our current knowledge database. *Int J Cancer*. 2015;136(9):2022-2036.

19. Papac RJ. Origins of cancer therapy. *Yale J Biol Med*. 2001;74(6):391-398.
20. Gilman EA, Knox EG. Childhood cancers: space-time distribution in Britain. *J Epidemiol Community Health*. 1995;(March 1994):158-163.
21. Percy C, Young JL, Muir C, et al. Introduction. *Cancer*. 1995;75(1 S):140-146.
22. Clegg LX, Reichman ME, Miller B a, et al. Impact of socioeconomic status on cancer incidence and stage and end results: National Longitudinal Mortality Study. *Cancer Causes Control*. 2009;20(4):417-435.
23. https://www.who.int/news-room/fact-sheets/detail/cancer.
24. Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin*. 2021;71(3):209-249.
25. Thun MJ, DeLancey JO, Center MM, Jemal A, Ward EM. The global burden of cancer: Priorities for prevention. *Carcinogenesis*. 2009;31(1):100-110.
26. Augustin LSA, Gallus S, Negri E, La Vecchia C. Glycemic index, glycemic load and risk of gastric cancer. *Ann Oncol*. 2004;15(4):581-584.
27. Donaldson MS. Nutrition and cancer: A review of the evidence for an anti-cancer diet. *Nutr J*. 2004;3:1-21.
28. Calle E, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, Obesity, and Mortality from Cancer in a Prospectively Studied Cohort of U.S. Adults. *N Engl J Med*. 2003;348:687-696.
29. Duffield-Lillico AJ, Reid ME, Turnbull BW, et al. Baseline characteristics and the effect of selenium supplementation on cancer incidence in a randomized clinical trial: A summary report of the Nutritional Prevention of Cancer Trial. *Cancer Epidemiol Biomarkers Prev*. 2002;11(7):630-639.
30. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer prevention: A review. *J Am Diet Assoc*. 1996;96(10):1027-1039.
31. Choi SW, Friso S, Ghandour H, Bagley PJ, Selhub J, Mason JB. Vitamin B-12 Deficiency Induces Anomalies of Base Substitution and Methylation in the DNA of Rat Colonic Epithelium. *J Nutr*. 2004;134(4):750-755.
32. Zhang SM, Willett WC, Selhub J, et al. Plasma Folate , Vitamin B 6 , Vitamin B 12 , Homocysteine , and Risk of Breast Cancer. 2003;95(5):373-380.
33. White MC, Holman DM, Boehm JE, Peipins LA, Grossman M, Jane Henley S. Age and cancer risk: A potentially modifiable relationship. *Am J Prev Med*. 2014;46(3 SUPPL. 1):S7-S15.
34. https://www.cancer.gov/about-cancer/causes-prevention/risk/age.
35. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin*. 2016;66(1):7-30.
36. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. *CA Cancer J Clin*. 2017;67(1):7-30.
37. Tevfik Dorak M, Karpuzoglu E. Gender differences in cancer susceptibility: An inadequately addressed issue. *Front Genet*. 2012;3(NOV):1-11.
38. Jeffrey Wang, Ying Huang. Pharmacogenomics of Sex Difference in Chemotherapeutic Toxicity. *Curr Drug Discov Technol*. 2007;4(1):59-68.
39. Anand P, Kunnumakara AB, Sundaram C, et al. Cancer is a preventable disease that requires major lifestyle changes. *Pharm Res*. 2008;25(9):2097-2116.
40. https://www.cancer.gov/about-cancer/causes-prevention/risk/infectious-agents.
41. Nagai H, Kim YH. Cancer prevention from the perspective of global cancer burden patterns. *J Thorac Dis*. 2017;9(3):448-451.

42. Bruno S, Crosignani A, Maisonneuve P, Rossi S, Silini E, Mondelli MU. Hepatitis C virus genotype 1b as a major risk factor associated with hepatocellular carcinoma in patients with cirrhosis: A seventeen-year prospective cohort study. *Hepatology*. 2007;46(5):1350-1356.
43. Moreno-Smith M, Lutgendorf SK, Sood AK. Impact of stress on cancer metastasis. *Futur Oncol*. 2010;6(12):1863-1881.
44. Huff J. Chemicals and cancer in humans: First evidence in experimental animals. *Environ Health Perspect*. 1993;100:201-210.
45. Ames BN, Gold LS. Paracelsus to parascience: The environmental cancer distraction. *Mutat Res - Fundam Mol Mech Mutagen*. 2000;447(1):3-13.
46. Newcomb PA, Carbone PP. The health consequences of smoking: Cancer. *Med Clin North Am*. 1992;76(2):305-331.
47. Sasco AJ, Secretan MB, Straif K. Tobacco smoking and cancer: A brief review of recent epidemiological evidence. *Lung Cancer*. 2004;45(SUPPL. 2).
48. Mozdarani H. Biological complexities in radiation carcinogenesis and cancer radiotherapy: Impact of new biological paradigms. *Genes (Basel)*. 2012;3(1):90-114.
49. Gilbert ES. Ionising radiation and cancer risks: What have we learned from epidemiology? *Int J Radiat Biol*. 2009;85(6):467-482.
50. Vaiserman A, Koliada A, Zabuga O, Socol Y. Health Impacts of Low-Dose Ionizing Radiation: Current Scientific Debates and Regulatory Issues. *Dose-Response*. 2018;16(3):1-27.
51. Key TJA. Hormones and cancer in humans. *Mutat Res - Fundam Mol Mech Mutagen*. 1995;333(1-2):59-67.
52. Boshuizen J, Peeper DS. Rational Cancer Treatment Combinations: An Urgent Clinical Need. *Mol Cell*. 2020;78(6):1002-1018.
53. Baskar R, Lee KA, Yeo R, Yeoh KW. Cancer and radiation therapy: Current advances and future directions. *Int J Med Sci*. 2012;9(3):193-199.
54. Einhorn N, Tropé C, Ridderheim M, Boman K, Sorbe B, Cavallin-Ståhl E. A systematic overview of radiation therapy effects in ovarian cancer. *Acta Oncol (Madr)*. 2003;42(5-6):562-566.
55. Hiller JG, Perry NJ, Poulogiannis G, Riedel B, Sloan EK. Perioperative events influence cancer recurrence risk after surgery. *Nat Rev Clin Oncol*. 2018;15(4):205-218.
56. Bergman PJ. Cancer Immunotherapies. *Vet Clin North Am - Small Anim Pract*. 2019;49(5):881-902.
57. Kroschinsky F, Stölzel F, von Bonin S, et al. New drugs, new toxicities: Severe side effects of modern targeted and immunotherapy of cancer and their management. *Crit Care*. 2017;21(1):1-11.
58. Igarashi Y, Sasada T. Cancer Vaccines: Toward the Next Breakthrough in Cancer Immunotherapy. *J Immunol Res*. 2020;2020.
59. Ali S, Mondal N, Choudhry H, et al. Current management strategies in breast cancer by targeting key altered molecular players. *Front Oncol*. 2016;6(MAR):1-9.
60. Sakai M, Elhilali M, Papadopoulos V. The GnRH Antagonist Degarelix Directly Inhibits Benign Prostate Hyperplasia Cell Growth. *Horm Metab Res*. 2015;47(12):925-931.
61. Groom AG, Younis T. Endocrine therapy for breast cancer prevention in high-risk women: Clinical and economic considerations. *Expert Rev Pharmacoeconomics*

Outcomes Res. 2016;16(2):245-255.
62. Li F, Dou J, Wei L, Li S, Liu J. The selective estrogen receptor modulators in breast cancer prevention. *Cancer Chemother Pharmacol*. 2016;77(5):895-903.
63. Chlebowski RT, Manson JE, Anderson GL, et al. Estrogen plus progestin and breast cancer incidence and mortality in the women's health initiative observational study. *J Natl Cancer Inst*. 2013;105(8):526-535.
64. Roland KB, Benard VB, Greek A, Hawkins NA, Lin L. Changes in knowledge and beliefs about human papillomavirus and cervical cancer screening intervals in low-income women after an educational intervention. *J Prim Care Community Heal*. 2016;7(2):88-95.
65. Palumbo MO, Kavan P, Miller WH, et al. Systemic cancer therapy: Achievements and challenges that lie ahead. *Front Pharmacol*. 2013;1-9.
66. Finch G., Burns-Naas L. *In Encyclopedia of Toxicology (Third Edition), 2014, Cancer Chemotherapeutic Agentse*.; 2014.
67. Hall AG, Tilby MJ. Mechanisms of action of, and modes of resistance to, alkylating agents used in the treatment of haematological malignancies. *Blood Rev*. 1992;6(3):163-173.
68. Robak T. Purine nucleoside analogues in the treatment of myeloid leukemias. *Leuk Lymphoma*. 2003;44(3):391-409.
69. Peters GJ, Van Der Wilt CL, Van Moorsel CJA, Kroep JR, Bergman AM, Ackland SP. Basis for effective combination cancer chemotherapy with antimetabolites. *Pharmacol Ther*. 2000;87(2-3):227-253.
70. Denny W, Baguley B. Dual Topoisomerase I / II Inhibitors in Cancer Therapy. *Curr Top Med Chem*. 2005;3(3):339-353.
71. Jacob S, Aguado M, Fallik D, Praz F. The role of the DNA mismatch repair system in the cytotoxicity of the topoisomerase inhibitors camptothecin and etoposide to human colorectal cancer cells. *Cancer Res*. 2001;61(17):6555-6562.
72. Pommier Y. Topoisomerase I inhibitors: Camptothecins and beyond. *Nat Rev Cancer*. 2006;6(10):789-802.
73. Liu YM, Chen HL, Lee HY, Liou JP. Tubulin inhibitors: A patent review. *Expert Opin Ther Pat*. 2014;24(1):69-88.
74. Gao Y, Shang Q, Li W, et al. Antibiotics for cancer treatment: A double-edged sword. *J Cancer*. 2020;11(17):5135-5149.
75. Yeh ETH, Tong AT, Lenihan DJ, et al. Cardiovascular complications of cancer therapy: diagnosis, pathogenesis, and management. *Circulation*. 2004;109(25):3122-3131.
76. Arcamone F, Cassinelli G, Faktini G, et al. Antitumor Antibiotic from. *Biotechnol Bioeng*. 1969;11:1101-1110.
77. Wang X, Tian X, Wu Y, Shen X, Yang S, Chen S. Enhanced doxorubicin production by Streptomyces peucetius using a combination of classical strain mutation and medium optimization. *Prep Biochem Biotechnol*. 2018;48(6):514-521.
78. Muggia FM, Green MD. New anthracycline antitumor antibiotics. *Crit Rev Oncol Hematol*. 1991;11(1):43-64.
79. Young R, Ozols R, Myers C. The anthracycline antineoplastic drugs. *Nex Engl J Med*. 1981;305:139-153.
80. Aubel-Sadron G, Londos-Gagliardi D. Daunorubicin and doxorubicin, anthracycline antibiotics, a physicochemical and biological review. *Biochimie*. 1984;66(5):333-352.

81. Seitz DE, Ezcurra JE, Guttman-Carlisle DL. Synthesis and chemical properties of a series of doxorubicin enaminomalonyl-β-alanine derivatives. *Tetrahedron Lett*. 1995;36(9):1413-1416.
82. https://pubchem.ncbi.nlm.nih.gov/compound/Doxorubicin#section=Names-and-Identifiers.
83. Speth PAJ, van Hoesel QGCM, Haanen C. Clinical Pharmacokinetics of Doxorubicin. *Clin Pharmacokinet*. 1988;15(1):15-31.
84. Robert J, Gianni L. Pharmacokinetics and metabolism of anthracyclines. *Cancer Surv*. 1993;17:219-252.
85. Kumar A, Gautam B, Dubey C, Tripathi PK. A Review: Role of Doxorubicin in Treatment of Cancer. *Int J Pharm Sci Res*. 2014;5(10):4117-4128.
86. Singal PK, Li T, Kumar D, Danelisen I, Iliskovic N. Adriamycin-induced heart failure: Mechanism and modulation. *Mol Cell Biochem*. 2000;207(1-2):77-86.
87. Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem Pharmacol*. 1999;57(7):727-741.
88. Fornari FA, Randolph JK, Yalowich JC, Ritke MK, Gewirtz DA. Interference by Doxorubicin with DNA Unwinding in MCF-7 Breast Tumor Cells. *Mol Pharmacol*. 1994;45:649 – 656.
89. Pigram WJ, Fuller W, Hamilton LD. Stereochemistry of intercalation Intercalation of daunomycin with DNA. *Nature*. 1972;235:17-19.
90. Cummings J, Anderson L, Willmott N, Smyth JF. The molecular pharmacology of doxorubicin in vivo. *Eur J Cancer Clin Oncol*. 1991;27(5):532-535.
91. Anderson R, Berger N. Mutagenicity and carcinogenicity of formaldehyde. *Mutat Res Mutagen Relat Subj*. 1986;164(4):288-289.
92. Šimůnek T, Štěrba M, Popelová O, Adamcová M, Hrdina R, Gerši V. Anthracycline-induced cardiotoxicity: Overview of studies examining the roles of oxidative stress and free cellular iron. *Pharmacol Reports*. 2009;61(1):154-171.
93. Alfarouk KO, Stock CM, Taylor S, et al. Resistance to cancer chemotherapy: Failure in drug response from ADME to P-gp. *Cancer Cell Int*. 2015;15(1):1-13.
94. Gottesman MM, Pastan I. Multidrug Resistance ,Multidrug Transporterl . *Annu Rev Biochem*. 1993;62:385-427.
95. Scheffer GL, Schroeijers AB, Izquierdo MA, Wiemer EAC, Scheper RJ. Lung resistance-related protein/major vault protein and vaults in multidrug-resistant cancer. *Curr Opin Oncol*. 2000;12(6):550-556.
96. Marie JP. Drug resistance in hematologic malignancies. *Curr Opin Oncol*. 2001;13(6):463-469.
97. Takano M, Yumoto R, Murakami T. Expression and function of efflux drug transporters in the intestine. *Pharmacol Ther*. 2006;109(1-2):137-161.
98. Mansoori B, Mohammadi A, Davudian S, Shirjang S, Baradaran B. The different mechanisms of cancer drug resistance: A brief review. *Adv Pharm Bull*. 2017;7(3):339-348.
99. Harbottle A, Daly AK, Atherton K, Campbell FC. Role of glutathione S-transferase P1, P-glycoprotein and multidrug resistance-associated protein 1 in acquired doxorubicin resistance. *Int J Cancer.* 2001;92(6):777-783.
100. Altieri P, Barisione C, Lazzarini E, et al. Testosterone antagonizes doxorubicin-induced senescence of cardiomyocytes. *J Am Heart Assoc*. 2016;5(1):1-13.
101. Warpe VS, Mali VR, Arulmozhi S, Bodhankar SL, Mahadik KR. Cardioprotective effect of ellagic acid on doxorubicin induced cardiotoxicity in wistar rats. *J Acute*

Med. 2015;5(1):1-8.
102. QuanJun Y, GenJin Y, LiLi W, et al. Protective effects of dexrazoxane against doxorubicin-induced cardiotoxicity: A metabolomic study. *PLoS One*. 2017;12(1):1-18.
103. Iarussi D, Indolfi P, Casale F, Martino V, Di Tullio MT, Calabr?? R. Anthracycline-Induced Cardiotoxicity in Children with Cancer. *Pediatr Drugs*. 2005;7(2):67-76.
104. Carvalho C, Santos R, Cardoso S, et al. Doxorubicin: The Good, the Bad and the Ugly Effect. *Curr Med Chem*. 2009;16(25):3267-3285.
105. Asensio-López MC, Soler F, Sánchez-Más J, Pascual-Figal D, Fernández-Belda F, Lax A. Early oxidative damage induced by doxorubicin: Source of production, protection by GKT137831 and effect on Ca2+ transporters in HL-1 cardiomyocytes. *Arch Biochem Biophys*. 2016;594:26-36.
106. Badkoobeh P, Parivar K, Kalantar SM, Hosseini SD, Salabat A. Effect of nano-zinc oxide on doxorubicin- induced oxidative stress and sperm disorders in adult male Wistar rats. *Int J Reprod Biomed*. 2013;11(5):355-364.
107. Spallarossa P, Garibaldi S, Altieri P, et al. Carvedilol prevents doxorubicin-induced free radical release and apoptosis in cardiomyocytes in vitro. *J Mol Cell Cardiol*. 2004;37(4):837-846.
108. Wang S, Konorev EA, Kotamraju S, Joseph J, Kalivendi S, Kalyanaraman B. Doxorubicin induces apoptosis in normal and tumor cells via distinctly different mechanisms: Intermediacy of H2O2- and p53-dependent pathways. *J Biol Chem*. 2004;279(24):25535-25543.
109. Bilginoğlu A, Aydin D, Özsoy Ş, Aygün H. Protective effect of melatonin on adriamycin-induced cardiotoxicity in rats. *Turk Kardiyol Dern Ars*. 2014;42(3):265-273.
110. Chatterjee K, Zhang J, Honbo N, Karliner JS. Doxorubicin cardiomyopathy. *Cardiology*. 2010;115(2):155-162.
111. Mandziuk S, Gieroba R, Korga A, et al. The differential effects of green tea on dose-dependent doxorubicin toxicity. *food Nutr Res*. 2015;59:29754.
112. Hanna AD, Lam A, Tham S, Dulhunty AF, Beard NA. Adverse effects of doxorubicin and its metabolic product on cardiac RyR2 and SERCA2A. *Mol Pharmacol*. 2014;86(4):438-449.
113. Bigotte L, Arvidson B, Olsson Y. Cytofluorescence localization of adriamycin in the nervous system - II. Distribution of the drug in the somatic and autonomic peripheral nervous systems of normal adult mice after intravenous injection. *Acta Neuropathol*. 1982;57(2-3):130-136.
114. Jansen CE, Dodd MJ, Miaskowski CA, Dowling GA, Kramer J. Preliminary results of a longitudinal study of changes in cognitive function in breast cancer patients undergoing chemotherapy with doxorubicin and cyclophosphamide. *Psychooncology*. 2008;17(12):1189-1195.
115. Yang XL, Fan CH, Zhu HS. Photo-induced cytotoxicity of malonic acid [C(60)]fullerene derivatives and its mechanism. Toxicol In Vitro. 2002;16(1):41-46.
116. Olney HJ, Mitelman F, Johansson B, Mrózek K, Berger R, Rowley JD. Unique balanced chromosome abnormalities in treatment-related myelodysplastic syndromes and acute myeloid leukemia: Report from an international workshop. *Genes Chromosom Cancer*. 2002;33(4):413-423.
117. http://www.inchem.org/documents/iarc/suppl7/adriamycin.html.
118. https://ntp.niehs.nih.gov/ntp/roc/content/profiles/adriamycin.pdf.

119. IARC. Adriamycin. In Chemicals, Industrial Processes and Industries Associated with Cancer in Humans, IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, suppl. 4. Lyon, France: International Agency for Research on Cancer. In: ; 1982:29-31.
120. Adriamycin (Group 2A). IARC monographs on the evaluation of carcinogenic risks to humans; (Suppl. 7). In: ; 1987:82-83.
121. Bertazzoli C, Chieli T, Solcia E. Different incidence of breast carcinomas or fibroadenomas in daunomycin or adriamycin treated rats. *Experientia*. 1971;27(10):1209-1210.
122. Marquardt H, Philips FS, Sternberg SS. Tumorigenicity in Vivo and Induction of Malignant Transformation and Mutagenesis in Cell Cultures by Adriamycin and Daunomycin. *Cancer Res*. 1976;36(6):2065-2069.
123. Bucciarelli E. Mammary Tumor Induction in Male and Female Sprague-Dawley Rats by Adriamycin and Daunomycin. 1981;66(1):81-84.
124. Jang JJ, Takahashi M, Hasegawa R, et al. Mammary and renal tumor induction by low doses of adriamycin in sprague-dawley rats. *Carcinogenesis*. 1987;8(8):1149-1153.
125. Ohtani M, Fukushima S, Okamura T, et al. Effects of lntravesical instillation of antitumor chemotherapeutic agents on bladder carcinogenesis in rats treated with N-butyl-N-(4-hydroxybutyl)nitrosamine. *Cancer*. 1984;54(8):1525-1529.
126. Quiles JL, Huertas JR, Battino M, Mataix J, Ramírez-Tortosa MC. Antioxidant nutrients and adriamycin toxicity. *Toxicology*. 2002;180(1):79-95.
127. Bridges B. .Short term screening tests for carcinogens. Nature 261, 195–200. *Nature*. 1976:195-200.
128. Au, W. W, Butler MA, Matney TS, Loo TL. Comparative Structure-Genotoxicity Study of Three Aminoanthraquinone Drugs and Doxorubicin. *Cancer Res*. 1981;41(2):376-379.
129. Bhuyan BK, Zimmer DM, Mazurek JH, et al. Comparative Genotoxicity of Adriamycin and Menogarol, Two Anthracycline Antitumor Agents. *Cancer Res*. 1983;43(11):5293-5297.
130. Babudri N, Pani B, Tamaro M, Monti-Bragadin C, Zunino F. Mutagenic and cytotoxic activity of doxorubicin and daunorubicin derivatives on prokaryotic and eukaryotic cells. *Br J Cancer*. 1984;50(1):91-96.
131. Yourtee DM, Elkins LL, Nalvarte EL, Smith RE. Amplification of doxorubicin mutagenicity by cupric ion. *Toxicol Appl Pharmacol*. 1992;116(1):57-65.
132. Clements J, Phillips M, Todd N. Mutagenicity of adriamycin in Drosophila melanogaster. *Mutat Res Toxicol*. 1984;135(3):175-179.
133. Musilova J, Michalova K, Urban J. Sister-chromatid exchanges and chromosal breakage in patients treated with cytostatics. *Mutat Res*. 1979;67:289--294.
134. Martins RA, Minari AL, Chaves MD, Santos RWT dos, Barbisan LF, Ribeiro DA. Exercise preconditioning modulates genotoxicity induced by doxorubicin in multiple organs of rats. *Cell Biochem Funct*. 2012;30(4):293-296.
135. Laky B, Janda M, Cleghorn G, Obermair A. Comparison of different nutritional assessments and body-composition measurements in detecting malnutrition among gynecologic cancer patients. *Am J Clin Nutr*. 2008;87(6):1678-1685.
136. Thomasset SC, Berry DP, Garcea G, Marczylo T, Steward WP, Gescher AJ. Dietary polyphenolic phytochemicals - Promising cancer chemopreventive agents in humans? A review of their clinical properties. *Int J Cancer*. 2007;120(3):451-458.

137. Hardy TM, Tollefsbol TO. Epigenetic diet: Impact on the epigenome and cancer. *Epigenomics*. 2011;3(4):503-518.
138. Alves JM, Munari CC, de Azevedo Bentes Monteiro Neto M, et al. In vivo protective effect of Copaifera langsdorffii hydroalcoholic extract on micronuclei induction by doxorubicin. *J Appl Toxicol*. 2013;33(8):854-860.
139. Santos GC, Almeida MR, Antunes LMG, Bianchi MLP. Effect of bixin on DNA damage and cell death induced by doxorubicin in HL60 cell line. *Hum Exp Toxicol*. 2016;35(12):1319-1327.
140. Rastegar H, Ashtiani HA, Anjarani S, Bokaee S, Khaki A, Javadi L. The role of milk thistle extract in breast carcinoma cell line (MCF-7) apoptosis with doxorubicin. *Acta Med Iran*. 2013;51(9):591-598.
141. Naga MA, El-Aziz MAA, Zeid SM, Daba M-HY, El-Gamal NK. Amelioration of Doxorubicin-induced genotoxicity in isolated cultured human lymphocytes by Thymoquinone. *Appl Sci Rep*. 2013;4:210-218.
142. Bokulić A, Garaj-Vrhovac V, Brajša K, Durić K, Glojnarić I, Šitum K. The effect of apigenin on cyclophosphamide and doxorubicin genotoxicity in vitro and in vivo. *J Environ Sci Heal - Part A Toxic/Hazardous Subst Environ Eng*. 2011;46(5):526-533.
143. Yalçin E, Oruç E, Çavuşoğlu K, Yapar K. Protective role of grape seed extract against doxorubicin-induced cardiotoxicity and genotoxicity in albino mice. *J Med Food*. 2010;13(4):917-925.
144. Petersen M, Simmonds MSJ. Rosmarinic acid. *Phytochemistry*. 2003;62(2):121-125.
145. Scarpati M, Oriente G. Isolamento e costituzione dell'acido rosmarinico (dal rosmarinus off.). *Ric Sci*. 1958;28:2329–33.
146. Domitrović R, Potočnjak I, Crnčević-Orlić Ž, Škoda M. Nephroprotective activities of rosmarinic acid against cisplatin-induced kidney injury in mice. *Food Chem Toxicol*. 2014;66:321-328.
147. Nadeem M, Imran M, Gondal TA, et al. therapeutic potential of rosmarinic acid : A comprehensive review. *ApplSci*. 2019;9(3139).
148. Osakabe N, Yasuda A, Natsume M, Yoshikawa T. Rosmarinic acid inhibits epidermal inflammatory responses: Anticarcinogenic effect of Perilla frutescens extract in the murine two-stage skin model. *Carcinogenesis*. 2004;25(4):549-557.
149. Scheckel KA, Degner SC, Romagnolo DF. Rosmarinic acid antagonizes activator protein-1-dependent activation of cyclooxygenase-2 expression in human cancer and nonmalignant cell lines. *J Nutr*. 2008;138(11):2098-2105.
150. Tao L, Wang S, Zhao Y, et al. Phenolcarboxylic acids from medicinal herbs exert anticancer effects through disruption of COX-2 activity. *Phytomedicine*. 2014;21(11):1473-1482.
151. Paluszczak J, Krajka-Kuźniak V, Baer-Dubowska W. The effect of dietary polyphenols on the epigenetic regulation of gene expression in MCF7 breast cancer cells. *Toxicol Lett*. 2010;192(2):119-125.
152. Tai J, Cheung S, Wu M, Hasman D. Antiproliferation effect of Rosemary (Rosmarinus officinalis) on human ovarian cancer cells in vitro. *Phytomedicine*. 2012;19(5):436-443.
153. Pereira P, Tysca D, Oliveira P, Brum LFDS, Picada JN, Ardenghi P. Neurobehavioral and genotoxic aspects of rosmarinic acid. *Pharmacol Res*. 2005;52(3):199-203.
154. Lamponi S, Baratto MC, Miraldi E, Baini G, Biagi M. Chemical profile,

antioxidant, anti-proliferative, anticoagulant and mutagenic effects of a hydroalcoholic extract of tuscan rosmarinus officinalis. *Plants*. 2021;10(1):1-16.
155. Lazalde-Ramos BP, Zamora-Perez AL. DNA protective effect of rosmarinus officinalis total extract in mouse peripheral blood. *MOJ Toxicol*. 2018;4(2):75-79.
156. De Oliveira NCD, Sarmento MS, Nunes EA, et al. Rosmarinic acid as a protective agent against genotoxicity of ethanol in mice. *Food Chem Toxicol*. 2012;50(5):1208-1214.
157. Razavi-Azarkhiavi K, Behravana J, Mosaffa F, Sehatbakhsh S, Shirani K, Karimi G. Protective effects of aqueous and ethanol extracts of rosemary on H_2O_2-induced oxidative DNA damage in human lymphocytes by comet assay. *J Complement Integr Med*. 2014;11(1):27-33.
158. Vattem DA, Levin R, Shetty K. Synergism of cranberry phenolics with ellagic acid and rosmarinic acid for antimutagenic and DNA protection functions. *J Food Biochem*. 2006;30(413):98-116.
159. Nakazawa T, Ohsawa K. Metabolism of rosmarinic acid in rats. *J Nat Prod*. 1998;61(8):993-996.
160. Granja A, Frias L, Neves AR, Pinheiro MS, Reis S. Therapeutic potential of epigallocatechin gallate. *Int J Green Pharm*. 2017;11(3):S364-S370.
161. Raza H, John A. In vitro effects of tea polyphenols on redox metabolism, oxidative stress, and apoptosis in PC12 cells. *Ann N Y Acad Sci*. 2008;1138:358-365.
162. Botten D, Fugallo G, Fraternali F, Molteni C. Structural Properties of Green Tea Catechins. *J Phys Chem B*. 2015;119(40):12860-12867.
163. Cione E, Torre C La, Cannataro R, Caroleo MC, Plastina P, Gallelli L. Quercetin, epigallocatechin gallate, curcumin, and resveratrol: From dietary sources to human MicroRNA modulation. *Molecules*. 2020;25(1).
164. Henning SM, Niu Y, Lee NH, et al. Bioavailability and antioxidant activity of tea flavanols after consumption of green tea, black tea, or a green tea extract supplement. *Am J Clin Nutr*. 2004;80(6):1558-1564.
165. Mereles D, Hunstein W. Epigallocatechin-3-gallate (EGCG) for clinical trials: More Pitfalls than Promises? *Int J Mol Sci*. 2011;12(9):5592-5603.
166. Albassam AA, Markowitz JS. An Appraisal of Drug-Drug Interactions with Green Tea (Camellia sinensis). *Planta Med*. 2017;83(6):496-508.
167. Lecumberri E, Dupertuis YM, Miralbell R, Pichard C. Green tea polyphenol epigallocatechin-3-gallate (EGCG) as adjuvant in cancer therapy. *Clin Nutr*. 2013;32(6):894-903.
168. Yang CS, Wang X, Lu G, Picinich SC. Cancer prevention by tea: animal studies, molecular mechanisms and human relevance. *Nat Rev Cancer*. 2009;9(6):429-439.
169. De Pace RCC, Liu X, Sun M, et al. Anticancer activities of (-)-epigallocatechin-3-gallate encapsulated nanoliposomes in MCF7 breast cancer cells. *J Liposome Res*. 2013;23(3):187-196.
170. Wu AH, Yu MC, Tseng CC, Hankin J, Pike MC. Green tea and risk of breast cancer in Asian Americans. *Int J Cancer*. 2003;106(4):574-579.
171. Wu AH, Spicer D, Stanczyk FZ, Tseng CC, Yang CS, Pike MC. Effect of 2-month controlled green tea intervention on lipoprotein cholesterol, glucose, and hormone levels in healthy postmenopausal women. *Cancer Prev Res*. 2012;5(3):393-402.
172. Luo T, Wang J, Yin Y, et al. (-)-Epigallocatechin gallate sensitizes breast cancer cells to paclitaxel in a murine model of breast carcinoma. *Breast Cancer Res*. 2010;12(1):1-10.
173. Carlson JR, Bauer BA, Vincent A, Limburg PJ, Wilson T. Reading the tea leaves: Anticarcinogenic properties of (-)- epigallocatechin-3-gallate. *Mayo Clin Proc*.

2007;82(6):725-732.
174. Paschka AG, Butler R, Young CYF. Induction of apoptosis in prostate cancer cell lines by the green tea component, (-)-epigallocatechin-3-gallate. *Cancer Lett*. 1998;130(1-2):1-7.
175. Gupta S, Hastak K, Afaq F, Ahmad N, Mukhtar H. Essential role of caspases in epigallocatechin-3-gallate-mediated inhibition of nuclear factor kappaB and induction of apoptosis. *Oncogene*. 2004;23(14):2507-2522.
176. Mittal A, Pate MS, Wylie RC, Tollefsbol TO, Katiyar SK. EGCG down-regulates telomerase in human breast carcinoma MCF-7 cells, leading to suppression of cell viability and induction of apoptosis. *Int J Oncol*. 2004;24(3):703-710.
177. Nihal M, Ahmad N, Mukhtar H, Wood GS. Anti-proliferative and proapoptotic effects of (-)-epigallocatechin-3- gallate on human melanoma: Possible implications for the chemoprevention of melanoma. *Int J Cancer*. 2005;114(4):513-521.
178. Biray Avcı Ç, Göker Bağca B. Epigallokateşin-3- gallat uygulamasına bağlı olarak kronik miyeloid lösemi hücrelerinde genetik ve epigenetik otofaji regülatörlerinin ekspresyon değişimlerinin değerlendirilmesi. *SDÜ Tıp Fakültesi Derg*. 2019;26(1):57-66.
179. Khan N, Afaq F, Mukhtar H. Cancer chemoprevention through dietary antioxidants: Progress and promise. *Antioxidants Redox Signal*. 2008;10(3):475-510.
180. Roy M, Chakrabarty S, Sinha D, Bhattacharya RK, Siddiqi M. Anticlastogenic, antigenotoxic and apoptotic activity of epigallocatechin gallate: A green tea polyphenol. *Mutat Res - Fundam Mol Mech Mutagen*. 2003;523-524:33-41.
181. Morley N, Clifford T, Salter L, Campbell S, Gould D, Curnow A. The green tea polyphenol (-)-epigallocatechin gallate and green tea can protect human cellular DNA from ultraviolet and visible radiation-induced damage. *Photodermatol Photoimmunol Photomed*. 2005;21(1):15-22.
182. De Flora S, Izzotti A. Mutagenesis and cardiovascular diseases. Molecular mechanisms, risk factors, and protective factors. *Mutat Res - Fundam Mol Mech Mutagen*. 2007;621(1-2):5-17.
183. da Silva RMG, de Sousa NC, Graf U, Spanó MA. Antigenotoxic effects of Mandevilla velutina (Gentianales, Apocynaceae) crude extract on cyclophophamide-induced micronuclei in Swiss mice and urethane-induced somatic mutation and recombination in Drosophila melanogaster. *Genet Mol Biol*. 2008;31(3):751-758.
184. Bedir A, Bilgici B, Yurdakul Z, Gürsel B. DNA Hasarı Analizinde µ-FADU ve COMET Yöntemlerinin Karşılaştırılması. *Türk Klin Biyokim Derg*. 2004;2(3):97-103.
185. Zeiger E. History and rationale of genetic toxicity testing: An impersonal, and sometimes personal, view. *Environ Mol Mutagen*. 2004;44(5):363-371.
186. Custer L, Sweder K. The Role of Genetic Toxicology in Drug Discovery and Optimization. *Curr Drug Metab*. 2008;9(9):978-985.
187. Albertini RJ, Anderson D, Douglas GR, et al. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. *Mutat Res - Rev Mutat Res*. 2000;463(2):111-172.
188. Mortelmans K, Zeiger E. The Ames Salmonella/microsome mutagenicity assay. *Mutat Res*. 2000;455:29-60.
189. McCann J, Spingarn NE, Kobori J, Ames BN. Detection of carcinogens as mutagens: bacterial tester strains with R factor plasmids. *Proc Natl Acad Sci U S*

A. 1975;72(3):979-983.
190. Maron DM, Ames BN. Revised methods for the Salmonella mutagenicity test. *Mutat Res Mutagen Relat Subj*. 1983;113(3-4):173-215.
191. Ames BN, Durston WE, Yamasaki E, Lee FD. Carcinogens are mutagens: a simple test combining liver homogenates for activation and bacteria for detection. *Proc Natl Acad Sci U S A*. 1973;70(8):2281-2285.
192. Ames BN, Lee FD, Durston WE. An improved bacterial test system for the detection and classification of mutagens and carcinogens. *Proc Natl Acad Sci U S A*. 1973;70(3):782-786.
193. Ames Bn, Mccann J, Yamasakı E. Methods for detecting carcinogens and mutagens with the salmonella/ mammalian- microsome mutagenicity test. *Mutat Res*. 1975;31:347-364.
194. Vanparys P, Vermeiren F, Sysmans M, Temmerman R. The micronucleus assay as a test for the detection of aneugenic activity. *Mutat Res Lett*. 1990;244(2):95-103.
195. Stopper H, Müller SO. Micronuclei as a biological endpoint for genotoxicity: A minireview. *Toxicol Vitr*. 1997;11(5):661-667.
196. Kirsch-Volders M, Elhajouji A, Cundari E, Van Hummelen P. The in vitro micronucleus test: A multi-endpoint assay to detect simultaneously mitotic delay, apoptosis, chromosome breakage, chromosome loss and non-disjunction. *Mutat Res - Genet Toxicol Environ Mutagen*. 1997;392(1-2):19-30.
197. Fenech M, Morley AA. Cytokinesis-block micronucleus method in human lymphocytes: effect of in vivo ageing and low dose X-irradiation. *Mutat Res - Fundam Mol Mech Mutagen*. 1986;161(2):193-198.
198. Żelazna K, Tejs S, Rudnicka K. In vitro micronucleus test assessment of polycyclic aromatic hydrocarbons. *Environ Biotechnol*. 2011;7(2):70-80.
199. Mateuca R, Lombaert N, Aka P V., Decordier I, Kirsch-Volders M. Chromosomal changes: induction, detection methods and applicability in human biomonitoring. *Biochimie*. 2006;88(11):1515-1531.
200. Tice RR, Agurell E, Anderson D, et al. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen. 2000;35(3):206-221.
201. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res*. 1988;175(1):184-191.
202. Olive PL. Cell proliferation as a requirement for development of the contact effect in Chinese hamster V79 spheroids. *Radiat Res*. 1989;117(1):79-92.
203. Collins AR, Duthie SJ, Dobson VL. Direct enzymic detection of endogenous oxidative base damage in human lymphocyte dna. *Carcinogenesis*. 1993;14(9):1733-1735.
204. Singh NP, Stephens RE, Schneider EL. Modifications of alkaline microgel electrophoresis for sensitive detection of DNA damage. *Int J Radiat Biol*. 1994;66(1):23-28.
205. Şekeroğlu ZA, Şekeroğlu V, Kolören Z. The in vitro alkaline comet assay in genetic toxicology. *J Appl Biol Sci*. 2011;5(1):49-54.
206. Yıldız Dinçer, Selin Kankaya. DNA Hasarının Belirlenmesinde Comet Assay. *Turkiye Klin J Med Sci*. 2010;30(4):1365-1373.
207. Riss TL, Moravec RA. Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. *Assay Drug Dev Technol*. 2004;2(1):51-62.

208. Galluzzi L, Aaronson SA, Abrams J, et al. Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. *Cell Death Differ*. 2009;16(8):1093-1107.
209. Kim SI, Kim HJ, Lee HJ, et al. Application of a non-hazardous vital dye for cell counting with automated cell counters. *Anal Biochem*. 2016;492(3):8-12.
210. Kamiloglu S, Sari G, Ozdal T, Capanoglu E. Guidelines for cell viability assays. *Food Front*. 2020;1(3):332-349.
211. Yin LM, Wei Y, Wang Y, Xu YD, Yang YQ. Long term and standard incubations of WST-1 reagent reflect the same inhibitory trend of cell viability in rat airway smooth muscle cells. *Int J Med Sci*. 2012;10(1):68-72.
212. Niles AL, Moravec RA, Eric Hesselberth P, Scurria MA, Daily WJ, Riss TL. A homogeneous assay to measure live and dead cells in the same sample by detecting different protease markers. *Anal Biochem*. 2007;366(2):197-206.
213. Corey MJ, Kinders RJ, Brown LG, Vessella RL. A very sensitive coupled luminescent assay for cytotoxicity and complement-mediated lysis. *J Immunol Methods*. 1997;207(1):43-51.
214. Sedlackova L, Korolchuk VI. Mitochondrial quality control as a key determinant of cell survival. *Biochim Biophys Acta - Mol Cell Res*. 2019;1866(4):575-587.
215. Ishihara G, Kawamoto K, Komori N, Ishibashi T. Molecular hydrogen suppresses superoxide generation in the mitochondrial complex I and reduced mitochondrial membrane potential. *Biochem Biophys Res Commun*. 2020;522(4):965-970.
216. Bankoglu EE, Schuele C, Stopper H. Cell survival after DNA damage in the comet assay. *Arch Toxicol*. 2021;95(12):3803-3813.
217. Reimann H, Bankoglu EE, Stopper H, Hintzsche H. In vitro evaluation of chromosomal damage and DNA strand breaks after treatment with the poppy seed alkaloid thebaine. *Mutat Res - Genet Toxicol Environ Mutagen*. 2021;870-871(August):503393.
218. Queisser N, Oteiza PI, Stopper H, Oli RG, Schupp N. Aldosterone induces oxidative stress, oxidative DNA damage and NF-κB-activation in kidney tubule cells. *Mol Carcinog*. 2011;50(2):123-135.
219. Charehsaz M, Reis R, Helvacioglu S, et al. Safety evaluation of styrax liquidus from the viewpoint of genotoxicity and mutagenicity. *J Ethnopharmacol*. 2016;194:506-512.
220. Resende FA, Vilegas W, Dos Santos LC, Varanda EA. Mutagenicity of flavonoids assayed by bacterial reverse mutation (Ames) test. *Molecules*. 2012;17(5):5255-5268.
221. Praphasawat R, Suttajit M, Charoensin S, et al. Cytotoxicity evaluation of leaf-extract from Moringa oleifera, Cratoxylum formosum, and Mangifera indica. *Naresuan Phayao.*2016; 9(2):5-8.
222. Varamini P, Doroudchi M, Mohagheghzadeh A, Soltani M, Ghaderi A. Cytotoxic evaluation of four Haplophyllum species with various tumor cell lines. *Pharm Biol*. 2007;45(4):299-302.
223. Sebaugh JL. Guidelines for accurate EC50/IC50 estimation. *Pharm Stat*. 2011;10(2):128-134.
224. Weerapreeyakul N, Nonpunya A, Barusrux S, Thitimetharoch T, Sripanidkulchai B. Evaluation of the anticancer potential of six herbs against a hepatoma cell line. *Chinese Med (United Kingdom)*. 2012;7:1-7.
225. Razak NA, Abu N, Ho WY, et al. Cytotoxicity of eupatorin in MCF-7 and MDA-MB-231 human breast cancer cells via cell cycle arrest, anti-angiogenesis and

induction of apoptosis. *Sci Rep*. 2019;9(1):1-12.
226. Xu SP, Sun GP, Shen YX, Peng WR, Wang H, Wei W. Synergistic effect of combining paeonol and cisplatin on apoptotic induction of human hepatoma cell lines. *Acta Pharmacol Sin*. 2007;28(6):869-878.
227. Damon LE, Cadman EC. Advances in rational combination chemotherapy. *Cancer Invest*. 1986;4(5):421-444.
228. Popova D, Forsblad A, Hashemian S, Jacobsson SO. Non-Serotonergic Neurotoxicity by MDMA (Ecstasy) in Neurons Derived from Mouse P19 Embryonal Carcinoma Cells. *PLoS One.* 2016;11(11):e0166750.
229. Lazutka JR, Margolin BH. Replication index in cultured human lymphocytes: Methods for statistical analysis and possible role in genetic toxicology. *Environ Mol Mutagen*. 1991;17(3):188-195.
230. Aardema MJ, Galloway S, Zeiger E, Cimino MC, Hayashi M. Guidance for understanding solubility as a limiting factor for selecting the upper test concentration in the OECD In Vitro Micronucleus Assay Test Guideline No. 487. *Mutat Res - Genet Toxicol Environ Mutagen*. 2011;722(1):89-90.
231. Fenech M. Cytokinesis-block micronucleus cytome assay. *Nat Protoc*. 2007;2(5):1084-1104.
232. Piasek A, Bartoszek A, Namieśnik J. Phytochemicals that counteract the cardiotoxic side effects of cancer chemotherapy. *Post x0229py Hig i Med doświadczalnej*. 2009;63:142-158.
233. Chacko SM, Nevin KG, Dhanyakrishnan R, Kumar BP. Protective effect of p-coumaric acid against doxorubicin induced toxicity in H9c2 cardiomyoblast cell lines. *Toxicol Reports*. 2015;2:1213-1221.
234. Hajra S, Patra AR, Basu A, Bhattacharya S. Prevention of doxorubicin (DOX)-induced genotoxicity and cardiotoxicity: Effect of plant derived small molecule indole-3-carbinol (I3C) on oxidative stress and inflammation. *Biomed Pharmacother*. 2018;101(October 2017):228-243.
235. Gentile JM, Rahimi S, Zwiesler J, Gentile GJ, Ferguson LR. Effect of selected antimutagens on the genotoxicity of antitumor agents. *Mutat Res - Fundam Mol Mech Mutagen*. 1998;402(1-2):289-298.
236. Aparecida Resende F, de Andrade Barcala CAM, da Silva Faria MC, Kato FH, Cunha WR, Tavares DC. Antimutagenicity of ursolic acid and oleanolic acid against doxorubicin-induced clastogenesis in Balb/c mice. *Life Sci*. 2006;79(13):1268-1273.
237. Furtado RA, Rodrigues ÉP, Araújo FRR, et al. Ursolic Acid and Oleanolic Acid Suppress Preneoplastic Lesions Induced by 1,2-Dimethylhydrazine in Rat Colon. *Toxicol Pathol*. 2008;36(4):576-580.
238. Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid Med Cell Longev*. 2009;2(5):270-278.
239. Brglez Mojzer E, Knez Hrnčič M, Škerget M, Knez Ž, Bren U. Polyphenols: Extraction Methods, Antioxidative Action, Bioavailability and Anticarcinogenic Effects. *Molecules*. 2016;21(7).
240. Kondratyuk TP, Pezzuto JM. Natural Product Polyphenols of Relevance to Human Health. *Pharm Biol*. 2004;42(sup1):46-63.
241. Nagarathna PKM, Johnson Wesley M, Sriram Reddy P, Reena K. Review on genotoxicity, its molecular mechanisms and prevention. *Int J Pharm Sci Rev Res*. 2013;22(1):236-243.
242. Lorge E, Moore MM, Clements J, et al. Standardized cell sources and recommendations for good cell culture practices in genotoxicity testing. *Mutat Res*

- *Genet Toxicol Environ Mutagen*. 2016;809:1-15.
243. Delvaeye M, Verovski V, De Neve W, Storme G. DNA breakage, cytotoxicity, drug accumulation and retention in two human ovarian tumor cell lines AZ224 and AZ364 treated with adriamycin, modulated by verapamiltle. *Anticancer Res*. 1993;13(5A):1533–1538.
244. Ganesh CJ, Ponemone V. Bael, Aegle marmelos (L.) Correa, an Indian medicinal plant protects V79 cells against the genotoxic effect of doxorubicin. *Int J Genet Mol Biol*. 2015;7(5):32-46.
245. Chandra Jagetia G. Alteration in the Doxorubicin-Induced DNA Damage in Cultured V79 Cells by Aegle marmelos (L.) Correa (Bael): A Comet Assay Study. *Int J Neurorehabilitation*. 2015;02(05).
246. Lorenzo Y, Costa S, Collins AR, Azqueta A. The comet assay, DNA damage, DNA repair and cytotoxicity: Hedgehogs are not always dead. *Mutagenesis*. 2013;28(4):427-432.
247. Liao W, McNutt MA, Zhu WG. The comet assay: A sensitive method for detecting DNA damage in individual cells. *Methods*. 2009;48(1):46-53.
248. Pfuhler S, Fellows M, Van Benthem J, et al. In vitro genotoxicity test approaches with better predictivity: Summary of an IWGT workshop. *Mutat Res - Genet Toxicol Environ Mutagen*. 2011;723(2):101-107.
249. Passi S, Picardo M, Nazzaro-Porro M. Comparative cytotoxicity of phenols in vitro. *Biochem J*. 1987;245(2):537-542.
250. Karakaya S. Bioavailability of phenolic compounds. *Crit Rev Food Sci Nutr*. 2004;44(6):453-464.
251. Kanadzu M, Lu Y, Morimoto K. Dual function of (-)-epigallocatechin gallate (EGCG) in healthy human lymphocytes. *Cancer Lett*. 2006;241(2):250-255.
252. Valcic S, Burr JA, Timmermann BN, Liebler DC. Antioxidant chemistry of green tea catechins. New oxidation products of (-)-epigallocatechin gallate and (-)-epigallocatechin from their reactions with peroxyl radicals. *Chem Res Toxicol*. 2000;13(9):801-810.
253. Furukawa A, Oikawa S, Murata M, Hiraku Y, Kawanishi S. (-)-Epigallocatechin gallate causes oxidative damage to isolated and cellular DNA. *Biochem Pharmacol*. 2003;66(9):1769-1778.
254. Sutherland BA, Rahman RMA, Appleton I. Mechanisms of action of green tea catechins, with a focus on ischemia-induced neurodegeneration. *J Nutr Biochem*. 2006;17(5):291-306.
255. Johnson MK, Loo G. Effects of epigallocatechin gallate and quercetin on oxidative damage to cellular DNA. *Mutat Res - DNA Repair*. 2000;459(3):211-218.
256. Lu LY, Ou N, Lu Q Bin. Antioxidant Induces DNA damage, cell death and mutagenicity in human lung and skin normal cells. *Sci Rep*. 2013;3:1-11.
257. Aydin S, Tokaç D, Başaran N, Başaran AA. Effect of epigallocatechin gallate on oxidative DNA damage in human lymphocytes. *Turkish J Pharm Sci*. 2015;12(1):19-28.
258. Glei M, Pool-Zobel BL. The main catechin of green tea, (-)-epigallocatechin-3-gallate (EGCG), reduces bleomycin-induced DNA damage in human leucocytes. *Toxicol Vitr*. 2006;20(3):295-300.
259. Fenech M. The in vitro micronucleus technique. *Mutat Res - Fundam Mol Mech Mutagen*. 2000;455(1-2):81-95.
260. Jagetia G., Vijayashree N. Micronuclei-induction and its correlation to cell survival in HeLa cells treated with different doses of adriamycin. *Cancer Lett*. 1996;110:123-128.

261. Chondrou V, Trochoutsou K, Panayides A, Efthimiou M, Stephanou G, Demopoulos NA. Combined study on clastogenic, aneugenic and apoptotic properties of doxorubicin in human cells in vitro. *J Biol Res*. 2018;25(1):1-13.
262. Dhawan A, Kayani MA, Parry JM, Parry E, Anderson D. Aneugenic and clastogenic effects of doxorubicin in human lymphocytes. *Mutagenesis*. 2003;18(6):487-490.
263. Khan F, Sherwani AF, Afzal M. Chromosomal aberration and micronucleus studies of two topoisomerase (II) targeting anthracyclines. *J Environ Biol*. 2009;30(3):409-412.
264. Hintzsche H, Montag G, Stopper H. Induction of micronuclei by four cytostatic compounds in human hematopoietic stem cells and human lymphoblastoid TK6 cells. *Sci Rep*. 2018;8(1):1-11.
265. Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med*. 1996;20(7):933-956.
266. Sugisawa A, Umegaki K. Physiological concentrations of (-)-epigallocatechin-3-O-gallate (EGCg) prevent chromosomal damage induced by reactive oxygen species in WIL2-NS cells. *J Nutr*. 2002;132(7):1836-1839.
267. Siddique YH, Ali F. Protective effect of epigallocatechin gallate against N-nitrosodiethylamine (NDEA) induced toxicity in rats. *Cogent Biol*. 2016;2(1):1141451.
268. Taghavi SM, Shadboorestan A, Sabzevari S, et al. Protective Role of EGCG against Malathion Induced Genotoxicity Using Human Lymphocytes. *Drug Res (Stuttg)*. 2020;70(8):360-366.
269. Yuki K, Ikeda N, Nishiyama N, Kasamatsu T. The reconstructed skin micronucleus assay in EpiDerm™: Reduction of false-positive results - a mechanistic study with epigallocatechin gallate. *Mutat Res - Genet Toxicol Environ Mutagen*. 2013;757(2):148-157.
270. Takumi-Kobayashi A, Ogura R, Morita O, Nishiyama N, Kasamatsu T. Involvement of hydrogen peroxide in chromosomal aberrations induced by green tea catechins in vitro and implications for risk assessment. *Mutat Res - Genet Toxicol Environ Mutagen*. 2008;657(1):13-18.
271. Del Baño MJ, Castillo J, Benavente-García O, et al. Radioprotective-antimutagenic effects of rosemary phenolics against chromosomal damage induced in human lymphocytes by γ-rays. *J Agric Food Chem*. 2006;54(6):2064-2068.
272. Pasqualetti V, Locato V, Fanali C, et al. Comparison between in vitro chemical and ex vivo biological assays to evaluate antioxidant capacity of botanical extracts. *Antioxidants*. 2021;10(7).
273. Almeida TP, Ferreira J, Vettorazzi A, Azqueta A, Rocha E, Ramos AA. Cytotoxic activity of fucoxanthin, alone and in combination with the cancer drugs imatinib and doxorubicin, in CML cell lines. *Environ Toxicol Pharmacol*. 2018;59(December 2017):24-33.
274. Shafiei SS, Solati-Hashjin M, Samadikuchaksaraei A, Kalantarinejad R, Asadi-Eydivand M, Abu Osman NA. Epigallocatechin gallate/layered double hydroxide nanohybrids: Preparation, characterization, and in vitro anti-tumor study. *PLoS One*. 2015;10(8):1-18.
275. Braicu C, Pilecki V, Balacescu O, Irimie A, Neagoe IB. The relationships between biological activities and structure of flavan-3-ols. *Int J Mol Sci*. 2011;12(12):9342-9353.

276. Li S, Wu L, Feng J, et al. In vitro and in vivo study of epigallocatechin-3-gallate-induced apoptosis in aerobic glycolytic hepatocellular carcinoma cells involving inhibition of phosphofructokinase activity. *Sci Rep*. 2016;6(June):1-18.
277. Esmaeelpanah E, Razavi BM, Vahdati Hasani F, Hosseinzadeh H. Evaluation of epigallocatechin gallate and epicatechin gallate effects on acrylamide-induced neurotoxicity in rats and cytotoxicity in PC 12 cells. *Drug Chem Toxicol*. 2018;41(4):441-448.
278. Rahbardar MG, Eisvand F, Rameshrad M, Razavi BM, Hosseinzadeh H. In Vivo and In Vitro Protective Effects of Rosmarinic Acid against Doxorubicin-Induced Cardiotoxicity. *Nutr Cancer*. 2022;74(2):747-760.
279. Zhao L, Liu S, Xu J, et al. A new molecular mechanism underlying the EGCG-mediated autophagic modulation of AFP in HepG2 cells. *Cell Death Dis*. 2017;8(11).
280. Li L, Takemura G, Li Y, et al. Preventive effect of erythropoietin on cardiac dysfunction in doxorubicin-induced cardiomyopathy. *Circulation*. 2006;113(4):535-543.
281. Yeh YC, Lai HC, Ting CT, et al. Protection by doxycycline against doxorubicin-induced oxidative stress and apoptosis in mouse testes. *Biochem Pharmacol*. 2007;74(7):969-980.
282. Al-Shdefat RI, Abd-Elaziz MA, Al-Saikhan FI. Genoprotective and genotoxic effects of thymoquinone on doxorubicin-induced damage in isolated human leukocytes. *Trop J Pharm Res*. 2014;13(12):2015-2020.
283. Nagi MN, Mansour MA. Protective effect of thymoquinone against doxorubicin-induced cardiotoxicity in rats: A possible mechanism of protection. *Pharmacol Res*. 2000;41(3):283-289.
284. Patil L, Balaraman R. Effect of green tea extract on doxorubicin induced cardiovascular abnormalities: Antioxidant action. *Iran J Pharm Res*. 2011;10(1):89-96.
285. Zhang X, Zhu JX, Ma ZG, et al. Rosmarinic acid alleviates cardiomyocyte apoptosis via cardiac fibroblast in doxorubicin-induced cardiotoxicity. *Int J Biol Sci*. 2019;15(3):556-567.
286. Kim DS, Kim HR, Woo ER, Hong ST, Chae HJ, Chae SW. Inhibitory effects of rosmarinic acid on adriamycin-induced apoptosis in H9c2 cardiac muscle cells by inhibiting reactive oxygen species and the activations of c-Jun N-terminal kinase and extracellular signal-regulated kinase. *Biochem Pharmacol*. 2005;70(7):1066-1078.
287. Zhang YR, Li YY, Wang JY, et al. Synthesis and characterization of a rosmarinic acid derivative that targets mitochondria and protects against radiation-induced damage in vitro. *Radiat Res*. 2017;188(3):264-275.
288. Gottlieb E, Armour SM, Harris MH, Thompson CB. Mitochondrial membrane potential regulates matrix configuration and cytochrome c release during apoptosis. *Cell Death Differ*. 2003;10(6):709-717.
289. Williams R V., DeMarini DM, Stankowski LF, et al. Are all bacterial strains required by OECD mutagenicity test guideline TG471 needed? *Mutat Res - Genet Toxicol Environ Mutagen*. 2019;848(August):503081.
290. Seino Y, Nagao M, Yahagi T, Hoshi A, Kawachi T, Sugimura T. Mutagenicity of Several Classes of Antitumor Agents to Salmonella typhimurium TA98, TA100, and TA92. *Cancer Res*. 1978;38(7):2148-2156.

291. Žegura B, Dobnik D, Niderl MH, Filipič M. Antioxidant and antigenotoxic effects of rosemary (Rosmarinus officinalis L.) extracts in Salmonella typhimurium TA98 and HepG2 cells. *Environ Toxicol Pharmacol*. 2011;32(2):296-305.
292. Vattem DA, Levin R, Shetty K. Acid and Rosmarinic Acid for Antimutagenic and DNA Protection Functions. *J. Food Biochem.* 2005;30(413):98-116.
293. Narayanan S, Ramesh K. Epigallocatechin Gallate, a Green Tea Polyphenol Inhibits Mycobacterium smegmatis: In silico and In vitro Studies. *Indian J Pharm Sci*. 2017;79:625-632.
294. Hayatsu H, Inada N, Kakutani T, et al. Suppression of genotoxicity of carcinogens by (-)-epigallocatechin gallate. *Prev Med (Baltim)*. 1992;21(3):370-376.
295. Choudhary A, Kaur B, Singh VS, Shah K, Kalsi V, Suttee A. EGCG: The shield against genotoxicity caused by cisplatin. *Int J Toxicol Pharmacol Res*. 2016;8(3):125-129.
296. Šmerák P, Šestáková H, Polívková Z, et al. Antimutagenic effect of epigallocatechin gallate and its effect on the immune response in mice. *Czech J Food Sci*. 2006;24(4):180-192.
297. Kwiatkowska E, Wojtala M, Gajewska A, Soszyński M, Bartosz G, Sadowska-Bartosz I. Effect of 3-bromopyruvate acid on the redox equilibrium in non-invasive MCF-7 and invasive MDA-MB-231 breast cancer cells. *J Bioenerg Biomembr*. 2016;48(1):23-32.
298. Mladkova J, Sanda M, Matouskova E, Selicharova I. Phenotyping breast cancer cell lines EM-G3, HCC1937, MCF7 and MDA-MB-231 using 2-D electrophoresis and affinity chromatography for glutathione-binding proteins. *BMC Cancer*. 2010;10.
299. Jokar F, Mahabadi JA, Salimian M, et al. Differential expression of HSP90β in MDA-MB-231 and MCF-7 cell lines after treatment with doxorubicin. *J Pharmacopuncture*. 2019;22(1):28-34.
300. Radwan EM, Abdullah R, Al-Qubaisi MS, et al. Effect of recombinant human erythropoietin and doxorubicin in combination on the proliferation of MCF-7 and MDA-MB231 breast cancer cells. *Mol Med Rep*. 2016;13(5):3945-3952.
301. Marinello PC, Panis C, Silva TNX, et al. Metformin prevention of doxorubicin resistance in MCF-7 and MDA-MB-231 involves oxidative stress generation and modulation of cell adaptation genes. *Sci Rep*. 2019;9(1):1-11.
302. Tassone P, Tagliaferri P, Perricelli A, et al. BRCA1 expression modulates chemosensitivity of BRCA1-defective HCC1937 human breast cancer cells. *Br J Cancer*. 2003;88(8):1285-1291.
303. Wen S hsuan, Su S chiang, Liou B huang, Lin C hao, Lee K rong. Sulbactam-enhanced cytotoxicity of doxorubicin in breast cancer cells. *Cancer Cell Int*. 2018;18(1):1-18.
304. Wan X, Hou J, Liu S, et al. Estrogen Receptor α Mediates Doxorubicin Sensitivity in Breast Cancer Cells by Regulating E-Cadherin. *Front Cell Dev Biol*. 2021;9: 583572.
305. Yang X, Taylor L, Yu J, Fenton MJ, Polgar P. Different effects of genistein on molecular markers related to apoptosis in two phenotypically dissimilar breast cancer cell lines. *J Cell Biochem*. 2001;82(1):78-88.
306. Devarajan E, Sahin AA, Chen JS, et al. Down-regulation of caspase 3 in breast cancer: A possible mechanism for chemoresistance. *Oncogene*. 2002;21(57):8843-8851.
307. Pilco-Ferreto N, Calaf GM. Influence of doxorubicin on apoptosis and oxidative

stress in breast cancer cell lines. *Int J Oncol*. 2016;49(2):753-762.
308. Al-Qubaisi M, Rozita R, Yeap SK, Omar AR, Ali AM, Alitheen NB. Selective cytotoxicity of goniothalamin against hepatoblastoma HepG2 cells. *Molecules*. 2011;16(4):2944-2959.
309. de Oliveira PF, Alves JM, Damasceno JL, et al. Cytotoxicity screening of essential oils in cancer cell lines. *Rev Bras Farmacogn*. 2015;25(2):183-188.
310. Goodin MG, Fertuck KC, Zacharewski TR, Rosengren RJ. Estrogen receptor-mediated actions of polyphenolic catechins in vivo and in vitro. *Toxicol Sci*. 2002;69(2):354-361.
311. Roy AM, Baliga MS, Katiyar SK. Epigallocatechin-3-gallate induces apoptosis in estrogen receptor-negative human breast carcinoma cells via modulation in protein expresssion of p53 and Bax and caspase-3 activation. *Mol Cancer Ther*. 2005;4(1):81-90.
312. Hong OY, Noh EM, Jang HY, et al. Epigallocatechin gallate inhibits the growth of MDA-MB-231 breast cancer cells via inactivation of the β-Catenin signaling pathway. *Oncol Lett*. 2017;14(1):441-446.
313. Yesil-Celiktas O, Sevimli C, Bedir E, Vardar-Sukan F. Inhibitory effects of rosemary extracts, carnosic acid and rosmarinic acid on the growth of various human cancer cell lines. *Plant Foods Hum Nutr*. 2010;65(2):158-163.
314. Messeha SS, Zarmouh NO, Asiri A, Soliman KFA. Rosmarinic acid-induced apoptosis and cell cycle arrest in triple-negative breast cancer cells. *Eur J Pharmacol*. 2020;885:173419.
315. Kapadia GJ, Rao GS, Ramachandran C, Iida A, Suzuki N, Tokuda H. Synergistic cytotoxicity of red beetroot (Beta vulgaris L.) extract with doxorubicin in human pancreatic, breast and prostate cancer cell lines. *J Complement Integr Med*. 2013;10(1):113-122.
316. Wang W, Chen D, Zhu K. SOX2OT variant 7 contributes to the synergistic interaction between EGCG and Doxorubicin to kill osteosarcoma via autophagy and stemness inhibition. *J Exp Clin Cancer Res*. 2018;37(1):1-16.